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ARGONNE CANCER RESEARCH HOSPITAL
950 EAST FIFTY-NINTH STREET • CHICAGO • ILLINOIS 60637

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1964

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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THE EFFECT OF ERYTHROPOIETIN UPON HEME SYNTHESIS BY MARROW CELLS IN VITRO*

By

S. B. Krantz,[†] O. Gallien-Lartigue[‡] and E. Goldwasser

In order to study the chemical mechanism of action of the hormone, erythropoietin, it would be desirable to work with an in vitro system capable of responding to it. Since this hormone occurs to exert its effect on the primitive precursor cells of erythroid tissue, causing them to give rise to distinctive, differentiated cells (normoblasts, reticulocytes, erythrocytes),¹⁻⁴ such a system would be useful for the general study of the chemical events concerned with the process of differentiation. Additionally, an in vitro system, with appropriate sensitivity, would be a welcome substitute for the current lengthy and somewhat inaccurate animal assay methods for following the purification of erythropoietin, and in studying the relationship of its chemical properties to its biological action. This paper describes a cell culture method of studying the action of erythropoietin which we think will serve as one basis both for an improved assay method and as a step toward the investigation of its mode of action. Several reports of effects of erythropoietin on marrow cells in vitro have appeared recently, but the effects seen were much smaller than would be expected from the relatively large amounts of hormone used.⁵⁻⁸

EXPERIMENTAL PROCEDURE

Materials and Methods. Cells were obtained from the femora and tibiae of 6- to 7-week-old Sprague-Dawley male rats, by splitting the scraped bones and removing the marrow with a thin spatula. The cells were suspended in the medium by multiple passages through a pipet until the easily visible aggregates had been dispersed. Examination of the suspension under the microscope showed that most of the cells were singly dispersed, although a few clumps containing about 10 to 20 cells were still present. About 60-70 per cent of the total cells were nucleated. The medium consisted of 60 per cent NCTC-109[§] with 50 units of penicillin added per ml, and 40 per cent plasma containing 250 to 300 units of heparin per ml. The plasma was taken from male Sprague-Dawley rats (160 to 200 g) the day before use and stored at 4° after the cells had been removed. Samples of anemic plasma with elevated titers of erythropoietin were prepared by the method of Hodgson⁹ and contained from 0.6 to 4.6 units per ml. Plasma iron determinations were done by the method of Peters *et al.*;¹⁰ these values were used to calculate total iron incorporation into heme, and from this the amount of heme synthesized.

The suspension of cells, at a concentration of from 2000 to 6000 cells per mm³, in a final volume of 2.8 ml, was placed in sterile plastic tissue culture dishes (60 x 15 mm)[¶] and incubated

* This report is taken from a paper appearing in J. Biol. Chem. 238:4085, 1963. ✓

[†] U.S. Public Health Service. Post doctoral Fellow.

[‡] North Atlantic Treaty Organization Fellow, present address: Collège de France, Laboratoire d'Embryologie Experimentale, Paris, France.

[§] Microbiological Associates, Inc.

[¶] Falcon Plastics.

at 37° in an atmosphere of 5 per cent CO₂, 95 per cent air.

Radioactive iron in the form of Fe⁵⁹Cl₃* was added to the complete medium at a concentration of 5 µc per ml and pre-incubated for 12 hours at 37° before addition of approximately 1 µc to each dish.

At the end of the indicated incubation intervals, the contents of the dishes were transferred to centrifuge tubes and the cells collected and washed twice with isotonic sodium chloride-phosphate buffer at pH 7.4. The second saline wash was virtually free of Fe⁵⁹. The packed cells were lysed by addition of 1 ml of water and 0.5 ml of Drabkin's solution,¹¹ and allowed to stand in the cold overnight. After centrifugation at 14,500 g for 20 minutes, the heme was extracted from the supernatant with butanone-2 by the method of Teale,¹² and counted in a well type scintillation counter.

Erythropoietin activity of plasmas and partially purified fractions was determined by the fasted rat assay¹³ using both CoCl₂ and a sheep plasma fraction as standards.^{14†}

Reversed-phase paper chromatography of the Fe⁵⁹-labeled heme was done using siliconized Whatman No. 1 paper, and both the water-propanol-pyridine system described by Chu,¹⁵ and a modified water-lutidine system described by Mauzerall.¹⁶

All glassware used with the cells during preparation for incubation was cleaned as described by Paul.¹⁷

RESULTS AND DISCUSSION

When several of the commercially available tissue culture media (Eagle's, NCTC-199, F-4, NCTC-109) were tested, using the incorporation of Fe⁵⁹ into the butanone extractable fraction as an indication of heme synthesis, we found that the medium prepared with NCTC-109 and rat plasma gave the greatest incorporation under the conditions we chose to use.

The routine use of the simple and rapid butanone extraction procedure as an indication of heme synthesis was justified by our findings that there was no radioactivity in the butanone layer at zero time, and that after a period of incubation the Fe⁵⁹ which was butanone extractable was identified as heme iron in two different chromatographic solvents (Figure 1). Unless the marrow cell lysate was acidified, essentially none of the label was extractable into butanone, making it probable that all the labeled heme is firmly bound to protein. In both control and stimulated cultures less than 4 per cent of the total heme was bound to the stroma, which contained, however, an appreciable quantity of non-heme Fe⁵⁹.

We have used the incorporation of iron over a 4-hour period as an indicator of the rate of synthesis of heme. The data are presented as rates of incorporation of iron rather than specific activities, because there is a large pool of pre-existent, unlabeled hemoglobin which would tend to make large differences in newly formed hemoglobin appear very small. One experiment in which the total heme was determined after incubation both with and without erythropoietin is summarized in Table 1. Here it is readily apparent that the presence of erythropoietin caused an appreciable net increase in heme, which more than compensated for the loss of heme seen in

* Abbott Laboratories.

† We are grateful to the United States Public Health Service Study Section on Hematology for supplying us with sheep plasma erythropoietin concentrates.

Figure 1. Chromatography of butanone extracts of cells cultured in the presence of 0.27 units per ml of erythropoietin for 55 hours, with the Fe^{59} present during the last 8 hours. A, represents chromatography in water: propanol:pyridine 5.5:0.1:0.4; heme R_f is 0.49; B, represents chromatography in 2,6 lutidine:water:1.1 N NH_4OH 10:2.8:4.2; heme R_f is 0.58. Upper part of each shows photograph of benzidine stained chromatogram, lower part shows radioactivity of 0.5 cm sections of the chromatogram.

Table 1
NET SYNTHESIS OF HEME BY MARROW CELL
CULTURES IN THE PRESENCE
OF ERYTHROPOIETIN*

Sample	Total heme μg
Zero time control	8.7
52-hour incubation no erythropoietin	6.6
52-hour incubation 0.4 units per ml erythropoietin	13.3

* Cultures started with 4100 nucleated cells per mm^3 from fasted rats; total volume 3.0 ml; erythropoietin was an anemic sheep plasma fraction containing 257 units per mg protein. Total heme was determined as cyanferriprotoporphyrin in the butanone extract.

the control during the 52 hours of incubation. The net amount of heme synthesized in this period is equivalent to approximately 10^7 red blood cells.

The changes in rate of heme synthesis by marrow cells, derived from normal rats, with

time of incubation, and the effect of erythropoietin on this system, are shown in Figure 2. In both stimulated and control cultures there was a fall in rate of heme synthesis from the 4th to about the 10th hour of incubation. While the controls continued to fall throughout the remainder of the period studied, the erythropoietin containing cultures showed a rapid increase in rate of heme synthesis, so that by 29 hours the stimulated cultures were synthesizing heme at about 3 times

Figure 2. Effect of erythropoietin on normal rat marrow. Cultures started with 3100 nucleated cells per mm^3 , total volume 3.0 ml, controls (●) had no added erythropoietin, stimulated (○) had 1.6 units per ml of erythropoietin as anemic plasma. Each point is the mean of duplicates and indicates the middle of the 4-hour incubation with radioiron.

the rate of the control cultures, and at almost twice their original rate. At 49 hours both rates had declined considerably, yet the rate in the stimulated cultures was 6 times that of the controls. These data indicate clearly that the loss of function by the marrow in culture can be temporarily arrested by some constituent, presumably erythropoietin, of the anemic plasma.

We reported some time ago that *in vivo* there is a heightened response to exogenous erythropoietin when the normal rate of red cell formation is depressed¹³ and have offered an explanation in terms of the mechanisms controlling erythropoiesis.¹⁸ The successful use of fasted rats for the routine assay of erythropoietin suggested that the same effect should hold *in vitro*, and we studied the rate of heme synthesis by marrow cells derived from fasted rats, with the results seen in Figure 3. While the amount of erythropoietin activity used in this experiment was much less than in the previously described one (0.21 units per ml in contrast to 1.6 units per ml), the stimulated rate of heme synthesis was still about 3 times greater than the control value after 29 hours of incubation. The continued increase in rate from 29 to 49 hours seen in this experiment was not seen in other experiments and may not be a typical response of this system. The shape of this curve may possibly be due to a somewhat later maximum response than seen earlier, the peak response being between 29 and 48 hours, where no samples were taken.

Factors other than erythropoietin may be present in anemic plasma and may have been re-

sponsible for the effects seen. Without a pure hormone, it is impossible to perform an unequivocal experiment to test the identity of erythropoietin with the factor in anemic plasma which stimulates heme synthesis in vitro, but some information regarding the possible identity of the two factors may be obtained with preparations of partially purified erythropoietin. When a time study, similar to those already discussed, was done using a partially purified sheep plasma ery-

Figure 3. Effect of erythropoietin on marrow from fasted rats. Cultures started with 3750 nucleated cells per mm^3 , total volume 3.0 ml, controls (●) had no added erythropoietin, stimulated (○) had 0.21 units per ml of erythropoietin as anemic plasma. Each point is the mean of duplicates and indicates the middle of the 4-hour incubation with radioiron.

thropoietin fraction (Step III, 3.3 units per mg protein)¹⁴ the results (Figure 4) were in good qualitative agreement with those already discussed: after 29 hours of incubation the stimulated rate was more than 20 times greater than the control rate. This increased ratio was due to a lower control value as well as to a higher absolute rate of synthesis.

More highly purified erythropoietin derived from a Step V fraction,¹⁹ and having a potency of 950 units per mg protein, * still showed the same activity while another fraction shown to be devoid of erythropoietin activity by the in vivo assay was also without effect on the marrow cell culture. As is also the case with the in vivo assay, trypsin treatment destroys the activity of erythropoietin in the marrow cell cultures.^{20,21} These data, summarized in Table 2, strongly suggest that the effect seen in vitro is in reality due to erythropoietin and not to another factor present in the preparations.

If this type of in vitro response is to be used as the basis for the assay of erythropoietin, one obviously necessary condition is a graded dose-response relationship. We have found, as would be expected for a hormone assay, a log dose-response curve which has a central linear

* E. Goldwasser and C. Kung, unpublished observations.

Figure 4. Effect of partially purified erythropoietin on fasted rat marrow. Cultures started with 3650 nucleated cells per mm^3 , total volume 3.0 ml, controls (●) had no added erythropoietin, stimulated (○) had 0.18 units per ml as the Step III fraction.¹⁴ Each point is the mean of duplicates and indicates the middle of the 4-hour incubation with radioiron.

Table 2

EFFECT OF HIGHLY PURIFIED ERYTHROPOIETIN
ON HEME SYNTHESIS BY MARROW CELLS
FROM FASTED RATS*

	Incorporation of Fe^{59} into heme %
Control	0.13
Sheep plasma erythropoietin fraction UF (0.17 units per ml)	0.86
Sheep plasma side fraction	0.20
Fraction UF + trypsin	0.19
Trypsin	0.12

* Cultures started with 4250 nucleated cells per mm^3 ; total volume 3.0 ml; Fe^{59} added at 27 hours and incubation stopped at 31 hours. Erythropoietin was UF fraction; side fraction contained the same amount of protein as UF fraction (0.18 μg per ml). Trypsin incubation was done in 109 medium containing 3 μg trypsin per ml at 37° for 5 hours. Similar incubations were done for fractions without trypsin and for trypsin alone.

portion that can be used for the assay of unknown fractions (Figure 5). The sensitivity of the marrow cell cultures to erythropoietin is considerably greater than that of the fasted rat assay, and may be more sensitive than the polycythemic mouse assay method.²² Since a rat weighing 150 g responds to 1 unit of exogenous erythropoietin when the response is measured in the fasting animal by incorporation of iron into red cells, and since the concentration of erythropoietin

Figure 5. Erythropoietin dose-response curve for marrow cells *in vitro*. Cultures were carried out in 35 x 10 mm dishes with total volume of 0.8 ml containing 5900 nucleated cells per mm³. Fe⁵⁹ was added after 24 hours of incubation and cultures terminated after 32 hours. (o) anemic plasma (4.6 units per ml); (●) sheep plasma erythropoietin fraction UF (950 units per mg protein). Each point is the mean of triplicates.

in such a rat is about 0.13 units per ml of blood, we should expect marrow cells *in vitro*, if they are behaving as they do *in vivo*, to respond to a similar concentration of hormone. In fact, the data in Figure 5 clearly demonstrate a response to erythropoietin concentrations as low as 0.05 units per ml. We take this as evidence that the cells are responding in a physiological manner to erythropoietin.

The *in vitro* system, however, has one aspect which may limit its potential usefulness as an assay method. The cells seem to be sensitive to inhibitors or toxic materials present in crude erythropoietin preparations. With large amounts of crude sheep plasma preparations there is a decided inhibitory effect upon the incorporation of iron into heme, as well as the incorporation of glucosamine into red cell stroma.*

The lag period seen in the response of the marrow cells to erythropoietin (Figures 3-5) may

* P. P. Dukes, F. Takaku and E. Goldwasser, unpublished observations.

represent the period of time which is necessary before heme synthesis starts, after an early action of the hormone on some other process. It may, on the other hand, be due solely to the adjustment of the cells to the conditions of in vitro cultivation, as is the case with many animal cells.^{23,24} In the artificially polycythemic mouse there is a lag period of 12 to 18 hours between the introduction of erythropoietin and the first clear indication of erythropoiesis.* These considerations would suggest that the observed lag in the stimulation of heme synthesis is inherent in the action of erythropoietin and may represent the time during which the enzymes concerned with heme synthesis are being elaborated.

In our experience, erythropoietin seemed to have no discernible direct effect upon heme synthesis by either reticulocytes or avian red cells,[†] nor does it have any effect upon the conversion of porphobilinogen to uroporphyrin I by an enzyme system from spinach leaves.[‡] Doehr and Orten,²⁵ however, have reported that in a preparation from bird erythrocytes, erythropoietin causes an increase in coproporphyrin and protoporphyrin.

If we accept the premise that the observed synthesis of heme is equivalent to the synthesis of hemoglobin, and that the synthesis of hemoglobin is a characteristic of those cells which have differentiated into the erythroid lineage, then a substance which initiates the synthesis of heme may be intimately involved in the process of differentiation. There is ample evidence from in vivo studies[‡] that erythropoietin can initiate the whole process of red cell formation in tissues which can normally form red cells but have been put into a condition totally devoid of identifiable erythroid cells. While the experiments reported in this paper have not been done with such completely suppressed tissues, it is not implausible to predict that similar effects will be demonstrated in vitro when the investigations now underway have been completed.

In regard to the chemical study of differentiation, the marrow systems offer several distinct advantages. There is at least one well-defined protein, hemoglobin, which is absent from the primitive precursor cells but is present in the end-product cells, and there is a specific hormone, erythropoietin, which plays an important role in the early stages of the induction of red cell formation. From the observations presented in this paper, we can conclude that the rapid loss of functional ability (hemoglobin synthesis) by marrow cells cultured in vitro can be temporarily arrested by the presence of erythropoietin. The fact that the cultures still lose this function at a somewhat later time, may point to the need for still other conditions which have to be met in vitro before the process of differentiation can be maintained in a continuous manner. We believe that this system may be very useful in the study of the detailed chemical mechanism of development, and would suggest, tentatively, that the loss of functional capacities of cells in culture may, in general, be due to a deficiency of specific hormonal type factors, such as erythropoietin, and may be avoided or reversed by the addition of the appropriate factor to the culture medium.

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STUDIES ON IRON ABSORPTION. II. THE ABSORPTION OF IRON IN
EXPERIMENTAL ANEMIAS OF DIVERSE ETIOLOGY^{*†}

By

G. A. Mendel,[‡] R. J. Weiler[§] and A. Mangalik

When physiologic quantities are involved, the amount of iron absorbed from the gastrointestinal tract is influenced by several factors among which are body iron content¹⁻³ and rate of red cell production.³⁻⁵ The effect of anemia is less clear, although there is general agreement that absorption is increased with iron lack anemia and hemolytic anemias of diverse etiology. Iron lack,⁶ like the accelerated erythropoiesis which accompanies many hemolytic states, can be associated with increased absorption in the absence of anemia.⁵ The precise role of anemia per se on iron absorption, independent of alterations in body iron content and the rate of erythropoiesis, remains controversial.⁶⁻⁹

Studies in this laboratory indicate that atmospheric hypoxia results in enhanced mucosal iron transport when body iron content and the rate of erythropoiesis remain unaltered.^{5,10} The present studies were undertaken to define more precisely the effect of anemia on the absorption of iron in mice having various forms of experimentally induced anemia: hypoplastic, iron lack, hemolytic and post-hemorrhagic. Our results indicate that anemia per se can markedly influence iron absorption, and invite further speculation on the physiology of this process.

MATERIALS AND METHODS

Cf No. 1 female mice, ten to eleven weeks old, maintained on a Rockland mouse pellet diet were used in all experiments except as indicated in Tables 4 and 5, where mice 16 to 20 weeks old were used.

Iron lack anemia was produced by maintenance on a diet of 28 per cent milk powder supplemented with iron-free Jones-Foster salt mixture and thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, choline, methyl-napthoquinone, and oleum percomorphum. After 8 weeks on this diet there was a decline in hematocrit to approximately 80 per cent of the control value, and the circulating red blood cells showed the hypochromasia and microcytosis characteristic of iron deficiency. These changes could be completely corrected by the administration of iron.

Anemia due to bone marrow hypoplasia was produced by removal of the spleen (an erythropoietic organ in this strain of mouse) and administration of 2 μ c per g of strontium-89 chloride as previously described.^{5,11}

2 Oct. C-10-9-63

^{*} This report is taken from a paper appearing in Blood 22:450, 1963.

[†] Some of these studies were presented at the Ninth Congress of the International Society of Hematology in Mexico City, September 12, 1962, and appeared in abstract form in J. Clin. Invest. 41:1384, 1962.

[‡] Schweppe Foundation Fellow.

[§] Present address: United States Naval Medical Research Unit No. 3, c/o American Embassy, Cairo, Egypt.

Hemolytic anemia was induced by the daily subcutaneous injection 0.6 mg of phenylhydrazine for the 3 days preceding the test dose of radioiron, or by a single intraperitoneal injection of 2 or 7.5 μ l of an antiserum to mouse red blood cells, given 2 days prior to the feeding of radioiron. The antiserum was collected from rabbits previously injected with washed mouse red blood cells by multiple routes 3 times a week for 3 weeks. This serum agglutinated mouse red blood cells in a dilution of 1:4096 and hemolyzed in a dilution of 1:64 in vitro after incubation at 37° C for 90 minutes.

Acute blood loss anemia was produced by withdrawal of approximately 0.2 ml of whole blood from the retro-ocular venous plexus on two consecutive days. Six days later the test dose of radioiron was administered. Where indicated (Table 3) the iron lost by bleeding was replaced by hemolyzing the blood removed by the freeze-thaw technique and injecting it intraperitoneally.

Iron absorption was measured as formerly described,⁵ except that radioiron in the form of Fe⁵⁹Cl₃ was used and 7 μ g of ferric ammonium sulfate was added as carrier.

The procedures used for the production of atmospheric hypoxia, measurement of incorporation of intravenously administered radioiron into circulating red blood cells, hematocrit determinations and reticulocyte counts are as previously reported.⁵

RESULTS

Effect of phenylhydrazine-induced anemia on iron absorption in normal mice and mice with bone marrow hypoplasia: as indicated in Table 1, iron absorption in normal mice treated with phenylhydrazine increased from a mean of 8.8 per cent to 40.5 per cent. Red cell production also increased, as indicated by the rise in incorporation of intravenously administered radioiron into circulating red blood cells from a mean of 34.3 per cent to 66.1 per cent, and the increase in reticulocyte count from 3.8 per cent to 12.5 per cent. Mice with erythroid hypoplasia produced

Table 1

THE EFFECT OF PHENYLHYDRAZINE INDUCED ANEMIA ON IRON ABSORPTION IN NORMAL AND SPLENECTOMIZED-Sr⁸⁹ TREATED MICE

Treatment	Fe ⁵⁹ absorbed from G-I tract (72 hours) %	IV administered Fe ⁵⁹ incorporated into circulating R.B.C.'s (24 hours) %	At time of Fe ⁵⁹ feeding	
			Mean reticulocyte count %	Mean hematocrit %
Normal controls	8.8 $\pm$ 1.61* (7) [†]	34.3 $\pm$ 2.69* (6) [†]	3.8 $\pm$ 0.37*	51
Phenylhydrazine 0.8 mg x 3	40.5 $\pm$ 4.28 (7)	66.1 $\pm$ 2.35 (6)	12.5 $\pm$ 1.11	34
Splenectomy + Sr ⁸⁹	11.8 $\pm$ 1.56 (6)	3.5 $\pm$ 0.33 (6)	1.1 $\pm$ 0.18	35
Splenectomy + Sr ⁸⁹ + phenylhydrazine 0.8 mg x 3	51.5 $\pm$ 12.65 (4)	3.3 $\pm$ 0.41 (7)	1.1 $\pm$ 0.11	20
Splenectomy + Sr ⁸⁹ + phenylhydrazine 0.2 mg x 3	25.9 $\pm$ 5.37 (7)	3.9 $\pm$ 0.82 (5)	0.8 $\pm$ 0.10	30

* Standard error of mean.

[†] Number of animals in each group.

by splenectomy and Sr^{89} administration showed an increase in iron absorption from 11.8 per cent to 51.5 per cent when treated with sufficient phenylhydrazine to reduce the hematocrit from 35 per cent to 20 per cent. The mean absorption value showed a lesser increase to 25.9 per cent when a smaller dose of phenylhydrazine was given and the hematocrit was 30 per cent at the time of feeding. In phenylhydrazine-treated mice previously splenectomized and given radiostrontium, no measurable increase in red cell production could be detected by the reticulocyte count or the incorporation of intravenously administered radioiron into circulating red blood cells. Although in normal animals treated with phenylhydrazine the hematocrit rapidly returned to pretreatment levels, the splenectomized radiostrontium-treated animals did not recover from the acute anemia induced by phenylhydrazine.

Thus, progressive increase in iron absorption was found to occur with increasing degrees of anemia produced by phenylhydrazine, and in the absence of significant alteration in red cell production.

Effect of anemia produced by a rabbit hemolysin on iron absorption in normal mice and mice with bone marrow hypoplasia: because phenylhydrazine may have a direct effect on absorption apart from its anemia and methemoglobin producing effects, an antiserum directed against mouse red blood cells was prepared from rabbits and its effect on iron absorption assessed. As indicated in Table 2, normal mice treated with this antiserum showed an increase in absorption

Table 2

THE EFFECT OF ANEMIA PRODUCED BY A RABBIT HEMOLYSIN ON IRON
ABSORPTION AND ERYTHROPOIESIS IN NORMAL AND
SPLENECTOMIZED- Sr^{89} TREATED MICE

Treatment	Fe ⁵⁹ absorbed from G-I tract (96 hours) %	IV administered Fe ⁵⁹ incorporated into circulating R.B.C.'s (24 hours) %	At time of Fe ⁵⁹ feeding	
			Mean reticulocyte count %	Mean hematocrit %
Normal controls	14.5 ± 1.63* (16) [†]	29.1 ± 1.59* (12) [†]	3.84 ± 0.26*	50
7.5 µl rabbit hemolysin	21.3 ± 3.22 (16)	37.7 ± 2.43 (14)	5.30 ± 0.98	27
Splenectomy + Sr^{89}	8.7 ± 1.52 (12)	3.4 ± 0.52 (13)	0.46 ± 0.10	37
Splenectomy + Sr^{89} + 2.0 µl rabbit hemolysin	21.3 ± 4.60 (10)	4.1 ± 0.90 (14)	0.20 ± 0.54	26

* Standard error of mean.

[†] Number of animals in each group.

from 14.5 per cent to 21.3 per cent coincident with a fall in hematocrit from a mean of 50 per cent to 27 per cent. Mice with erythroid hypoplasia produced by radiostrontium and splenectomy showed an increase in absorption from a mean of 8.7 per cent to 21.3 per cent when treated with sufficient antiserum to produce a decline in mean hematocrit from 37 per cent to 26 per cent. In contrast to the normal mice, there was no evidence of increased red cell production in the splenectomized radiostrontium-treated mice on further treatment with the rabbit hemolysin, and they did not recover from the increased anemia produced by the hemolysin.

Thus, an increase in iron absorption was found to occur coincident with increased anemia produced by a hemolysin, and in the absence of measurable alteration in red cell production.

Effect of anemia produced by hemorrhage on iron absorption in mice with bone marrow hypoplasia: splenectomized Sr^{89} -treated mice were bled approximately 0.2 ml on 2 consecutive days, and iron absorption was measured 6 days later. Table 3 shows that before bleeding mean absorption was 19 per cent and the hematocrit was 36 per cent. Six days after bleeding, absorption had increased to a mean of 31.9 per cent and the hematocrit was 29 per cent. That this difference was not due to the iron lost by hemorrhage is suggested by a comparable increase in

Table 3

THE EFFECT OF ANEMIA PRODUCED BY HEMORRHAGE ON IRON ABSORPTION
AND ERYTHROPOIESIS IN SPLENECTOMIZED- Sr^{89} TREATED MICE

Treatment	Fe ⁵⁹ absorbed from G-I tract (120 hours) %	IV administered Fe ⁵⁹ incorporated into circulating R.B.C.'s (24 hours) %	At time of Fe ⁵⁹ feeding	
			Mean reticulocyte count %	Mean hematocrit %
Normal controls	22.8 ± 2.11* (14)†	24.4 ± 2.30* (14)†	3.95 ± 0.26*	52
Splenectomy + Sr^{89}	19.0 ± 0.87 (16)	3.2 ± 0.68 (16)	0.74 ± 0.05	36
Splenectomy + Sr^{89} + 0.2 ml bleed x 2	31.9 ± 5.68 (8)	5.1 ± 1.86‡ (12)	1.57 ± 0.46	29
Splenectomy + Sr^{89} + 0.2 ml bleed x 2 with reinjection of hemolysate	29.1 ± 3.16 (9)	3.7 ± 0.75 (7)	1.15 ± 0.12	29

* Standard error of mean.

† Number of animals in each group.

‡ One of the animals in this group showed an incorporation of 24.5% which was greater than 7 times the mean of the others.

iron absorption in mice that were bled and subsequently re-injected with a hemolysate of the removed blood. The apparent increase in reticulocyte count in the bled groups was not statistically significant and was not followed by a rise in hematocrit. Incorporation of intravenously administered radioiron into circulating red blood cells remained low in the bled splenectomized Sr^{89} -treated animals.

Effect of decreased erythropoiesis produced by splenectomy and radiostrontium administration on iron absorption in iron deficient mice: the effect of splenectomy and administration of radiostrontium on iron absorption in iron deficient mice is illustrated in Table 4 which shows that the mean absorption value in the iron deficient mice was 51.3 per cent compared to the mean value of 9.5 per cent in the normal controls. In these mice, mean absorption was 39.6 per cent despite the decrease in erythropoiesis indicated by decline in the reticulocyte count (from 4.6 per cent to 0.6 per cent), decrease in the incorporation of intravenously administered radioiron into circulating red blood cells (from 60.1 per cent to 9.5 per cent) and decline of hematocrit (from 42 per cent to 29 per cent).

Thus, iron absorption in the iron deficient mice remained more than 4 times the control

value despite the marked impairment of erythropoiesis produced by splenectomy and radiostrontium administration.

Table 4

THE EFFECT OF DECREASED ERYTHOPOIESIS INDUCED BY SPLENECTOMY
AND RADIOSTRONTIUM ADMINISTRATION OF IRON ABSORPTION
IN NORMAL AND IRON-DEFICIENT MICE

Treatment	Fe ⁵⁹ absorbed from G-I tract (72 hours) %	IV administration Fe ⁵⁹ incorporated into circulating R.B.C.'s (24 hours) %	At time of Fe ⁵⁹ feeding	
			Mean reticulocyte count %	Mean hematocrit %
Normal controls	9.5 ± 1.3* (7)†	37.8 ± 4.6* (7)†	3.2 ± 0.47*	53
Splenectomy + Sr ⁸⁹	9.4 ± 1.1 (8)	15.1 ± 6.7 (5)	0.9 ± 0.09	37
Iron deficient	51.3 ± 7.6 (8)	60.1 ± 2.5 (6)	4.6 ± 0.41	42
Iron deficient + splenectomy + Sr ⁸⁹	39.6 ± 4.7 (11)	9.5 ± 2.0 (9)	0.6 ± 0.02	29

* Standard error of mean.

† Number of animals in each group.

Effect of hypoxia on iron absorption in iron deficient mice: iron deficient mice were made hypoxic and the effects of hypoxia on iron absorption and erythropoiesis were determined. As indicated in Table 5, mean iron absorption in the iron deficient group was 53.6 per cent while the normal control value was 19.7 per cent. In the hypoxic iron deficient group, mean iron absorption increased from 53.6 per cent to 66.8 per cent without increased incorporation of intravenously administered iron into circulating blood cells, and without statistically significant change in the reticulocyte count.

Table 5

THE EFFECT OF HYPOXIA ON IRON ABSORPTION IN IRON-DEFICIENT MICE

Treatment	Fe ⁵⁹ absorbed from G-I tract (72 hours) %	IV administered Fe ⁵⁹ incorporated into circulating R.B.C.'s (24 hours) %	At time of Fe ⁵⁹ feeding	
			Mean reticulocyte count %	Mean hematocrit %
Normal controls	19.7 ± 2.7* (7)†	39.2 ± 3.6* (7)†	4.2 ± 0.40*	54
Iron deficient	53.6 ± 4.3 (8)	69.1 ± 2.8 (8)	6.5 ± 0.59	42
Iron deficient + hypoxia	66.8 ± 3.5 (8)	67.1 ± 2.4 (5)	9.7 ± 2.80‡	41

* Standard error of mean.

† Number of animals in each group.

‡ One animal in this group demonstrated a reticulocytosis of 30.5% which is greater than four times the mean of the others.

DISCUSSION

Existing data on the gastrointestinal absorption of iron in hypoplastic anemias are conflicting, both increased and decreased absorption having been described.^{3,12} The splenectomized CF No. 1 mouse treated with radiostrontium develops a moderate anemia because of bone marrow hypoplasia.¹¹ While erythropoiesis is not entirely eradicated in this preparation, it is greatly suppressed, and the ability to increase red blood cell production in response to hemorrhage or hemolysis is much impaired. Previous studies have shown an inability to respond with an increase in red cell production to erythropoietin administration and atmospheric hypoxia in this preparation.⁵ When the degree of anemia present in the splenectomized Sr⁸⁹-treated animal is increased by hemorrhage, phenylhydrazine, or by treatment with a hemolysin of rabbit origin, iron absorption shows a sharp increase although there is no measurable increase in red cell production and no alteration in body iron content.* Thus, it would appear that anemia *per se* has a marked influence on the quantity of iron absorbed from the gastrointestinal tract in animals with erythroid hypoplasia. It might effect this through a final common pathway (shared with atmospheric hypoxia), by producing a reduction in oxygen tension at some site, such as the bowel mucosa or the liver. Iron overload has been documented in some patients with hepatic cirrhosis.¹³ It is conceivable that portal hypertension results in bowel hypoxia which is responsible for augmentation of dietary iron absorption. It is equally plausible to suggest that the liver, as one of the principal sites of iron storage and as the organ through which iron must initially pass after absorption, may play a major role in the regulation of absorption and that it is here that a hypoxic stimulus might act.

In contrast to the marked inhibition of iron absorption which occurs in mice with erythropoiesis suppressed by hypertransfusion, reduction in erythropoiesis due to splenectomy and radiostrontium administration is not accompanied by consistent changes in iron absorption. It is likely that hypertransfusion has three effects which act in an additive fashion to depress iron absorption. This procedure suppresses red cell production, raises the hemoglobin level and increases body iron content. We would suggest that radiostrontium has two antagonistic effects on absorption. The hemoglobin reduction which develops following radiostrontium administration (which of itself would increase absorption) counteracts the effect of the erythropoietic suppression (which of itself would decrease absorption). The net result is the absorption of quantities of iron which do not vary significantly from normal.

In human subjects with refractory anemias associated with erythroid hyperplasia, absorption is increased as compared with subjects with comparable degrees of anemia, but having erythroid hypoplasia.¹² Similarly, absorption is much increased in those hemolytic anemias associated with ineffective erythropoiesis.¹⁴ These observations are in accord with the view that the effects of the rate of erythropoiesis† (albeit ineffective erythropoiesis) and anemia on the absorptive process are to some extent additive. It is also of interest that, with few exceptions,¹⁵⁻¹⁷ secondary hemochromatosis occurs chiefly in those anemias associated with hypercellular bone

* Where radioiron was given intravenously and total-body radioactivity determined daily, no increased loss of radioactivity above control values was detected in mice treated with phenylhydrazine or hemolysin.

† Studies to assess whether the effects of the rate of erythropoiesis on absorption are mediated through the plasma iron-transferrin system are now in progress.

marrows and presumably high degrees of ineffective erythropoiesis.^{18,19} The iron load in these instances is frequently greater than can be accounted for by administered blood,^{19,20} indicating that iron absorption from the gastrointestinal tract is increased.

Our own studies indicate that iron loading can counteract the augmenting effect of hypoxia and absorption.²¹ Thus, the quantity of iron absorbed in subjects with hypoplastic anemia may be the resultant of the individual effects of anemia, the rate of erythropoiesis, and the body iron load acting on the absorptive process.

These experiments confirm the results of others on the enhancing effects of phenylhydrazine-induced hemolytic anemia on iron absorption²² and demonstrate that hemolysis produced by an immune mechanism also acts to augment iron absorption. They further show that hemolysis results in augmented iron absorption in the absence of any acceleration of erythropoiesis, and that the degree of enhancement is roughly proportional to the degree of anemia produced, in those instances where the rate of erythropoiesis is maintained at a constant level. The enhancing effect of phenylhydrazine on the gastrointestinal absorption of iron seems greater than that of hemolysin. The hematocrit of the phenylhydrazine-treated groups may not be an accurate reflection of the oxygen carrying capacity of the blood, since phenylhydrazine administration results in the formation of significant quantities of methemoglobin. The possibility that phenylhydrazine has an effect on the absorptive process that is independent of its lowering of the oxygen carrying capacity of the blood, is not excluded.

These studies also demonstrate that the increased iron absorption produced by hemorrhage occurs independently of any increase in red cell production. A time lag of several days is required before the enhancing effects of hemorrhage on absorption is seen,* even in the experimental situation where the normal bone marrow response to hemorrhage is prevented.²¹ It is possible that this delay is related to the renewal time of intestinal epithelial cells which has been estimated at 2 days in the mouse.²³ That the augmentation in the bled animal is not produced as a result of the iron lost by bleeding is indicated by the observation that this occurs even when the lost blood is returned to the animal parenterally as a hemolysate.

The absorption of greater than normal amounts of iron under conditions of chronic iron deficiency is well documented. Ferrokinetic studies in iron deficiency anemia have demonstrated a rapid and almost quantitative incorporation of intravenously administered iron into circulating red blood cells.^{24,25} The high percentage of intravenously-administered radioiron incorporated into circulating red blood cells observed in the iron deficient mice in these experiments is consistent with these data. It has also been suggested on the basis of ferrokinetic data that there is *in situ* destruction of maturing red blood cells in the bone marrow.²⁵ These studies show that iron absorption remains elevated in the animal with iron deficiency anemia when there is erythropoietic suppression, and suggest that mechanisms other than that associated with accelerated erythropoiesis, remain operative.

In previously reported studies it was demonstrated that the effect of atmospheric hypoxia in augmenting iron absorption was independent of the rate of red cell production by using splenec-

* A similar interpretation might explain the unaccounted for inconsistency in Table 2 where normal mice treated with hemolysin showed a mean absorption level identical to the splenectomized-Sr⁸⁹ treated mice with comparative hematocrit levels, although erythropoiesis is accelerated in the normal group: a longer interval between the injection of hemolysin and the test dose of iron might have resulted in differences in absorption.

tomy followed by strontium-89 to render animals incapable of an erythropoietic response to hypoxia.⁵ This observation has been confirmed by using iron deficiency to limit the rate of erythropoiesis and by exposing iron deficient animals to a hypoxic stimulus. Again, augmentation of iron absorption occurs without a measurable increase in red cell production. This observation is also in accord with the suggestion that the effects of changes in body iron content, hypoxia and rate of erythropoiesis on the absorptive process are to some extent additive. Whether these factors have a cumulative effect on a single rate limiting step in the absorptive process, such as the availability of an intramucosal carrier molecule, or act at different points on the absorptive mechanism, remains unknown.

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AN UNUSUAL CASE OF RADIUM TOXICITY*

By

H. F. Lucas,[†] R. E. Rowland,[‡] C. E. Miller,[†] R. B. Holtzman,[†]
R. J. Hasterlik, and A. J. Finkel[§]

An unusual case of radium toxicity was discovered in the course of a study of radium levels in bone in known cases of osteogenic sarcoma in man. Bone from individuals with such sarcomas were obtained from the collection at the Mayo Clinic, Rochester, Minnesota, through the courtesy and cooperation of Dr. D. C. Dahlin. Normal total body radium levels, as a result of ingestion of food and water, are in the range of 10^{-5} to 10^{-3} $\mu\text{c Ra}^{226}$. The first estimate for this case yielded a total skeletal content of 0.14 μc , a value well above the normal environmental range, and presented an extremely interesting problem. The results of further investigations are described here as a separate study.

At the time of the initial radiochemical analysis of the bone sample that had been acquired after a mid-femoral amputation of the leg, the patient was still alive although in very poor health, and we were able to make total body gamma-ray and radon breath measurements, and to collect a series of fecal samples. After her death which occurred a short time later, a piece of bone from the opposite femur was made available for study.

CASE HISTORY

The patient, a 54-year old married white woman, was studied by our group in 1961 during the last five months of her life. She was born in South Dakota and at the age of 4 moved to western Oregon where she spent the remainder of her life. She had never been pregnant. Previous medical history included appendectomy at 18 years, unilateral ovariectomy at 23, complete hysterectomy at 35, and excision of a benign tumor of the posterior neck at 48.

Painful discomfort in the left knee developed during the summer of 1958 (age 51). Radiography disclosed a destructive lesion on the posterior aspect of the lower femur immediately superior to the medial epicondyle. Surgical excision of the lesion and replacement of the defect by bone chips was performed in November, 1958. Because of recurrent pain, the affected area was biopsied and the lesion was again thought to be non-malignant. Pain increased, the lesion was explored a third time in June, 1959, and the graft was removed by curettement. Within a month, radiography showed massive proliferation of aberrant bone at the site of the original lesion which was now thought to be an osteosarcoma. Amputation through the upper end of the femur was performed in October 1959 at the Mayo Clinic. The pathologic report, kindly furnished by Dr. Dahlin, gave a diagnosis of "grade 3 osteoblastic osteogenic sarcoma." By October 1960,

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[†] Radiological Physics Division, Argonne National Laboratory, Argonne, Ill.

[‡] Present address: University of Rochester, Rochester, N.Y.

[§] Health Division, Argonne National Laboratory, Argonne, Ill.

pulmonary metastases were present, and the patient died on October 28, 1961.

We interviewed the patient and measured her for whole-body radium content on June 7, 1961 in the home of her physician. She had no occupational history of exposure to radium, having worked as a waitress, as a store clerk, and in an office. She could not recall ever taking radium by mouth or receiving it by injection.

X-ray films for the period 1959 to 1961 were made available to us. Apart from the tumor, films of the left knee and leg taken in June 1959 revealed definite coarsening of trabeculation of the tibia and fibula, multiple areas of bone resorption measuring up to 2 cm in length in the shaft of the tibia, and a 1 cm area of bone resorption in the distal femur 5 cm proximal to the tumor. Areas of bone resorption and marked thickening of the calvarium were seen in the lateral films of the skull taken in June 1961. Multiple areas of bone resorption measuring up to 1 cm in diameter were also noted at this time in the medial epicondyle of the left humerus. In addition, there was marked thickening of the cortices of the radius and ulna bilaterally. These radiographic findings are typical of those seen following long term deposition of radium in the skeleton at levels in the whole body above $0.4 \mu\text{c}$.^{1,2} The observation that the old distal epiphyseal line of the left radius was accentuated and sclerotic led to the impression that the patient might have received radium at about 18 years of age.

Shortly before the patient died she recalled that she had consulted a dermatologist in a large city in the Pacific Northwest in 1922 or 1923 (age 15 or 16) for the treatment of psoriasis. She received a series of weekly injections and had a prescription for a pink liquid that was taken by mouth. We made an attempt to trace the records of the dermatologist, who had died in 1948, but were not successful; nor were we able to determine if radium had been prescribed for the patient by a search of the records of the pharmacy where the prescription had been filled. Consequently, we have no direct evidence bearing on the source of the radium that was measured in vivo and in various specimens of bone that were made available to us.

WHOLE-BODY GAMMA RAY MEASUREMENTS

The patient was measured for radium content in the home of her physician by means of portable equipment which consisted of a 3 inch diameter x 5 inch long NaI(Tl) crystal, a 256-channel transistorized analyzer,* and a Hewlett-Packard printer.[†]

Three different whole body measuring techniques were used. Physical descriptions of these techniques, and their probable accuracies, have been published elsewhere.³ The first arrangement was an adaptation of the tilting chair technique used in the whole body counter at Argonne National Laboratory. The patient was seated in an ordinary lawn chair with plastic webbing, and the 3" x 5" crystal was located 42 cm above the seat of the chair and 42 cm in front of the back of the chair. The axis of the crystal was tilted 45 degrees from the vertical and pointed at the patient's pelvis (Figure 1, crystal position 1). Since the counting rate obtained with this technique in an unshielded room was very little above background, two additional measuring techniques were used in an effort to improve counting statistics.

In the second technique, the patient was seated in the same lawn chair but the crystal was

* Technical Measurements Corporation, Model CN-110.

† Ibid., Model HP-561B.

placed behind the chair at the level of the lumbar spine. The crystal was positioned with its axis parallel to the patient's vertebral column 10 cm from the back of the chair and with the top of the crystal 60 cm from the floor (Figure 1, crystal position 2).

Figure 1. Geometrical arrangement of crystals and patient for gamma-ray spectroscopy.

The third technique is a modification of a four crystal array in that four separate measurements were made with the same crystal. The crystal with its axis vertical and with its face 21 cm above the surface of the bed was placed at each of four locations above the mid-line of the supine patient. These four positions were centered over the nose, the sternum, the pelvis, and the patella.

It was necessary to measure normal subjects in the same position as the patient, since her body shielded the crystal from the naturally present gamma-ray background. While these persons were uncontaminated with radium, they contained Cs^{137} and K^{40} , and this procedure corrected for the gamma ray contributions from these two elements. Inasmuch as these elements may contribute as many gamma ray counts as $0.01 \mu\text{C RaB}$ and RaC , this correction was considered to be of importance.

The calibration factors used to calculate the amount of radium by the chair techniques were determined by counting two other patients whose body content of radium had been accurately measured in the whole body counters at Argonne National Laboratory and at Massachusetts Institute of Technology. The portable equipment was set up in each of these patient's homes where the patient and an uncontaminated person were measured. The calibration factor in counts-per-minute per $\mu\text{C RaB}$ and RaC was found by dividing the counting rate of the contaminated patient (after subtracting that of the uncontaminated subject) by the amount of radium known to be present from previous measurements. For the third technique, similar calibration factors were derived for each of the four crystal positions by measuring two supine subjects within the low-background, shielded room at Argonne National Laboratory. One of these patients had also been measured in the two previously mentioned whole body counters. The quantity of RaB and RaC expressed in μC calculated by each crystal position technique is given in Table 1.

The values given in Table 1 for the three techniques agree reasonably well. The first and

third yield values within 11 per cent of their average of 0.152 μc of RaB and RaC. The second technique yielded a value of 0.115 μc or 25 per cent less than the average of the other two methods.

Table 1
RaB AND RaC CONTENT MEASURED
BY γ -RAY SPECTROMETRY

Technique 1		0.169 μc
Technique 2		0.115
Technique 3		
Over nose	0.105	
Over sternum	0.161	
Over pelvis	0.152	
Over knee	0.127	
Mean		0.136
Mean of Three Techniques		0.140 μc

On the basis of these whole body measurements we estimate that this patient contained about 0.15 μc of RaB and RaC in her body. If on the basis of previous studies we assume that 69 per cent of the radon formed in her body was lost by exhalation, then the body content of Ra²²⁶ was 0.49 μc . Correction for the absent leg as a result of mid-thigh amputation would bring this value to 0.60 μc Ra²²⁶ for the entire body. This calculation is based on an estimated loss of 18 per cent of the mass of the skeleton as a result of the surgery.⁴

Previous experience has shown that the first and third techniques have inherent uncertainties of about 10 per cent due to variability in radium deposition in the human skeleton,³ while the second method has an error at times as large as twofold, and is therefore less reliable.

RADIOCHEMICAL ANALYSIS

The first sample of bone was a complete cross-section of the mid-femur taken from an apparently normal area of bone adjacent to the tumor. This sample (410B-1) had been stored in formalin for about one year and was still wet on receipt. Negligible leaching of radium by the formalin had occurred. The specimen was dried at 110° C, split into three longitudinal sections, and the marrow washed from the bone with ether and a small brush. One of these pieces (approximately a one-third cross-section) was ashed at 600° C in a platinum crucible, dissolved in 14.7 ml concentrate HNO₃, and diluted with water to 40 ml. The thorium was extracted into dibutyl phosphate by the technique of Stehney, et al.^{5,6} and the Ra²²⁶ was determined by the emanation technique and counting system of Lucas.^{7,8} RaD was determined on a section of wet-ashed bone by plating the RaD daughter, Po²¹⁰, onto silver and counting the deposited alpha activity.^{9,10} The second sample (410B-2) obtained from the contralateral femur after death was analyzed in a manner identical to the first.

The data from these analyses are summarized in Table 2. The Th²²⁸ content of 0.002 pc/g ash is nearly identical to the 0.003 pc/g ash found in bone from persons whose only source of Ra²²⁶ and Ra²²⁸ is in food.¹¹ This very low content of Th²²⁸ is further evidence that the very

large concentration of Ra^{226} in the patient could not have been obtained from natural food and water sources.

The concentration of Ra^{226} in the second sample obtained after death is nearly five times that of the 8.3 g piece of the first sample. This difference between homologous bones is attributable to the nonuniform distribution of the radium in the bone as shown by autoradiography (*vide infra*).

Table 2
 Ra^{226} AND Th^{228} RAD CONTENT OF BONE

Sample number	Date taken	Sample size (g ash)	RaD pc/g ash $\pm$ E*	Th^{228} pc/g ash $\pm$ E*	Ra^{226} pc/g ash $\pm$ E*	Ra^{226} Extrapolated whole body† (μg)
410B-1	10/5/59	8.32		0.002 ± 0.001	53.1 ± 0.5	0.14
	"	0.204	42.1 ± 1.1	-	149 ± 2	0.39
	"	0.254	35.0 ± 0.7	-	122 ± 2	0.32
	"	0.197	26.4 ± 0.5	-	88 ± 2	0.23
410B-2	10/28/61	1.72	-	-	267 ± 5	0.69
						Mean 0.4

* 0.9 statistical counting error.

† Assuming 2600 g ash per skeleton.

The RaD was found to vary by a factor of about two for the three small portions of bone. The ratio of RaD to Ra^{226} was, however, remarkably constant at 0.28 with a standard deviation of ± 0.01 . This value, when considered along with previously published measurements of RaD in persons with a high radium content,¹² is consistent with the estimate that the radium had been acquired 35 to 40 years previous to measurement and that the average radon retention was about 40 per cent. The latter figure is in fair agreement with other earlier estimates of whole body radon retention of 31 per cent found after 30 to 35 years.¹³

Fecal samples were obtained for a five-day period in August 1961, approximately two month before death. The Ra^{226} content of these samples is summarized in Table 3. Each sample represents a single bowel movement except No. 100F1 which represents two. The average daily Ra^{226} excretion (35.4 pc/day) divided by the observed body burden of about $0.5 \mu\text{c}$, gives a coefficient of daily elimination of 7×10^{-5} . This is almost identical to the average of ten cases for which Norris found a value of 8×10^{-5} .¹⁴ The dry weight and calcium content of these fecal samples, although somewhat low, are within normal limits.

The RaD present in these fecal samples averaged about 1 pc/day and is within the normal limits of 1 to 2 pc/day obtained from food and water.⁹ The contribution of a whole body RaD content of $0.2 \mu\text{c}$ would not be detectable in the feces unless the RaD content of the food intake were very accurately known.

In order to measure the amount of radon being exhaled from the patient's body, a single 10-liter breath sample was collected from both the patient and from a control subject. A net radon concentration of 1.74 pc/l was found after correcting for a room air blank of 0.09 pc/l. This

Table 3
 Ra^{226} IN FECAL SAMPLES CASE NO. 03-216

Sample number	Date of collection	Weight dry	ash (g)	Calcium (g)	Ra^{226} pc $\pm$ E*	RaD pc $\pm$ E*
100F1	8/15/61	23.1	1.60	0.104	52.1 $\pm$ 1.1	0.96 $\pm$ 0.04
100F2	8/16/61	15.7	1.17	0.113	36.6 $\pm$ 0.9	1.11 $\pm$ 0.04
100F3	8/17/61	11.4	1.11	0.124	33.3 $\pm$ 0.7	1.17 $\pm$ 0.05
100F4	8/18/61	10.0	0.77	0.074	26.2 $\pm$ 0.5	0.76 $\pm$ 0.04
100F5	8/19/61	19.7	1.02	-	28.8 $\pm$ 0.4	-
100F6	Blank	8.7	0.00	-	0.01 $\pm$ 0.01	-
Average		14.4	1.13	0.104	35.4	1.00 $\pm$ 0.18

* 0.9 Statistical counting error.

radon content normally would indicate a total body radium content of about $0.17 \mu\text{c}$ of Ra^{226} .¹⁵ This breath sample therefore indicated a body content one-third of that found by gamma ray measurements. Breath samples obtained from other patients have demonstrated that the radon concentration in any single sample of this type may vary from the average by a factor of 2.

AUTORADIOGRAPHIC AND MICRORADIOGRAPHIC STUDIES

The microradiographs of material cut from a one-third segment of a cross-section of the femur verified that this was in fact bone from a patient suffering from radium poisoning.¹⁶ Many plugged haversian canals, hypercalcified areas, and typical "moth-eaten" areas that characterize radium-containing bone microradiographically were present. However, these microradiographic signs were not uniformly distributed throughout the bone sections, some of the areas showing very few such signs, others many.

The radium content of the diffusely labelled areas was determined from autoradiographic alpha track plates exposed for 28 days. From such measurements a good estimate of total skeletal radium content can be made, since the diffuse level in other radium cases averages about 50 per cent of the hypothetically uniform label, i.e., the total skeletal radium content divided by the total skeletal mass.¹⁷ These first measurements of diffuse and hot-spot radium concentration are listed in Table 4.

From these measurements it was calculated that this diffuse distribution was characteristic of a total body radium content of about $2 \mu\text{c}$. In addition, it has been possible to estimate from the observed data the route of acquisition of the radium, the total quantity acquired, and the length of time over which it was acquired. It would appear that this patient received Ra^{226} , probably via the intravenous route, for a period of three months or less, and that she was given between 300 and $400 \mu\text{c}$ Ra^{226} .

These conclusions were based on the following observations: the "hot spot" to diffuse ratio of 180:1 is suggestive of a relatively short acquisition time. Thus the medical route is more likely than occupational exposure since no former radium dial painter has yet been studied who had a ratio this high. Two Elgin State Hospital patients, with acquisition times of 7 and 18 weeks, had ratios of 263:1 and 218:1, respectively.¹⁸ Subsequent examination of high resolution strip

films has given a more accurate estimate of the short acquisition period, for they show the alpha tracks to be concentrated in bands 50 to 80 microns wide. At a linear growth rate of 1 micron/day,¹⁹ the exposure would have been limited to less than 3 months.

Table 4

DIFFUSE AND HOT SPOT MEASUREMENTS OF BONE SAMPLE 410B-1

Section	Diffuse level pc Ra ²²⁶ /mg bone	Hot spot level pc Ra ²²⁶ /mg bone	Predicted body content μc^*
1-3	0.11	-	1.3
2-3	0.20	-	2.4
2-9	0.20	-	2.4
2-6	0.21	36.4	2.4
Hot spot to diffuse ratio = 180			

*Based on a 60 kg body mass, 6 kg bone, diffuse 50% of uniform label.

The maximum "hot spot" concentrations in the Elgin State Hospital patients studied average 14 pc/mg bone in persons who had received 10 μc Ra²²⁶ per week intravenously.¹⁸ The level of 36 pc/mg observed here is about three times as great, so that about 30 μc Ra²²⁶ per week intravenously would be required to have produced "hot spots" at this level. 30 μc per week for 12 weeks yields a total input of 360 μc . Had this isotope been given orally, a much greater quantity would have been required to produce the observed concentrations as a consequence of the discrimination factors controlling absorption of radium from the gastrointestinal tract.

The power law has been proposed as a valid description of radium retention.²⁰ On the assumption that the radium was acquired about 30 years ago, a total input of 360 μc would have left 1.6 μc terminally, according to a power function of $R = 0.54 t^{-0.52}$. This value is in good agreement with the level of 2 μc suggested by autoradiography.

A gross autoradiograph (Eastman Kodak Autoradiographic Type A) of a segment of the amputated femur was developed after an exposure of one year and is shown, with a microradiograph of the section, in Figure 2. It is apparent that the diffuse distribution of radium in this bone is not uniform.

Subsequently a complete cross-section from the amputated femur was obtained for autoradiographic study. When these autoradiographs (Figure 3) were examined, it was found that about one-half of the bone section contained a much lower diffuse radium level than the other. Alpha track counts over these two regions indicated that the lower level contained 4 per cent of the concentration found in the higher diffuse level.

It is concluded that considerable bone turnover had occurred during the interval between the acquisition of radium and the amputation. The new replacement bone contains radium at a very low level, determined by the level of the radium in the blood at the time the new bone was formed. The amount of bone turnover is remarkably high as indicated by the quantity of bone that is relatively radium free. Of the 30 or so radium cases studied autoradiographically in this laboratory,²¹ only one, No. 01-008, approached this level of bone turnover. The other cases appear to have bone turnover that was an order of magnitude lower for equivalent exposure intervals.

a

b

Figure 2. a) A microradiograph and b) an autoradiograph of 100 micron thick cross section from the amputated femur of case 03-216. The autoradiograph was exposed for one year on Type A autoradiographic plate.

It should be recalled that the chemical determination of the radium content of a complete cross-section of this bone (Sample 410B-2) gave a result 5 times as large as the first determination done on a one-third segment of a femoral cross-section (Table 2). From Figure 3 it is easy to visualize how a one-third segment could have missed the higher concentrations present in the complete cross-section and emphasizes the necessity of working with complete cross-sections of bone from as many skeletal sites as possible.

a

b

Figure 3. a) A microradiograph and b) an autoradiograph of a 150 micron thick section from the amputated femur of case 03-216. This one year autoradiograph shows considerable evidence of remodelling in several locations.

DISCUSSION

This case was pursued with tenacity because of the important implications of the available information. At the beginning of the investigation, the body content was estimated to be $0.14 \mu\text{c}$ by extrapolation from a radiochemical analysis of a small piece of bone. This value is much lower than that found in any other radium patient with a malignant bone tumor. Unfortunately, the patient and her physicians were unaware that she carried a large amount of radium and thus were not able to provide any information concerning its acquisition. There are still many hundreds of people in the United States who carry a significant amount of radium in their body. Consequently, the techniques described herein to establish the date, route, and amount of radium exposure will have application in the future.

The radium in the patient, exclusive of the amputated leg, was determined by whole body gamma-ray spectroscopy to be $0.5 \mu\text{c}$. Estimates have also been obtained by a variety of other methods. By extrapolating to the whole skeleton, from radiochemical measurements of the radium contained in small pieces of bone, estimates ranging from 0.14 to $0.69 \mu\text{c}$ are obtained. While comparable measurements have not been made for a single whole bone, a similar range of values has been found by Norris *et al.* on small samples from many different bones from the same individual.¹⁴ Only one radon breath sample was obtained from our patient and it is not possible to evaluate the accuracy of this determination. Estimates from autoradiographs were very much higher and ranged from 1.3 to $2.4 \mu\text{c}$. As will be discussed later, these high estimates are related to abnormally large fractions of the skeleton which have been removed and replaced with bone having very low levels of radium.

By elimination of various possible sources of exposure such as natural acquisition and industrial exposure, it was shown that the route must have been by intravenous injection. Study of the autoradiographs suggests that the radium was given in a period of about 3 months or less and that a total dose of about $360 \mu\text{c}$ Ra^{226} was administered. The diffuse radium levels that were observed in areas of bone not remodelled since the time of acquisition are characteristic of a terminal body content of $2 \mu\text{c}$ radium. This estimate is in agreement with what would be predicted by comparison with radium retentions observed in the Elgin series.¹³ These inferences were made before we received the information that the patient did indeed have a series of weekly injections of an unknown medication in 1922-23. Although we surmise that these injections may have been radium, this supposition is not supported by direct evidence.

The date of intake was estimated from several observations. The marked thickening of the cortices of the radius and ulna, and the accentuated epiphyseal line of the left radius are both characteristic of radiation damage during the period of rapid skeletal growth. The ratio of fecal radium to measured body radium content ($0.5 \mu\text{c}$) is essentially the same as that found in other patients who received radium 20-40 years earlier.^{14,22} On the assumption that RaD was entirely a daughter product of the Ra^{226} deposited in the bone, the $\text{RaD}/\text{Ra}^{226}$ ratio in the bone was also consistent with a 40 year residence time of radium in this patient.

If the estimate of terminal body content from the autoradiographs is high by a factor of about 3 ($2/0.6$) then the initial injected dose may have been $120 \mu\text{c}$ rather than $360 \mu\text{c}$. This amount could readily have been acquired by weekly injections of $10 \mu\text{c}$ (the usual treatment dose) over a three-month period and is a value that is reasonable since $10 \mu\text{c}$ ampules were then commercially available and are known to have been administered for a variety of medical conditions.²

On the other hand, another explanation of the apparent discrepancy may be obtained from the

study of the autoradiographs (Figure 2b and 3b). The diffusely labelled areas of bone are not as uniform as is found in the usual radium case. The radium contained in large areas of bone that appear to have little or no radium has been determined by alpha-track counting, and is 4 per cent of the diffuse and 0.02 per cent of the "hot spot" concentrations. Thus, large areas of bone appear to have been resorbed and replaced by essentially radium-free bone many years after radium intake. The remaining original bone has a radium content and distribution characteristic of a 2 μc total body radium. Presumably, sufficient bone throughout the skeleton had been resorbed and replaced to reduce the retained amount of radium to only 0.6 μc .

This high degree of bone remodelling may be related to the early surgical induction of menopause at the age of 35. Finally, the frequency of injection and the assumed early age at which the radium was administered may be significant factors. Thus, the damage in this case may have been more severe per unit of retained radium than in other cases studied. It appears that where-as the actual terminal body content was about 0.6 μc , the degree of radiographically observed damage was comparable to a content of 2 μc in other radium patients.

Although considerable attention is being given to terminal body content of radium, it may very well be that the initial amount of radium in the body is a more important factor in the carcinogenic process than terminal body content at the time the tumor is present.^{23,24} The significance of this case in evaluating the carcinogenicity of radium in man lies in its contribution to the total body of knowledge of this subject, especially in its demonstration of methods for recreating the past history of the exposure. This case, accordingly, has to be considered along with the large series of cases that are currently under investigation by several groups in the United States and abroad. It would be unwise to generalize from the data of this particular case alone. It is presented as an example of the many types of analyses that can be brought to bear on this interesting and important problem.

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RESULTS OF ULTRAFRACTIONATION RADIATION THERAPY IN BREAST CARCINOMA^{*}

By

M. Archambault,[†] M. L. Griem, and D. J. Lochman[‡]

Beginning in 1952, postoperative radiation therapy of carcinoma of the breast was modified at the University of Chicago Clinics to a technique employing the principles of ultrafractionation. Baclesse¹ and Lenz² had reported their experiences with this approach. A preliminary study was presented by one of us^{3,4} in 1955. In 1956, this ultrafractionated approach was adopted at Columbus Hospital, and this group is included in this study to answer the question of applicability of such a technique in general practice.

METHODS AND MATERIALS

Between January 1, 1952, and December 31, 1959, 168 female patients were referred for postoperative radiation therapy, 131 from The University of Chicago Clinics, and 37 from Columbus Hospital. All patients had had a radical mastectomy, none gave evidence of distant metastasis and all were in apparent good health. Included in the results as failures are all patients who did not finish therapy because of medical complications such as hepatitis (8); who died during therapy (5); who refused further treatment (3); who died of intercurrent disease (8); and who were lost to follow-up (2).

The radical mastectomies were performed by various surgeons, including residents in training. No standardized criteria of operability (such as those of Haagensen and Stout^{5,6}) were followed.

STAGING AND SELECTION OF CASES

The staging is based on a mixed classification utilizing both clinical (Portmann) and pathologic data and is as follows:

- Stage I - Localized lesion without skin involvement or fixation; no movable lymph nodes
- Stage II - Movable localized lesion in breast with skin not involved or only slightly edematous or ulcerated over tumor, tumor cells in axillary lymph nodes
- Stage III - Tumor diffusely infiltrating breast with fixation of tumor to chest wall or breast, or edema of breast or secondary tumor nodules; axillary lymph nodes may be fixed; no evidence of distant metastases
- Stage IV - Tumor and skin as above, but supraclavicular lymph nodes involved or distant metastases

As the surgeons referred largely patients with histologic evidence of lymph node invasion, there is a predominance of Stage II cases. Cases were distributed as shown in Table 1. All had

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[†]Department of Radiology, The University of Chicago.

[‡]The Columbus Hospital.

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evidence of supraclavicular metastases. The patients' ages ranged from 20 to 80 years, the majority being between 40 and 50 years, as shown in Table 2.

Table 1
STAGE DISTRIBUTION

	Total	University of Chicago Clinics	Columbus Hospital
Unclassified	4	4	0
Stage I	34	14	20
Stage II	121	107	14
Stage III	7	4	3
Stage IV	2	2	0

Table 2
AGE DISTRIBUTION

Decade	Total	University of Chicago Clinics	Columbus Hospital
20-29	2	1	1
30-39	32	23	9
40-49	53	42	11
50-59	45	39	6
60-69	26	20	6
70+	10	6	4

TREATMENT TECHNIQUE

Treatment was started about 10 to 14 days after surgery and was given to the adjacent lymph nodes, i.e., the internal mammary, axillary, supraclavicular and infraclavicular, and the inferior cervical lymph nodes as well as the chest wall. Five portals were used as shown in Figure 1: (1) a single anterior sternal field including both internal mammary chains and all intercostal spaces; (2) a field, without bolus material, with radiation applied tangentially from the medial and lateral sides of the patient, arranged to encompass the chest wall, extending laterally from the midaxillary line, medially to the adjacent lateral margins of the internal mammary chain, superiorly to the inferior margin of the shoulder portal (described below), and inferiorly to the lower extent of the incision. The treatment set-up for the lateral tangential port is shown in Figure 2; (3) anterior and posterior fields, also without bolus, cross-firing and encompassing the supraclavicular and infraclavicular, axillary and lower cervical areas. The arm was elevated and extended so that the supraclavicular and axillary areas were on the same plane, as shown in Figures 3 and 4.

The planned tumor dose was 6,000 rads throughout the thickness of the chest wall, to the

Figure 1. Photograph showing the portal areas of the sternum, chest wall and anterior supraclavicular region.

Figure 2. Photograph showing the treatment set-up of the lateral tangential chest wall.

Figure 3. Photograph showing the treatment portal of the anterior supraclavicular area.

Figure 4. Photograph showing the treatment portal of the posterior supraclavicular area.

lymphatic tissue of the shoulder area and the base of the neck, and 4,500 rads to the internal mammary lymph nodes. This dose was delivered with daily increments, 5 or 6 per week, in an over-all period of 11 to 14 weeks.

The technical factors at the University of Chicago were 250 kv focus skin distance 50 cm, half value layer 1.5 mm Cu to the chest wall, half value layer 3.0 mm Cu to the other two fields. At Columbus Hospital telecobalt was used at a source skin distance of 80 cm, treating the anterior shoulder field, the posterior field being omitted. Initially, telecobalt was also used for treating the sternal field at a source skin distance of 56 cm, but because of the frequency of troublesome esophageal and tracheal symptoms and the occurrence of 2 cutaneous recurrences, this was discontinued and conventional roentgen therapy was resumed. Prophylactic castration by irradiation or surgery was performed on 117 patients who were either still menstruating or within 5 years of menopause.

COMPLICATIONS

Immediate morbidity with this technique was seen in 17 patients. Ten patients experienced a severe dry epidermitis, 6 a wet epidermitis, and lymphangitis occurred in 1. Another complication noted during the latter part of the treatment course, often 6 to 8 weeks after completion of the therapy, was a mild to severe radiation pneumonitis in 13 patients. This responded readily to antibiotic or expectant therapy. There was no permanent disability. Early in the series, treatment was given to the supraclavicular portals with the arm at the side and with bolus; however, after revision of the field with extension and elevation of the arm and removal of bolus from these areas so that less lung tissue was included in the irradiated block, the incidence and severity of this complication decreased. Routine chest roentgenograms following therapy showed a minimal to a moderate degree of apical fibrosis.

In 4 cases nonmalignant ulcers occurred in the irradiated breast portal. One required surgery and grafting; another healed slowly with conservative management. The remaining 2 cases are of recent onset.

RESULTS

Survival curves derived by the actuarial method of Berkson and Gage⁷ show 70.1 per cent 3 year survivals, 54.3 per cent 5 year survivals and 41.6 per cent 10 year over-all survivals (Figure 5). Little difference was noted between the survival curves of The University of Chicago patients and those at Columbus Hospital; therefore both groups are considered together. At the time of the last follow-up, 70 patients were symptom free, 2 were ill without neoplasm, 10 were ill with neoplasm, and 7 were alive but their condition was unknown.

The second facet of the study was the evaluation of local recurrence. Only 8 patients developed histologically proven recurrences in a treated area, 7 on the chest wall (4.1 per cent), 2 in the supraclavicular fossa (1.2 per cent) and 1 in the axilla (0.6 per cent). These patients were treated by surgery and/or chemotherapy rather than by further radiation therapy. Two of the 8 are still alive with disease.

DISCUSSION

Untreated breast cancer patients have an 18 per cent 5 year survival rate from the onset of symptoms, as reported recently by Bloom *et al.*⁸ confirming Daland⁹ and Greenwood's statistics. About 3.8 per cent of these survive 10 years without treatment. Using these results as a base line, it is a challenge to the clinician to increase survival.

Halstead and Willy Meyer introduced the radical mastectomy in 1886 and reported a 28 per cent 5 year survival. Subsequently, further improvements have been reported with these operative approaches which currently can be divided into 4 primary attacks: (1) radical mastectomy with *en bloc* dissection of breast tissue and axillary lymph nodes; (2) radical mastectomy with dissection extended into the internal mammary and supraclavicular areas; (3) simple mastectomy with radical radiation therapy; and (4) radical mastectomy and postoperative radiation therapy.

The over-all survival rate, as reported by Haagensen and Stout,⁶ using their criteria of operability and radical mastectomy is 47.2 per cent; Urban¹⁰ with selection and an extended radical mastectomy reports 66 per cent; simple mastectomy, as recently reported by Kennedy and

Figure 5. Survival curve with standard deviation of the patients treated by postoperative ultrafractionated radiation therapy.

Miller,¹¹ resulted in an over-all 5 year survival rate of 45 per cent; McWhirter¹² with simple mastectomy and radiation therapy had a 42 per cent 5 year survival for his entire group, and 58 per cent for the operable cases; while DeMoor *et al.*¹³ and others using radical mastectomy and postoperative radiation therapy report a 45.5 per cent survival rate. Our over-all 5 year survival rate is 54.3 per cent (Table 3).

Of note is the control study by Kaae and Johansen¹⁴ of extended radical mastectomy versus the McWhirter method of treatment, which demonstrated no significant difference in over-all survival between Stage I (67 per cent) and Stage II (69 per cent) patients.

A comparison of the incidence of local recurrence after radical mastectomy and after various irradiation or surgical approaches is of interest. With the standard Halstead procedure, the skin flaps may be the site of cutaneous recurrence with an incidence reported as high as 45 per cent in the series of Peterson and Krippaehne;¹⁵ if skin grafts were used, the incidence was reduced to 28 per cent in their hands. Other authors report between 12 per cent and 23 per cent recurrences with various approaches. Kaae and Johansen¹⁴ noted a 10 per cent recurrence on the chest wall and 13 per cent in the axillary region with radical surgery alone. DeMoor *et al.*¹³ report 22 cases of 165, or 13 per cent, and 13 cases of 68, or 19 per cent, with chest wall recurrences when this area is not irradiated. With adjuvant radiation therapy, this was reduced to 5 per cent. Our series shows 4.2 per cent chest wall recurrences and compares favorably with that of DeMoor *et al.*¹³ (Table 4).

Table 3
RESULTS OF TREATMENT IN
OPERABLE PATIENTS

	No. of patients	5 Year survival (per cent)
Kennedy and Miller ¹¹	212	45
Urban ¹⁰	200	66
McWhirter ¹²	1,063	58
DeMoor <u>et al.</u> ¹³	313	45.5
Kaae and Johansen ¹⁴		
McWhirter Method	187	67
Extended Method	159	69
Present Series	168	54.3

Table 4
INCIDENCE OF CHEST WALL AND REGIONAL LYMPH NODE RECURRENCE

		Chest wall (per cent)		Regional lymph nodes (per cent)	
Kaae and Johansen ¹⁴	McWhirter Method	7		8	
	Extended Method	10		13	
DeMoor <u>et al.</u> ¹³	Surgery alone (165 patients)	(22)	13	(7)	4
	Peripheral field and chest wall irradiation	(11)	5	(6)	3
	Peripheral field irradiation only (68 patients)	(13)	19	(3)	4
Present series	(168 patients)	(7)	4.2	(3)	1.7

CONCLUSION

Ultrafractionated radiation therapy to the postoperative breast carcinoma patient adequately controls the disease in the operative site as well as in the regional lymph nodes, but does not modify the problem of dissemination of distant metastases. The immediate morbidity is less with this type of treatment than with short intensive courses of irradiation. On the basis of these findings, we feel that postoperative radiation therapy is a worthwhile procedure.

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CLINICAL SIGNIFICANCE OF PERCUTANEOUS ABSORPTION*

By

S. Rothman

In the following deliberations the term "percutaneous absorption" denotes the passage of substances from the outside through the entire thickness of the skin and into the circulation. This must be emphasized because many substances applied to the human skin surface are taken up by the loose outer half of the dead horny layer only. In this layer, called the stratum corneum disjunctum (Figure 1), there are large spaces between the horny lamellae about to be shed, which

Figure 1. Cross-section of human epidermis showing stratum disjunctum and stratum compactum of the horny layer on top. (Reprinted from Rothman, S., Physiology and pathology of keratinization, J. Cosmet. Chemists 7:576, 1956.)

can be easily entered by any large molecular aggregate. There, however, the substance may remain, as in fact a great number of substances do. Such superficial penetration has no systemic consequences, although the surface may be modified (stained, hardened, softened, etc.). The term "entering the skin" is certainly not synonymous with absorption.

The barrier at which many substances are halted is situated at the base of the lower, compact part of the horny layer (Figure 2). The presence of a relatively impermeable thin membrane

*This report has been taken from an article that appears in Triangle 6, No. 5, 1964.

or "barrier membrane" was first postulated in the twenties on the basis of classical electrophysiological experimentation by H. Rein and Ph. Keller. In recent years this barrier layer was isolated by A. Szakall who for this purpose used the method of stripping with adhesive tape. By this method one successively removes more and more rows of dry horny cells throughout the stratum corneum disjunctum and compactum until the surface appears shiny, glistening and slightly moist. The last strip represents the barrier membrane. After its isolation its physical and chemical properties were studied in great detail.

Figure 2. Diagrammatic scheme of cross-section of human skin indicating the site of the superficial barrier. (Reprinted from Malkinson, F. D., Percutaneous absorption of toxic substances in industry, AMA Arch. Industr. Health 21:87, 1960.)

The barrier layer hinders the entrance of particulate elements from the outside and presents a formidable, although not absolute barrier against the penetration of water in liquid form, of electrolytes and of other polar compounds. However, the epidermis is relatively easily permeable to substances with good solubility in lipid solvents and at the same time with a fairly good solubility in water. Thus, penetration through the epidermis has an optimal ether/water partition coefficient.

The relatively good protection against penetration of substances from the outside is rather surprising because the barrier layer as well as the upper layers of the skin in general are interrupted by canals (the hair follicles and the ducts of sweat glands) with surface openings wide enough to permit in and out movement of coarse particles. It seems that sweat gland ducts do not play any role in percutaneous absorption. The role of hair follicles can be briefly characterized as being the preferred route of penetration if the material is applied with sufficient pressure to produce either displacement of air to the surface, or to enforce absorption of air by the follicular epithelium. Under pressure, the material may enter the free space between the upper part of the hair shaft and the follicular wall, and from there penetrate the duct of the sebaceous gland and

the gland cells. These glands are easily permeable, and, thus, material—circumventing the epidermal barrier—may penetrate from here into the dermal connective tissue and from there into the blood vessels (Figure 3).

Figure 3. Routes of percutaneous absorption. (Reprinted from Malkinson, F. D., Percutaneous absorption of toxic substances in industry, AMA Arch. Industr. Health 21:87, 1960.)

Consequently, we must distinguish two routes of percutaneous absorption: the transepidermal and the transfollicular routes (Figure 3). There is a good deal of evidence that—quantitatively—the hair follicles are not of overwhelming significance as a break in the defense against penetration; for example, after the removal of the epidermal barrier there is a large increase in the penetration of any substance, irrespective of whether its original preferred route was transepidermal or transfollicular.

Percutaneous absorption in man has clinical significance in many respects. First of all, poisonous substances may be absorbed through the skin in sufficient amounts to cause systemic disease and eventually death. Among the compounds used in industry and agriculture the largest group carrying the risk of percutaneous absorption, with resulting harmful effects, are the insecticides. Most dangerous are those agents which act by inhibition of the enzyme cholinesterase, the latter being as vitally important for man and other mammals as for insects. Another notorious group of insecticides is represented by chlorinated hydrocarbon compounds. Among these DDT is relatively harmless if it settles on the human skin in dry powder form; but massive percutaneous absorption may occur after its contact with the skin in oily bases or in organic solvents. Other chlorinated hydrocarbon insecticides, such as lindane and chlordane are even more dangerous than DDT. Percutaneous absorption of insecticides containing a nicotine base have also caused much serious trouble in agriculture.

Among "war gases" again, those with anti-cholinesterase effect ("nerve gases") enter the

skin with great ease and cause systemic poisoning, while mustard gas and lewisite react with the skin tissue proper so violently that they are inactivated before reaching the general circulation.

Most substances in gaseous form penetrate the skin with great ease. Cases of severe and fatal poisoning due to percutaneous absorption of hydrogen cyanide gas have been reported as industrial accidents when the workers were protected against inhalation of gases by appropriate masks, but not against contact with the skin.

Concerning carcinogenic agents it is interesting to note that the hundreds of polycyclic aromatic hydrocarbon carcinogens occurring in tars and tarry products when in contact with the skin cause predominantly cutaneous cancers although most of these compounds are easily absorbable through the skin. In contrast, the aromatic amine and azo-compounds as well as benzidine and its derivatives when in contact with the skin are absorbed without any ill effect on the skin but produce cancers of the urinary bladder. It has been conclusively demonstrated that skin contamination, principally from work clothing is the main factor in producing these bladder cancers, and that better hygiene in this respect has greatly reduced the incidence of such unfortunate industrial accidents.

In addition to systemic poisoning, percutaneous absorption can also cause local irritative reactions, i.e. eczematous contact dermatitis on a primary toxic or on an allergic basis, and burn reactions as is the case with mustard gas and lewisite, already mentioned. Such reactions occur only if the substance can pass the subcorneal barrier and react with the living cells in the epidermis.

Many thousands of substances occurring in plants and in animal hairs and skins, as well as substances used in industry and in every day life, ingredients of utensils of all kinds, garments, drugs, cosmetics, etc. etc. will, after penetrating repeatedly into the epidermis, sensitize the whole skin surface and cause allergic eczematous dermatitis.

There is no need for massive penetration to cause sensitization. Heavy metals and metal salts, such as nickel and chromates, penetrate only in traces; still, they are vicious sensitizers as, for instance, many women who have sensitized themselves with garter-fasteners made of nickel will testify. Interestingly, drugs intended to be used internally are often strong sensitizers of the skin. Workers in penicillin manufacturing plants, and nurses preparing streptomycin solutions have suffered eczematous dermatitis on their fingers from these contacts.

On the other hand, many substances penetrate the skin with relative ease and enter the circulation without eliciting skin reactions, just as the aromatic amines mentioned before. Therefore, time and again it has been pointed out, and it has been "discovered" (and re-discovered) that drugs may be administered percutaneously for the purpose of achieving systemic effects. Some authors have gone even so far as to recommend percutaneous nutrition.

It is perfectly true that many drugs, for instance fat-soluble vitamins and steroid hormones, easily penetrate through intact skin and could thus reach therapeutic (and unfortunately even higher) blood levels in this way. However, as long as systemic effects are desired, the percutaneous route of administration has no advantage whatsoever over the oral or parenteral routes, and even has quite a few disadvantages. Among these are the individually extremely variable rate of absorption and the unavoidable uncertainty of the total dose administered.

The situation is entirely different if local effects in the skin are desired. Even substances which penetrate through the skin without difficulty, and therefore are likely to have systemic ef-

fects, may react primarily with the skin tissue at the site of application and only the excess amount will then reach the circulating blood. Many years ago M. E. Davis, M. W. Boynton, J. H. Ferguson and myself experimented on guinea pigs with local applications of diethylstilbestrol in sesame oil to one nipple and areola, while the nipple and the areola of the other side were used as a control and received topical application of sesame oil only. In one experiment we obtained unilateral enlargement of the nipple and unilateral hyperpigmentation of the areola on the experimental side and no effect on the control side (Figure 4). Applying locally a slightly higher dose of stilbestrol we obtained bilateral effects because of its systemic absorption and action from within.

Figure 4. Unilateral enlargement of nipple and hyperpigmentation of areola in area A after local application of diethylstilbestrol in the guinea pig. No change in the control area B. (Reprinted from Davis, et al., see references.)

It was often thought that in order to achieve local effects by percutaneous absorption one could obtain more rapid and more intense action if the material was introduced in aqueous solution by galvanic current ("iontophoresis," "electrophoresis"). While it is true that penetration of aqueous (polar and non-polar) solutions is greatly accelerated by the galvanic current, most of this absorption is not via the epidermis but through the follicular canals; in this way the material reaches the blood vessel walls and into the blood stream too rapidly. Thus, local effects on epidermal cells are not usually obtained except by using caustic concentrations. However, effects on cutaneous blood vessels with vasoconstrictors and vasodilators and on sweat glands with the corresponding autonomic drugs can be easily produced through an intact surface if these pharmacologic agents are introduced electrophoretically.

It was stated earlier that in order for substances acting from the outside to exert effects on epidermal cells they must pass the epidermal barrier. It is clear that topical drug therapy of skin lesions is based on this penetration.

In topical dermatotherapy two main kinds of processes are utilized: physical and chemical actions. The application of inert vehicles, water, lotions, dusting powders, emollient greases and creams act by their physical properties and do not require penetration through the barrier because they act on the surface film of the skin and the disjunct stratum corneum. However, if drug effects are involved penetration must occur, and in this case the risk of undesired systemic effects must always be kept in mind. This is particularly true for applications on large surfaces and/or relatively high concentrations of such common topical remedies as salicylic acid, elemental sulfur, resorcin, mercury and other heavy metal compounds, tars, chrysarobin etc. Even boric acid with its extremely mild astringent and antiseptic effect has been found to be not without serious risks in local applications to diseased skin.

Ammoniated mercury is a relatively poorly absorbed and therapeutically very useful mercury compound. This is also true for the modern hydrocortisone and related preparations for local application with the exception of fluoro-substituted derivatives which have been reported to cause severe undesired side effects such as salt retention.

The danger that systemic effects will follow locally applied medications is particularly great in infants and small children, and at all ages if the barrier is severely damaged by the disease process or broken by excoriations.

Conscientious dermatologists thoroughly check their patients periodically for untoward side effects of local applications. For instance, if coal tar ointments are applied to large surfaces or in unusually high concentrations, weekly urine analyses for albuminuria are usually done because damage to the kidney is one of the first detectable systemic signs of tar poisoning.

Elemental sulfur is reduced to sulfides in the tissues. Fortunately, high sulfur concentrations in ointments against scabies and other infestations are no longer needed since better and less toxic antiscabetic drugs have been introduced. In the past, when small children with scabies were treated with sulfur ointments severe and fatal poisonings with sulfhemoglobin formation occurred.

A paradoxical situation prevails if phenol is applied to the skin. Highly concentrated phenol solutions are relatively less well absorbed than more dilute ones. This is due to the caustic protein-precipitant effect of strong phenol solutions. The coagulated protein-phenol complex forms a protective coat in the form of a dry crust on the surface which hinders penetration. Thus, from the point of view of systemic absorption and phenol poisoning, application of weak phenol solutions is more dangerous than that of the concentrated liquefied carbolic acid.

In closing, I may note that in the phylogenetic development culminating in the amphibiae, the skin has been a vitally important organ for the exchange of substances with the outer world. In mammals both functions of cutaneous absorption and excretion have become rudimentary. Teleologically, for man, absorption through the skin brings about more calamity than blessings. With the stupendous technical progress of modern times, things in this respect have become worse rather than better.

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RADIATION ACCIDENTS AND EMERGENCIES*

By

L. H. Lanzl and J. H. Pingel†

A Symposium on Radiation Accidents and Emergencies in Medicine, Research and Industry was held in Chicago, December 19-20, 1963. While emphasis was most generally placed on local emergencies and small accidents with ionizing radiation, a few papers also dealt with major catastrophes, short of a wartime nuclear holocaust. The symposium covered all pertinent aspects of a pure emergency situation, including accident dosimetry, handling of spills, medical responsibility, mass survey problems, control of post-accident exposures, psychological and legal considerations, and public relations.

In most accident or emergency situations where prompt action is necessary, the intelligent attention and full capacity of the emergency worker should be directed to the following sequence of actions:

- (a) The saving of lives (rescue operations, protection from further injury, and directing the victims into the process of restoration to active useful lives);
- (b) containment measures and prevention of further injury or threat of injury;
- (c) salvage of equipment and materials;
- (d) release of the disaster site to authorities responsible for restoration.

The type of emergency action taken in an area where radiation has been released will depend on whether or not there is a reasonable expectation that individuals are present and alive. In either case, the course of action to be pursued should be determined by the person designated as responsible for the emergency action (E. Vallario and R. Catlin, U.S. Atomic Energy Commission, Washington). The risk to rescue workers should be weighed against the probable success of the rescue action. Attempts to rescue victims should be regarded in the same context as any other emergency action involving the rescue of victims, regardless of the type of hazard involved. Any rescue activity that may involve substantial personal risk should be performed by volunteers, and all emergency workers should be advised of such risks prior to their participation.

From the legal point of view, Forgotson (Walter E. Meyer Research Institute of Law, Washington, D.C.) pointed out that a particularly complicated situation is presented when, for the purpose of attempting or effecting a rescue of persons involved in a disaster, or of preventing a disaster, it becomes necessary to expose individuals to doses of radiation in excess of 3 rem per quarter of a year and 25 rem for a single accidental exposure. He discussed the effect of these dose limitations and concluded that, on the basis of Federal Radiation Council publications, exceeding these doses does not constitute negligence per se, and, in certain situations, even might

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† Argonne National Laboratory, Argonne, Illinois.

not constitute evidence of negligence. (He further discussed a number of liability questions, including the potential liability of a manufacturer or seller of a source. In this connection, he called special attention to the recent product liability case of Goldberg versus Kallsman Instrument Corporation, a case which marked a departure from the way the New York Court of Appeals or any other court has previously handled one of these decisions. In this case, the majority of the court held that someone, namely, the ultimate manufacturer is left in the role of a virtual insurer for the defective designs.)

The screening of persons exposed to radioactivity for medical attention, decontamination, or release is only a passing phase of the emergency situation, although a most important one. Some controversy exists in the case of a contaminated person who requires medical treatment—which should come first, medical aid or decontamination? This question, of course, does not have a simple answer.

Speaking on medical effects, G. Voelz (U.S. Atomic Energy Commission, Idaho) stated that: "The atomic energy industries to date have not experienced acute accidental exposures from internal emitters (any radioactive chemical entering the body either through the skin, pulmonary or gastrointestinal tract) resulting in an acute or dramatic radiation injury similar to the direct external radiation exposures which produce the dramatic acute radiation syndrome." The concern about internal emitters is related more to the continuing radiation dose which may produce late pathological effects.

Inhalation of radioactive particulates or aerosols by workers has been the most common and important source of internal deposition in atomic energy installations. In the case of contaminated wounds, excision has been practiced most frequently when plutonium-239 was the contaminant. To evaluate the nature and amount of internally deposited radioactive material, G. V. LeRoy (University of Chicago) remarked that at the outset it is most important to collect all urine passed by each person from the time that he escapes—or is removed—from the site of the accident. Depending on the circumstances, radioassay of the first urine voided may be of great value in estimating the accidental burden of radioactive material.

The art and science of methods for decontaminating equipment and materials have been vigorously pursued for the last two decades. L. Gemmell (Brookhaven National Laboratory) reviewed older as well as some new techniques such as shot blasting. Speaking on maximum permissible levels of surface contamination, W. R. Bush (Chalk River, Canada) pointed out that these varied by a factor of 1,000 for alphas and 100 for the betas among the various countries that use radioactive isotopes. He also developed data showing that, for a given surface contamination of a material, the inhalation hazard varied by a factor of 1,000,000, with carbon-14 at the lowest limit and plutonium-239 at the highest. J. Maloney (Edgewood Arsenal, Edgewood, Maryland) reported on new effective procedures of major outdoor decontamination under cold weather and winter conditions. (Many portable instruments for measuring radiation fail to operate while at low temperatures.)

The spread of alpha contamination which results from a non-nuclear explosion (chemical part) of atomic weapons has been a subject of concern for many years. The most extensive measurements ever made in this field were reported on for the first time by W. Johnson, Sr. (Eberline Instrument Company). The detonations were designed to simulate conditions of storage, transportation, and handling of plutonium-bearing weapons. Surveys were performed with both alpha and gamma instruments at distances up to 10 miles or 16 kilometers. As one might expect, drastic

changes in the contamination patterns were observed from one test shot to another.

The generation and disposal of waste in emergency decontamination is of little consequence in most accidents involving radioactivity (R. O'Brien, General Electric Company, Idaho). This is due in part to the well-established waste disposal channels. In unusual situations such as the SL-1 reactor incident where, after 5 months decay, about 3,500 curies of fission products were distributed as contamination throughout the reactor building and its environs, the use of a local waste disposal site saved more than 300 man-rem of exposure. It is estimated that the cumulative total whole body dose of 1,000 rem was received by those who were involved in the clean up. The cost of clean up was \$1,000,000, not counting the cost of volunteer and army workers.

R. Gallagher (Applied Health Physics, Pittsburgh) reported a wide range of restoration effort for the decontamination of radium spills in medical situations. In one situation an entire building had to be demolished. There is on the average one radium contamination incident a week in the United States.

Unlike many types of accidents, those involving radiation can go undetected for an extended period of time. Such delays can lead to considerable spread of the contamination.

The topic of public relations was assigned to the well-known science reporter, Robert S. Kleckner (Sun-Times, Chicago), and not to an institutional public relations officer. Kleckner stated that the first step in reporting an accident is to avoid any type of censorship and to get the facts to the people as quickly and simply as possible. If there is a hazard beyond the confines of an installation, it should be stated that this is so and how great it is. The public should be informed about precautions to be taken. There should be a steady flow of information to the news media until the story has been covered from all angles. There are reassurances even in bad radiation mishaps; the good as well as the bad should be brought out. The American public has never panicked when it knew the truth immediately.

Pre-planning and preparedness are the keys to the reduction of the deleterious effects of accidents. R. Landauer (Cook County Hospital, Chicago) and G. V. LeRoy (University of Chicago, Chicago) spoke on hospital preparedness, but differed greatly on the approach. LeRoy stressed pre-planning between a given radiation installation and a nearby hospital for the care of injuries that may occur. Landauer, on the other hand, stressed the need for a simple plan for all hospitals because accidents, especially transport accidents, may occur anywhere.

Emergency situations produce anxieties in those who are directly involved. D. Oken (Chicago), a psychiatrist, talked on mental preparedness of emergency personnel, both as individuals and as groups. Emergency teams must be suffused with a strong esprit de corps; members of a group with high morale become capable of carrying out tasks that are personally unappealing or even severely stressful. The group may admit to a certain degree of internal fear, but disparagement of the group itself or self-protective avoidance of responsibilities to one's co-workers is intolerable. Individuals who transgress these limits must be excluded. Panic, however, is rare: Little was seen at Hiroshima or Nagasaki. Emergency teams should be supplied with a maximum of correct information and be trained in the most helpful methods of communicating this to victims. The antidote to scare stories and rumors is information. Even if the news is bad, it is always reassuring to know that you know the worst. On the question of prevention, Oken pointed out that accidents tend to occur in clusters during periods in which other signs of psychological stress are evident: the accident syndrome. Subtle changes in the behavior patterns of an individual may be precursors to a major accident.

In the event of a radiation accident that cannot be handled by the organization in which it occurs, there are some private organizations that might be called in. In addition to these, the U.S. Atomic Energy Commission has a Radiological Assistance Program. Zintz (U.S. Atomic Energy Commission, Washington) and Brobst (U.S. Atomic Energy Commission, Chicago) reported on the program which is capable of responding to a radiological emergency upon request 24 hours a day anywhere in the United States. During the last three years 223 responses to requests for radiological assistance were made. Most of these (40 per cent) involved transportation incidents.

The Radiological Health Division of the United States Public Health Service has a somewhat broader program, although it too has Radiological Assistance Teams. R. Moore (U.S. Public Health Service, Dallas) and L. Thomas (U.S. Public Health Service, Chicago) outlined the role of the U.S. Public Health Service in the Radiation area along with its traditional role of protection of public health. An Inter-agency Radiological Assistance Plan involving twelve federal agencies has been formed. The members of the symposium were gratified to learn of the wide interest shown by the federal government.

The 350 individuals who attended the symposium came mainly from the midwestern states, but included visitors from 28 continental states, Hawaii, Puerto Rico, Canada, and Austria. Institutional representation was 28 per cent from industrial or commercial firms, 17 per cent from the U.S. Atomic Energy Commission or its contractors, 14 per cent from public health authorities, 12 per cent from space and defense agencies, 11 per cent from universities, and 7 per cent from hospitals. The remainder included representatives from insurance companies, labor unions, research institutions, and private individuals.

THE BIOSYNTHESIS OF 5-RIBOSYLURACIL-5'-MONOPHOSPHATE
IN TETRAHYMENA PYRIFORMIS*

By

R. L. Henrikson and E. Goldwasser

Since the discovery^{1,2} and elucidation of the structure of 5-ribosyluracil,³⁻⁵ increasing attention has been turned toward the metabolism of this compound. Pseudouridine,[†] along with the other newly discovered constituents of ribonucleic acid, has been shown to be present in highest proportions, if not exclusively, in soluble, or amino acid transfer RNA.^{2,6} Because of the central role of s-RNA in protein biosynthesis, investigations into the metabolic formation of Ψ -uridine and its incorporation into polynucleotide have become of importance not only in gaining a clearer insight into the chemical events of protein formation, but also in order to better understand the mode of synthesis of transfer RNA.

In preliminary experiments we observed that extracts from the pyrimidine requiring protozoan, *Tetrahymena pyriformis*, catalyzed the conversion of uracil and 5-phosphoribosylpyrophosphate to both uridine monophosphate and 5-ribosyluracil-5'-monophosphate.⁷ The present paper describes the separation and partial purification of the enzymes responsible for UMP and Ψ -UMP[†] biosynthesis in this organism. During the course of this investigation it was shown that whereas UMP synthesis proceeds via the conventional pyrophosphorylase pathway,⁸ Ψ -UMP is formed directly from uracil and ribose-5-phosphate by way of an entirely distinct mechanism involving a new enzyme which has been tentatively called Ψ -UMP-synthetase. This latter finding constitutes the first clearly defined description of the enzymatic formation of the carbon-carbon ribosyl linkage of Ψ -uridine and its derivatives. A preliminary account of some of this work has been published.⁹

EXPERIMENTAL PROCEDURE

Materials. Uridine, UMP, ATP, NADH and the magnesium salt of PP-ribose-P were obtained from Pabst Laboratories, Inc. The sodium salt of ribose-5-P, calcium-bis-para-nitrophenylphosphate, disodium para-nitrophenylphosphate and isocytosine were purchased from Sigma Chemical Company. Ribose-1,5-diphosphate was a gift from Dr. H. G. Khorana. Tricyclohexylammonium phospho-enol-pyruvate, 2-thiouracil-2-C¹⁴ and cytosine-2-C¹⁴ were obtained from the California Corporation for Biochemical Research. Uracil-2-C¹⁴ and uridine-2-C¹⁴ were purchased from Nuclear-Chicago Corporation. UMP-2-C¹⁴ was synthesized from uracil-2-C¹⁴ using UMP-pyrophosphorylase isolated from *Tetrahymena pyriformis* during the course of this investigation. Orotic acid-6-C¹⁴ was bought from Volk Radiochemical Company. 5-Ribosyluracil was a gift from Dr. Waldo Cohn. Lyophilized *Crotalus adamanteus* venom was pur-

* This paper appears in the Journal of Biological Chemistry, 239:1177, 1964. It is taken, in part, from the thesis submitted by Robert L. Henrikson (United States Public Health Service Fellow) in fulfillment of the requirements for the degree of Doctor of Philosophy, University of Chicago, Chicago, Illinois, 1963. Present address, The Rockefeller Institute, New York 21, New York.

† The abbreviations used are: Ψ -UMP, 5-ribosyluracil-5'-monophosphate; Ψ -uridine, 5-ribosyluracil.

chased from the Ross Allen Reptile Institute. Pyruvate kinase and lactate dehydrogenase were obtained from C. F. Boehringer and Sons. Cultures of Tetrahymena pyriformis (strain W) were kindly provided by Dr. Frank M. Child (Department of Zoology, University of Chicago). Crystalline bovine plasma albumin was purchased from Armour and Company. Superbrite glass beads were obtained from Minnesota Mining and Manufacturing Company, Catalogue #100. The Superbrite was washed with acid, rinsed exhaustively with distilled water and dried. Analytical grade Dowex anion exchange resins and Ecteola cellulose were obtained from the California Corporation for Biochemical Research. Sephadex gels were purchased from Pharmacia Laboratories, Box 1010, Rochester, Minnesota. All other reagents were the best chemically available grade.

Uridine kinase was prepared from mouse Ehrlich ascites tumor cells by a modification of the method described by Sköld.¹⁰ Fluid from 600 ascitic mice was collected and centrifuged at 600 x g. The cells were washed once with 0.05 M Tris buffer at pH 7.4 containing 0.11 M KCl and were then suspended in 80 ml of this medium. The suspension was passed through an hydraulic homogenizer¹¹ using an annular clearance of 18 μ . The homogenate was centrifuged at 100,000 x g and the supernatant fluid was fractionated with ammonium sulfate. The protein precipitating between 33 per cent and 50 per cent saturation was dissolved in and dialyzed against the Tris-KCl buffer mentioned above. The final clear, colorless preparation was centrifuged at 10,000 x g to clarify, and the supernatant fraction was assayed spectrophotometrically in the coupled system of Kornberg and Pricer.¹² The enzyme was stored for 2 months at -20° without loss of activity.

Orotidine-5'-phosphate pyrophosphorylase and orotidine-5'-phosphate decarboxylase were prepared from autolysates of dried brewer's yeast as described by Liebermann et al.¹³ The 25 to 60 per cent ethanol fraction containing both enzymes was dissolved in, and dialyzed against, 0.05 M Tris buffer at pH 8.0.

Snake venom phosphodiesterase free of 5'-nucleotidase was prepared from lyophilized Crotalus adamanteus venom according to the procedure of Williams et al.¹⁴ The protein precipitating between 42 and 50 per cent acetone at -17° (Step 1, precipitate 3) was dissolved in water and brought to pH 9 with dilute NaOH. This preparation rapidly hydrolyzed the phosphodiester bonds of calcium-bis-para-nitrophenylphosphate but was inactive toward disodium para-nitrophenylphosphate. Assays were carried out at 35° in Tris-buffered systems at pH 9.0 in the absence of magnesium.

Uridine phosphorylase assays were conducted as described by Pontis et al.¹⁵

Soluble RNA, used as a source of Ψ -UMP was prepared from pressed bakers yeast as described by Monier et al.¹⁶ One hundred mg of this material was hydrolyzed with the phosphodiesterase preparation which was added intermittently over a period of 24 hours, the pH being maintained at 9.0 ± 0.1 by the addition of dilute NaOH. The reaction was followed in a pH-stat titrometer. From the amount of the base added it was calculated that the extent of hydrolysis was approximately 60 per cent. The solution was boiled, filtered and the 5' nucleotides fractionated by anion-exchange chromatography as described below.

Methods. Protein was determined by a modification of the method of Lowry et al.¹⁷ developed by Dr. David Straus.¹⁸ Crystalline bovine plasma albumin was used as standard. Elution of protein from chromatographic columns was followed spectrophotometrically by the absorbance of the fraction at 280 $m\mu$.

Pentose was determined by the method of Dische and Schwartz.¹⁹ The standard used in

these determinations was the sodium salt of ribose-5-phosphate. Orthophosphate analyses were performed according to the procedure of Lowry and Lopez.²⁰

Radioactive samples were counted as infinitely thin layers (less than 100 μg per cm^2) without correction for self-absorption in the low background Tracerlab AC-2 Omni-Guard Automatic System.

Paper chromatographic separation of Ψ -uridine from uridine, uracil and nucleotides was done in the butanol-water system of Markham and Smith.²¹ UMP and Ψ -UMP were separated in the ethanol-ammonium acetate system first described by Paladini and Leloir²² and later used by Rabinowitz and Goldberg²³ to distinguish between these particular isomers.

Tetrahymena pyriformis was grown at room temperature under sterile conditions on 2 per cent proteose peptone (Difco) without aeration. Cells were grown in test tubes for 2 to 3 days in 3 ml sub-inoculum cultures. Each tube was then used to inoculate a large culture grown on 500 ml of medium in 1 liter Roux bottles. After 4 to 6 days growth, protozoa from 6 liters of medium were harvested by low-speed centrifugation in 50 ml conical glass centrifuge tubes. The cells were washed twice with cold distilled water and centrifuged for 5 minutes at 6,000 x g. The pellet was then quickly frozen in an acetone-dry ice bath. This procedure routinely yielded 10 to 15 cm^3 of packed cells per liter of growth medium. Cell rupture was most reproducibly accomplished by grinding the frozen pellet with twice its volume of chilled glass powder using a mortar and pestle precooled to -20° . Initially, the broken cells were extracted twice with 60 ml of 0.01 M Tris buffer at pH 8.0, and centrifuged at 10,000 x g and 100,000 x g. In more recent experiments, the extracting medium was supplemented with 20 per cent glycerol. The glycerol, when present, will be noted in all experiments described.

In many of the enzyme systems investigated during the course of this work both 5'-UMP and 5'- Ψ UMP were concomitantly synthesized and hence a reliable, quantitative method of separation of these isomers was desirable. A slight modification of the anion-exchange system described by Cohn and Volkin¹ for the separation of the mixed 3' and 2' isomers proved adequate in this respect. To test the method, a phosphodiesterase hydrolysate of RNA containing approximately 200 μmoles of 5' nucleotides was applied to a column Dowex AG1-x8 formate (20 cm x 0.7 cm^2). CMP and AMP were removed as described by Cohn and Volkin.¹ Uracil ribonucleotides were then eluted with 0.05 M ammonium formate plus 0.005 M formic acid at pH 4.6. Ψ -UMP was eluted between 12 and 21 resin bed volumes, UMP between 22 and 32 volumes. An example of the separation of Ψ -UMP from UMP may be seen in Figure 1.

Using this ion-exchange method, large scale reaction mixtures containing enzymes known to synthesize UMP were analyzed for the presence of Ψ -UMP. In assays employing crude enzyme systems, the Ψ -UMP and UMP peaks were invariably contaminated with an unlabeled, endogenous, ultraviolet-absorbing substance. Therefore the calculation of newly-formed nucleotide was based on radioactivity measurements. When the relative amounts of Ψ -UMP and UMP formed by a given enzyme preparation were known from analysis as described above, or when qualitative information regarding the nucleotide product was not crucial, assays were conducted according to the method of Crawford *et al.*⁸ This procedure affords a fairly rapid separation of uracil or uridine from UMP and Ψ -UMP. The assays were conducted for various periods of time at 30° . Reactions were terminated by heating the contents of the vessel to boiling for 60 seconds followed by filtration (large-scale mixtures) or centrifugation and analysis of the filtrates or supernatant solution for labeled uracil ribonucleotides.

Ψ -UMP was concentrated and desalted on small columns of Dowex-AG 1-x4 carbonate as described by Cohn.⁴

RESULTS

Several enzyme systems known to be involved in the metabolism of uridine and UMP were examined for activity toward the corresponding Ψ -uridine analog. The ability of uridine kinase isolated from Ehrlich ascites tumor to phosphorylate Ψ -uridine was examined. Under conditions in which uridine was phosphorylated, no corresponding phosphorylation of Ψ -uridine was detected (Figure 1). A second addition of substrate to the appropriate cuvettes had no effect on the respective reaction rates, but when uridine was added to the system containing Ψ -uridine, phosphorylation at a rate comparable to that in Figure 1-B was readily observed. This demonstrates not only

Figure 1. Specificity of uridine kinase. The complete system contained 33.0 μ moles of KCl, 66.0 μ moles of $MgCl_2$, 0.5 μ mole of NADH, 1.0 μ mole of PEP, 2.0 μ moles of ATP, 1.25 μ g of LDH, 5 μ g of PK, 100 μ moles of Tris buffer at pH 7.4, uridine kinase (0.20 mg of protein) and substrate in a total volume of 2.0 ml. Reaction mixtures were prepared as follows:

A Control: Complete system minus substrate.

B Uridine: Complete system containing 0.25 μ mole of uridine.

C Ψ -Uridine: Complete system containing 0.13 μ mole of Ψ -uridine.

A blank was prepared consisting of the complete system minus NADH and substrate. The various solutions (minus substrate) were incubated for 10 minutes at 37° after which time substrate was added to initiate reaction (zero time). The arrows at 10 minutes indicate a second addition of uridine and Ψ -uridine to cells B and C respectively. The arrow at 20 minutes designates the addition of 0.25 μ mole of uridine to system C.

that Ψ -uridine does not serve as a substrate for uridine kinase, but also that it does not inhibit the phosphorylation of uridine. In addition, we were unable to demonstrate any Ψ -uridine kinase activity in extracts prepared from a variety of yeast sources.

The yeast orotic acid pathway of UMP biosynthesis was investigated for possible concomitant formation of Ψ -UMP. An assay mixture* was prepared containing orotic acid, PP-ribose-P, and the yeast enzymes and the reaction products analyzed for Ψ -UMP. Analysis of the mixture on Dowex-1-formate indicated that no Ψ -UMP had been formed during the course of the reaction. A 60 per cent conversion of orotic acid-6-C¹⁴ to UMP-6-C¹⁴ was obtained in this experiment. Similar results were observed using as enzyme source the crude undialyzed autolysate prepared in the presence of 20 per cent glycerol.

Several experiments were performed to determine whether or not Tetrahymena pyriformis contained any of the alternate enzyme pathways of UMP biosynthesis. Reaction mixtures were prepared as described in Table 1, Experiments A and B and we found conversion of both uracil and uridine to nucleotide. These data, while not absolutely conclusive, suggest the presence of both UMP-pyrophosphorylase and uridine kinase Scheme 1 reactions I and III in the crude extract of T. pyriformis. In addition, these crude preparations were shown to contain uridine phosphorylase (Scheme 1 reaction II), confirming an earlier report by Friedkin.²⁴

Table 1

DEMONSTRATION OF UMP-PYROPHOSPHORYLASE AND
URIDINE KINASE IN CRUDE EXTRACTS

Experiment A: 0.1 μ mole of uracil-2-C¹⁴ (200,000 cpm per μ mole), 0.2 μ mole of PP-ribose-P, 10 μ moles of MgCl₂ and enzyme (100,000 x g supernatant extracted in 0.01 M Tris buffer containing 20 per cent glycerol pH 8.0; 0.6 mg of protein) in a total volume of 1.0 ml. 15 minutes incubation at 30°.

Experiment B: 0.2 μ mole of uridine-2-C¹⁴ (100,000 cpm per μ mole), 4.0 μ moles of ATP, 100 μ moles of MgCl₂ and 1.5 ml of 10,000 x g supernatant dialyzed against 0.01 M Tris buffer at pH 7.5 (6 mg of protein) in a total volume of 3.0 ml. 10 minute incubation at 30°.

Experiment	Enzyme	Total nucleotide formed m μ moles	Nucleotide formed per minute per mg protein m μ moles
A	UMP-pyrophosphorylase	2.1	0.23
B	Uridine kinase	1.2	0.02

Knowing the modes of UMP biosynthesis in T. pyriformis facilitated the investigation of these enzyme pathways for Ψ -UMP synthesis. When either the 10,000 x g or the 100,000 x g supernatant fractions were incubated with uracil-2-C¹⁴ and PP-ribose-P in the presence of

* The reaction mixture contained 50 μ moles of NaOH, 50 μ moles of orotic acid-6-C¹⁴ (2.8 mc per mmole), 150 μ moles of magnesium acetate, 100 μ moles of PP-ribose-P, 2.0 mmoles of Tris buffer, at pH 8.0 and the yeast preparation containing both orotidine-5'-phosphate-pyrophosphorylase and orotidine-5'-phosphate-decarboxylase (200 mg of protein) in a total volume of 75 ml. The solution was incubated 6 hours at 37°, boiled, filtered and the filtrate fractionated on a column of Dowex-1-formate.

Scheme 1

Mg⁺⁺ and the reaction mixtures analyzed for Ψ -UMP and UMP, 25 to 30 per cent of the total labeled nucleotides formed was routinely isolated as Ψ -UMP. These data are presented in Table 2, A and B. The fact that the specific radioactivity of the Ψ -UMP isolated from these systems was higher than that of UMP suggested that the latter was not a precursor of Ψ -UMP.

Table 2
SYNTHESIS OF Ψ -UMP AND UMP BY VARIOUS FRACTIONS
ISOLATED FROM *Tetrahymena pyriformis*

Experiment	Enzyme	Protein mg per ml	Specific activity for Ψ -UMP synthesis*	Specific activity for UMP synthesis*	Total nucleotide as	
					Ψ -UMP %	UMP
A	10,000 x g supernatant†	10.6	36.0	79.5	27	73
B	100,000 x g supernatant†	8.5	66.5	132.0	30	70
C	AS-II‡	6.4	28.8	56.3	34	66
D	AS-III‡	16.2	19.6	3.2	84	16
E	AS-IV‡	0.9	53.5	171.0	3	97
F	E-II‡	0.68	24.4	500.0	4	96
G	E-III‡	0.63	330.0	13.2	95	5

* Defined as μ moles of nucleotide formed per minute per mg of protein.

† Reaction mixtures contained 10 μ mole of uracil-2-C¹⁴ (200,000 cpm per μ mole), 10 μ moles of PP-ribose P; 0.8 mmoles MgCl₂ and 20 ml of the appropriate supernatant fraction (which had been isolated in and dialysed against 0.01 Tris buffer containing 20 per cent glycerol at pH 8.0) in a total volume of 80 ml. Incubation was for 15 minutes at 30° and the reaction products were separated on Dowex-1-formate.

‡ Reaction mixtures contained 5.0 μ moles of uracil-2-C¹⁴ (200,000 cpm per μ mole), 10 μ moles of PP-ribose P; 0.5 mmole of MgCl₂ and 10.0 ml of the appropriate enzyme mixture (see text) in a total value of 50 ml. The reaction mixtures were incubated 20 minutes at 30° and analyzed as in Figure 3.

The enzymatically formed Ψ -UMP obtained in this manner was concentrated and desalted prior to characterization. In experiments involving crude enzymes the labeled Ψ -UMP was contaminated by an unknown, non-labeled substance exhibiting an absorption maximum of 250 m μ which was almost entirely removed during the concentration procedure.⁴ The Ψ -UMP thus obtained was further identified with respect to its spectral characteristics at neutral and elevated pH and by paper chromatography in the system of Paladini and Leloir.²² A comparison of these data for the 5'- Ψ -UMP obtained by snake venom phosphodiesterase hydrolysis of RNA and the enzymatically formed 5'- Ψ -UMP-2-C¹⁴ is presented in Table 3. The spectral curves obtained for the enzymically formed Ψ -UMP were identical to those published by Goldberg and Rabinowitz for chemically synthesized 5'- Ψ -UMP.²⁴

Table 3
COMPARISON OF PROPERTIES OF 5'- Ψ -UMP OBTAINED BY
SNAKE VENOM PHOSPHODIESTERASE HYDROLYSIS OF RNA
AND ENZYMATICALLY SYNTHESIZED 5'- Ψ -UMP-2-C¹⁴

Source of 5'- Ψ -UMP	R_F^*	pH 4.5			pH 14		
		$\frac{A_{280}}{A_{260}}$	$\frac{A_{250}}{A_{260}}$	λ max m μ	$\frac{A_{280}}{A_{260}}$	$\frac{A_{250}}{A_{260}}$	λ max m μ
RNA	0.29	0.43	0.75	262	1.30	0.85	282
Enzymically formed	0.29	0.46	0.76	262	1.33	0.83	283

* In absolute ethanol—0.5 M ammonium acetate, pH 3.8 (5:2).²²

The Ψ -UMP formed in this enzyme system was further characterized by hydrolysis with crude snake venom which contains 5'-nucleotidase. A solution was prepared containing 0.17 μ mole of Ψ -UMP-2-C¹⁴ (200,000 cpm per μ mole), 10.0 μ moles of MgCl₂, 1.0 μ mole of Tris buffer at pH 8.7 and 0.1 mg of lyophilized snake venom in a total volume of 0.20 ml. The reaction mixture was incubated 1 hour at 37°, boiled, centrifuged and 30 μ l applied to Whatman No. 1 paper. Uracil, uridine and Ψ -uridine were similarly applied as reference compounds. The chromatogram was developed in the system of Markham and Smith²¹ and dried. Examination of the chromatogram under ultraviolet light revealed that the reaction mixture contained only Ψ -uridine, and an autoradiogram indicated that all radioactivity was associated with this spot. The Ψ -uridine-2-C¹⁴ was quantitatively eluted with water and characterized spectrally and in terms of specific radioactivity. Phosphorous determinations were performed on aliquots of the supernatant of the reaction mixture and on the venom used. The results indicated that 0.17 μ mole of Ψ -UMP yielded 0.15 μ mole of Ψ -uridine and 0.19 μ mole of orthophosphate.

Several lines of evidence indicated that the enzyme catalyzing the synthesis of Ψ -UMP was distinct from UMP-pyrophosphorylase. A purified preparation of the latter enzyme converted uracil and PP-ribose-P to UMP only; no Ψ -UMP formation was detected. In addition, when the 100,000 x g supernatant fraction from extracts of the disrupted *T. pyriformis* (no glycerol) was subjected to dialysis or protamine sulfate precipitation, a loss of activity in terms of Ψ -UMP

synthesis was observed whereas the formation of UMP was either unaffected or enhanced by such treatment.

These experiments demonstrated that there was a separate enzyme for Ψ -UMP synthesis but it was not certain whether the nucleotide was synthesized directly from uracil or from some intermediate derivative of the base. The formation of UMP and Ψ -UMP was therefore allowed to proceed in the presence and in the absence of added nonlabeled uridine in order to determine the effect of the nucleoside on the specific radioactivity of the nucleotide isomers. A comparison of the elution profiles for UMP and Ψ -UMP synthesized under these conditions indicated that whereas the specific radioactivity of the Ψ -UMP was the same in both reaction mixtures, that of UMP isolated from the system containing uridine was approximately half the control value.

A study was also made on the effect of UMP on uracil ribonucleotide biosynthesis in these crude preparations. The experiments described in Figure 2 indicate clearly that UMP is not a precursor of Ψ -UMP. The addition of unlabeled UMP diluted the specific radioactivity and reduced the total synthesis of UMP, whereas an increased yield of Ψ -UMP of high specific radioactivity was obtained. Further substantiation of these results was provided by the fact that no conversion of UMP-2-C¹⁴ to Ψ -UMP could be detected. This experiment demonstrates in addition the absolute dependence of Ψ -UMP synthesis on a ribose source, in this case PP-ribose-P.

Figure 2. The effect of added UMP on the synthesis and specific radioactivities of UMP and Ψ -UMP. Reaction mixture A contained 8 μ moles of uracil-2-C¹⁴ (200,000 cpm per μ mole), 10 μ moles of PP-ribose-P, 3 μ moles of UMP, 0.8 mmole of MgCl₂ and 20 ml of 100,000 $\times$ g supernatant from *T. pyriformis* in a total volume of 80 ml. The enzyme was extracted in and dialyzed against 0.01 M Tris buffer containing 20% glycerol at pH 8.0. System B was the same except that PP-ribose-P was omitted, labeled uracil was replaced by unlabeled uracil, and cold UMP was replaced with 1.5 μ moles of UMP-2-C¹⁴ (200,000 cpm per μ mole). Incubation and analysis were carried out as described in the text to Figure 3.

The loss of activity of Ψ -UMP-synthetase during dialysis could not be reconstituted by addition of the dialysate, an indication that the diminished activity was not due to the loss of dialyzable cofactors. The enzyme was not stabilized by the addition of sulfhydryl compounds. Because of the success achieved by Jarabak *et al.*²⁵ in enzyme stabilization with glycerol, the disrupted *T. pyriformis* were extracted with the Tris buffer system at pH 8.0 supplemented with 20 per cent glycerol. Supernatant fractions from these extracts retained Ψ -UMP synthetic activity after freezing and thawing and dialysis, and could be stored at -20° for 1 year without significant loss of activity. The presence of glycerol thus provided the stabilization necessary for the further purification of the Ψ -UMP-synthetase.

PARTIAL PURIFICATION OF Ψ -UMP SYNTHETASE: SEPARATION FROM UMP-PYROPHOSPHORYLASE

Preparation of the 100,000 x g supernatant. The Tris-glycerol extracts from *Tetrahymena pyriformis* prepared as described earlier were centrifuged for 30 minutes at 10,000 x g and the supernatant fractions were combined. The turbid 10,000 x g supernatant fluid was then centrifuged for 2 hours at 100,000 x g and the clear yellow supernatant fluids were collected to yield 100 to 110 ml of solution. The sediment was a clear, reddish-brown gel having no detectable activity in the synthesis of uracil ribonucleotides. The conversion of uracil to Ψ -UMP and UMP catalyzed by the dialyzed 10,000 and 100,000 x g supernatant fraction is presented in Table 2, A and B. When further purification was intended these supernatant fractions were not dialyzed.

Ammonium sulfate fractionation. The undialyzed 100,000 x g supernatant was fractionated into preparations AS-I, AS-II and AS-III. These fractions contain the protein precipitating between 0 and 1.14 M, 1.14 and 2.28 M and 2.28 and 3.42 M ammonium sulfate respectively. They were dissolved in 10 to 20 ml of 0.01 M Tris buffer containing 20 per cent glycerol at pH 8.0 and, together with the supernatant fraction from the final ammonium sulfate precipitation (AS-IV) were dialyzed against two 200-volume changes of buffer. Fraction AS-I was turbid and colorless; AS-II, AS-III and AS-IV were pale yellow to yellow in color and quite clear.

Assays of the 4 fractions (see Table 1, A) indicated that most of the nucleotide synthetic activity was in fractions AS-II, AS-III and AS-IV. These preparations were therefore assayed in large-scale reaction mixtures and the nature of the nucleotide formed determined by ion exchange chromatography. Quantitative data are presented in Table 2, C, D and E. These results showed that whereas the nucleotide formed by fraction AS-III was largely Ψ -UMP, that synthesized by fraction AS-IV was almost entirely UMP. Thus a partial separation of Ψ -UMP-synthetase and UMP-pyrophosphorylase was achieved by this method.

Fractionation of AS-III on Ecteola cellulose. Ecteola anion-exchange cellulose (Cellex E, .48 meq per g) was washed several times with 0.01 M Tris buffer containing 20 per cent glycerol at pH 8.0, and a column (8 cm x 1.2 cm²) of this material was equilibrated with the same buffer. A 3.0 ml aliquot of AS-III containing 50 mg of protein was applied to the column and washing was continued with the same Tris-glycerol buffer until the absorbance of the eluant at 280 m μ was less than 0.040. Elution was then carried out in stepwise fashion with 0.05 M, 0.10 M and 1.0 M Tris buffers at pH 8.0, each containing 20 per cent glycerol. The flow rate which was regulated by hydrostatic pressure head, was approximately 2 ml per minute. A typical elution profile is presented in Figure 3. Those fractions comprising each peak with an A_{280} value greater than

0.200 were pooled and assayed for nucleotide synthesis. Most of the activity was isolated in peaks II and III (fractions E-II and E-III), which were then assayed in large-scale reaction mixtures as before and the uracil ribonucleotide determined. The results are presented in Table 2, F and G. These data clearly show that of the total nucleotide formed in each case, fraction E-II gave rise primarily to UMP while the main product of the reaction catalyzed by fraction E-III was Ψ -UMP.

Figure 3. Chromatography of ammonium sulfate fraction AS-III on Ecteola-cellulose. Stepwise elution with Tris buffers containing 20 per cent glycerol at pH 8.0 at concentrations indicated along the abscissa.

Purification on Sephadex G-200. In some cases an alternate procedure was employed in the purification of Ψ -UMP-synthetase. Sephadex G-200 (medium) was allowed to swell in water for 1 week at room temperature. The gel was then equilibrated for 6 hours at 4° with 0.01 M Tris buffer containing 20 per cent glycerol at pH 8.0. A small amount of Sephadex G-25 (medium) was equilibrated with the same buffer and a thin layer (0.5 cm) of the gel was placed at the bottom of a column of cross sectional area 3.14 cm². A slurry of the Sephadex G-200 in the Tris-glycerol system was then added and slowly packed to give a column 24 cm in height. The column was washed with the same buffer for 2 hours with a hydrostatic pressure head. The sample of frac-AS-III was then added, followed by the same buffer system. The elution diagram is presented in Figure 4.

Although this procedure did not yield discrete peaks of protein material, separation based on $A_{260}:A_{280}$ ratios of the fractions was possible. Those tubes containing solutions having fairly uniform $A_{260}:A_{280}$ ratios were pooled to give Sephadex fractions S-I, S-II and S-III with average $A_{260}:A_{280}$ ratios 1.30, 0.89 and 0.68 respectively. Fraction IV represented nucleotide material. All the added protein was isolated in these first 3 fractions and most of the Ψ -UMP synthetase

Figure 4. Fractionation of ammonium sulfate fraction AS-III on Sephadex G-200. A 2.0 ml sample containing 30 mg of protein was added and eluted with 0.01 M Tris buffer containing 20 per cent glycerol at pH 8.0. Fractionation was based on regions of uniform A_{260}/A_{280} ratios. The void volume of the column was 28 cm³ and the flow rate average over the 12 hour period of separation was 9 ml per hour. The procedure was carried out at 5°.

activity was found in fraction S-III (20 ml, 0.5 mg of protein per ml).

Preparation S-III was then further fractionated on Ecteola-cellulose as described below. A great loss in yield was observed in this step: from the 10 mg of protein added, approximately 1 mg was eluted in fraction III (0.1 M Tris buffer containing 20 per cent glycerol at pH 8.0). This solution will be referred to as fraction SE-III. Treatment of the various active preparations with calcium phosphate and alumina (C- γ) gel failed to yield any purification. The absorption of protein by both these gels was accompanied by corresponding loss of activity in the supernatant fluid. Attempts to remove activity from the gel by treatment with glycerol-containing Tris buffers of increasing ionic strength were unsuccessful.

During the course of the characterization of fraction E-III, a control was prepared with ribose-5-P as a substrate in place of PP-ribose-P. The results of this experiment indicated that

ribose-5-P was nearly twice as efficient in effecting the synthesis of Ψ -UMP as PP-ribose-P. A time curve of Ψ -UMP formation using ribose-5-P and PP-ribose-P as ribose source is shown in Figure 5. When the concentration of ribose-5-P was reduced to 0.05 μ mole, the rate of Ψ -UMP formation was still greater than that produced by 0.2 μ mole of PP-ribose-P. Chromatographic analysis of a large-scale reaction mixture in which ribose-5-P was the ribose source revealed that the sole product of reaction was Ψ -UMP (0.53 μ mole); no UMP synthesis was detected.

Figure 5. Time curve of Ψ -UMP synthesis by Ecteola fraction E-III with ribose-5-P and PP-ribose-P as ribose source. Four reaction vessels were prepared containing 0.1 μ mole of uracil-2- C^{14} (200,000 cpm per μ mole), 0.2 μ mole of ribose source, 10 μ mole of $MgCl_2$ and 200 μ l of E-III in a total volume of 1.0 ml. Solutions were incubated at 30° for the times indicated and assayed for total nucleotide.

Thus the distinction between the modes of formation of UMP and Ψ -UMP in *T. pyriformis* is quite clear. Fraction E-II, containing UMP-pyrophosphorylase, has been shown to be absolutely dependent on PP-ribose-P as ribose source in UMP synthesis whereas Ψ -UMP-synthetase catalyzes the formation of Ψ -UMP from uracil and ribose-5-P.

Having determined the pathway and some of the conditions of Ψ -UMP synthesis, we assayed the 100,000 x g supernatant, ammonium sulfate fraction III, and Ecteola fraction III under standard conditions using ribose-5-P. A summary of the purification procedure is presented in Table 4. A 13-fold purification was obtained with a yield of 48.5 per cent.

CHARACTERIZATION Ψ -UMP-SYNTHETASE

The dependence of Ψ -UMP formation upon temperature, Mg^{++} concentration, enzyme concentration, and time was determined as described in Figure 6. Reaction mixtures were made up in a total volume of 1.0 ml and were incubated for 60 minutes at 30°, except for those experi-

Table 4

PARTIAL PURIFICATION OF Ψ -UMP-SYNTHETASE
FROM Tetrahymena pyriformis

The reaction vessels contained 0.1 μ mole of uracil-2-C¹⁴ (200,000 cpm per μ mole), 0.2 μ mole of ribose-5-P, 10 μ moles MgCl₂ and enzyme in a total volume of 1.0 ml.

- A. 100 μ l of 100,000 x g supernatant (in 0.01 M tris buffer containing 20% glycerol at pH 8.0; 0.93 mg of protein per ml). Reaction time = 15 minutes.
- B. 0.050 ml AS-III (in same buffer as A; 15.0 mg of protein per ml). Reaction time = 30 minutes.
- C. 200 μ l E-III (in 0.1 M Tris-glycerol, pH 8.0; 0.45 mg of protein per ml). Reaction time = 120 minutes.

Experiment	Enzyme fraction	Total protein mg	Specific enzyme activity	Total units	Yield %
A	100,000 x g supernatant	1022	50	51,200	100
B	AS-III	240	179	43,000	84
C	E-III	38	650	24,800	48.5

ments in which time and temperature were variable. The enzyme displayed a well-defined temperature optimum at 30° while exhibiting a broad maximal velocity between 0.01 M and 0.05 M Mg⁺⁺. The formation of Ψ -UMP was directly proportional to time from 0 to 120 minutes, and to the amount of enzyme added from 31 to 126 μ g of protein per ml.

The pH optimum for the formation of Ψ -UMP was determined with ammonium sulfate fraction III. The results shown in Figure 7 indicate maximal velocity between pH 8.0 and 8.5.

The Lineweaver-Burk plots²⁶ in Figure 8, A and B, demonstrate the dependence of the reaction velocity on the respective concentrations of uracil and ribose-5-P. No inhibition was observed at substrate concentrations up to 0.0002 M. The apparent Michaelis constants for uracil and ribose-5-P were 7.1×10^{-5} and 2.4×10^{-5} M respectively at near saturation concentrations of the latter substrate.

Ecteola fraction E-III was found to contain a maximum of 0.15 μ mole of phosphate per mg of protein. In the reaction described in Figure 2, 0.53 μ mole of Ψ -UMP were formed by the enzyme, which may have contained as much as 0.91 μ mole of phosphate. It therefore seemed possible that ribose-5-P might undergo activation prior to reaction with uracil. When aliquots were removed at various times from reaction mixtures catalyzed by E-III and subjected to phosphate analysis, no release of orthophosphate was detected. A similar experiment indicated no loss of pentose during Ψ -UMP synthesis except for that utilized in nucleotide formation. If activation of the ribose-5-P did occur, then it seemed most likely that it would take place at C₁. A ribose derivative which might plausibly serve as an intermediate in the synthesis of Ψ -UMP was ribose-1,5-diphosphate. However, when the rates of Ψ -UMP synthesis from ribose-5-P and ribose-1,5-diP were compared, the former compound was shown to be twice as efficient as the latter. That activity seen with ribose-1,5-diphosphate may have been due to its conversion to ribose-5-phosphate by the enzyme preparation.

Figure 6. Formation of Ψ -UMP by Ecteola fraction E-III under varying conditions of temperature, Mg^{++} concentration, enzyme concentration and time. **A** - Temperature: Reaction mixtures contained 0.1 μ mole of uracil-2- C^{14} (200,000 cpm per μ mole), 0.2 μ mole of ribose-5-P, 10 μ moles $MgCl_2$ and 200 μ l of E-III (0.126 mg of protein) in a total volume of 1.0 ml. Reaction was for 60 minutes at various temperatures designated; **B** - Mg^{++} : Reaction mixtures were prepared as in **A** except they contained Mg^{++} at the indicated concentrations. Reaction was for 60 minutes at 30°; **C** - Enzyme: Reaction mixtures were prepared as in **A** except they contained the indicated amounts of enzyme preparation E-III. Reaction was for 60 minutes at 30°; **D** - Time: Reaction mixtures were prepared as in **A** and were incubated at 30° for the times designated.

The stoichiometry of conversion of uracil and ribose-5-P was determined using the SE-III fraction of Ψ -UMP-synthetase (Table 5). It can be seen that equimolar quantities of uracil and ribose-5-P were consumed with the appearance of an equimolar amount of Ψ -UMP. Since the magnitude of change (ca 2 per cent) in ribose-5-P concentration during the formation of Ψ -UMP is less than the experimental error involved in the method, the figures presented in Table 5 for this compound do not have quantitative significance. They indicate only that the change in concentration is in the right direction, and of a magnitude which precludes extensive degradation of the ribose-5-P.

The experiment described in Table 6 was designed to demonstrate the reversibility of the reaction and the effect of phosphate, if any. It may be noted that uracil was not isolated from these mixtures as such, but as the non-nucleotide material not retained by the anion-exchange resin. In other words, no differentiation was made between uracil and Ψ -uridine which might arise by phosphomonoesterase or 5'-nucleotidase activity. Therefore a control was run (Exper-

Figure 7. Ψ -UMP formation by ammonium sulfate fraction AS-III as a function of pH. Reaction mixtures contained 0.1 μ mole of uracil-2- C^{14} (200,000 cpm per μ mole), 0.2 μ mole of ribose-5-P, 10 μ moles of $MgCl_2$, 0.2 mmole of Tris buffer at the indicated pH and 50 μ l of AS-III (in 0.01 M Tris buffer containing 20 per cent glycerol at pH 8.0) in a total volume of 1.0 ml. Solutions were incubated 30 minutes at 30° and analyzed for nucleotide on columns of Dowex-1-chloride. The pH of each reaction mixture was determined after incubation.

iment C, Table 6) containing UMP-2- C^{14} in place of its isomer. No uracil or nucleoside counts resulted from this parallel experiment, an indication that these interfering enzymes were absent. The presence of phosphate had no essential effect on the breakdown of Ψ -UMP. Equilibrium was apparently reached after 1 hour in Experiment A; similar values were obtained in both experiments (A and B) after 2 hours. From these studies on hydrolysis of Ψ -UMP it would seem that the equilibrium of the reaction favors the synthesis of the nucleotide, although equilibrium was never attained in the forward reaction. The highest conversion of uracil to Ψ -UMP observed was 23 per cent. The data presented therefore do not permit calculation of an equilibrium constant for the reaction; further work on this problem is in progress.

The specificity of Ψ -UMP-synthetase towards the pyrimidine substrate was determined for 3 uracil analogs (Table 7). The total nucleotides formed from 2-thiouracil and isocytosine were approximately one-half, and one-third respectively of that synthesized from uracil under similar conditions. No conversion of cytosine to nucleotide was observed.

DISCUSSION

The metabolic origin of the unique carbon-carbon ribosyl linkage of Ψ -uridine and its phosphorylated derivatives has recently been under active investigation in several laboratories. From experiments in yeast²⁷ and in the pyrimidine-requiring *Neurospora crassa* 36601,²⁸ Hall and Allen suggested that either the pathway of Ψ -UMP biosynthesis was as direct as that leading to

Figure 8. Lineweaver-Burk plot of Ψ -UMP formation by Ecteola fraction E-III as a function of substrate concentration. A - uracil; B - ribose-5-P. Reaction mixtures were prepared as in Table 5, Step III, varying the concentrations of appropriate substrate. Incubation was for 30 minutes at 30°. Enzyme activity is plotted on the ordinate at the number of μ moles of Ψ -UMP formed per 30 minutes.

Table 5

STOICHIOMETRY OF CONVERSION OF URACIL AND
RIBOSE-5-PHOSPHATE TO Ψ -UMP

Two reaction vessels were prepared containing 0.55 μ mole of uracil-2-C14 (200,000 cpm per μ mole), 3.27 μ moles of ribose-5-P, 100 μ moles of MgCl_2 and 2.0 ml of SE-III in a total volume of 6.0 ml. The control mixture was immediately boiled; the reaction mixture was incubated for 6.5 hours at 30° and boiled. The solutions were analyzed for uracil, ribose-5-P and Ψ -UMP on columns of Dowex-1-formate (2.0 cm x 1.0 cm²).

Remarks		Uracil μ moles	Ribose-5-P μ moles	Ψ -UMP μ moles
Control	Added	550	3270	0
	Isolated	548	3280	0
	Δ	-2	+10	0
Reaction	Added	550	3270	0
	Isolated	502	3215	49
	Δ	-48	-55	+49

Table 6

HYDROLYSIS OF Ψ -UMP BY ECTEOLA FRACTION III IN THE
PRESENCE AND ABSENCE OF ORTHOPHOSPHATE

Experiment A: 0.75 μ mole of Ψ -UMP-2-C¹⁴ (200,000 cpm per μ mole), 25 μ moles of MgCl₂, and 500 μ l of E-III in a total volume of 2.5 ml.

Experiment B: Same as A, containing in addition 2.5 μ moles of Na₂HPO₄ (pH 8.0).

Experiment C: Same as A, except containing 0.125 μ moles of UMP-2-C¹⁴ (200,000 cpm per μ mole) in place of Ψ -UMP.

Aliquots (500 μ l) from A and B were removed at 0, 30, 60 and 120 minutes. 1.0 ml quantities were removed from C at 0 and 120 minutes.

Time minutes	Experiment A		Experiment B		Experiment C	
	Uracil m μ moles	Ψ -UMP m μ moles	Uracil m μ moles	Ψ -UMP m μ moles	Uracil m μ moles	UMP m μ moles
0	0	14.2	0	14.4	0.6	50
30	0.8	13.5	0.8	13.4		
60	1.9	12.1	1.4	12.2		
120	1.8	12.1	1.7	12.0	0.8	51

Table 7

SPECIFICITY OF Ψ -UMP-SYNTHETASE
FOR PYRIMIDINE SUBSTRATE

Experiment A: 0.1 μ mole of uracil-2-C¹⁴ (200,000 cpm per μ mole), 0.2 μ mole of ribose-5-P, 10 μ moles of MgCl₂ and 200 μ l of fraction E-III in a total volume of 1.0 ml.

Experiment B: Same as A except containing 0.1 μ mole of cytosine-2-C¹⁴ (300,000 cpm per μ mole) in place of uracil.

Experiment C: Same as A except containing 0.1 μ mole of 2-thiouracil-2-C¹⁴ (365,000 cpm per μ mole) in place of uracil.

Experiment D: Same as A except containing 0.1 μ mole of isocytosine in place of uracil.

Experiment	Pyrimidine substrate	Total nucleotide formed m μ moles
A	Uracil	6.5
B	Cytosine	0.0
C	2-thiouracil	3.2
D	Isocytosine	2.0

UMP, or that a uridine phosphate might serve as an intermediate in the formation of Ψ -UMP. This latter hypothesis was questioned by Breitman²⁹ on the basis of labeling experiments in the intact rat in which he found a significantly higher incorporation of orotic acid into Ψ -UMP than into UMP.

Lis and Allen³⁰ postulated an interconversion of uridine and Ψ -uridine via a hypothetical 3,5-diribosyluracil intermediate. This mutase mechanism was derived from a study of the metabolism of a pseudomonad adapted to growth on Ψ -uridine. Support for this mechanism was provided by the studies of Pollack and Arnstein³¹ who isolated a compound, tentatively called 3,5-diribosyluracil, formed during Ψ -uridine synthesis by crude extracts of cells of Escherichia coli (W3301). A recent paper by Lis and Lis³² describes the isolation and characterization of a presumed 3,5-diribosyluracil which they obtained from commercial uridine, and as a by-product during the enzymic synthesis of 5-ribosylisocytosine by extracts of Penicillium brevi-compactum. This organism was grown on Ψ -uridine in a manner analogous to the growth of the pseudomonad previously described.³⁰ The cytoplasmic supernatant material from the Penicillium brevi-compactum was found to contain enzymes catalyzing the amination of Ψ -uridine to a presumed 5-ribosylisocytosine, as well as enzymes that catalyze the addition of ribose phosphates to isocytosine to form nucleotides containing 5-ribosylisocytosine and 3-ribosylisocytosine.³³ Lis and Lis³⁴ have also reported finding various nucleotide derivatives of 3,5-diribosyluracil in hydrolysates of RNA from Ehrlich ascites tumor cells.

Additional evidence in favor of the metabolic relationship of uridine and Ψ -uridine was presented by Robbins and Hammond³⁵ who showed that when yeast was grown on uridine doubly labeled in both the pyrimidine and ribose portions, both segments of the molecule served as direct precursors of Ψ -uridine isolated from the s-RNA. They suggested that Ψ -uridine might be formed via some sort of rearrangement of uridine (or a uridine derivative) at the mononucleotide or polynucleotide level. More recent experiments by Robbins and Kinsey³⁶ have suggested that the proposed rearrangement is intramolecular.

If the interconversion of uridine and Ψ -uridine via an intermediate 3,5-diribosyluracil represents a universal mechanism of synthesis of the ribosyl linkage of Ψ -uridine, and if incorporation of the nucleoside into RNA involves its phosphorylation to the di- or triphosphate level,^{37,38} then one might expect to be able to demonstrate an enzyme comparable to uridine kinase (if not uridine kinase itself) capable of phosphorylating Ψ -uridine. It has been shown in this paper that uridine kinase is inactive toward Ψ -uridine, and attempts to demonstrate a Ψ -uridine kinase were unsuccessful. This latter negative finding does not of course constitute evidence that such an enzyme might not exist. However phosphorylation of the nucleotide and incorporation into RNA have been shown to occur via known enzymic processes.^{24,39} It may well be that the nucleoside interconversions described above are not involved in the synthesis of the ribosyl bond in Ψ -uridine but represent catabolic transformations in these systems. In none of the papers dealing with this mutase mechanism has evidence for Ψ -uridine synthesis been presented in terms of clearly defined enzymes and substrates.

During the course of this investigation the biosynthetic pathways for UMP and Ψ -UMP in T. pyriformis were elucidated and some of the enzymes were partially purified and characterized. UMP synthesis in these organisms may occur directly via the condensation of uracil with PP-ribose-P, or indirectly through the formation and subsequent phosphorylation of uridine. No conversion of orotic acid to UMP was observed in extracts from these organisms. In addition to

these known enzyme systems a new enzyme, tentatively called Ψ -UMP-synthetase, was demonstrated, partially purified, and characterized. Stabilization of the Ψ -UMP-synthetase was achieved using buffers containing 20 per cent glycerol; the mode of enzyme stabilization by this compound is at present unknown but does not seem to be correlated with the maintenance of sulfhydryl structures.* Preparations containing glycerol were stable to dialysis and further purification, and retained full activity when stored as long as 1 year at -20° . In experiments with crude, undialyzed preparations it was observed that the presence of added uridine had no effect on the specific radioactivity of the Ψ -UMP formed from labeled uracil, but that of the UMP was reduced significantly (Figure 2). Although this evidence was not definitive, it suggested that synthesis of Ψ -UMP did not proceed via uridine as an intermediate. The reduction in the specific radioactivity of UMP observed in this study may be explained by the known presence of uridine kinase in the crude preparation. Further experiments indicated that UMP was not an intermediate in the synthesis of Ψ -UMP in these systems. The higher yield of the latter compound in the study described in Table 2, experiment D as compared with experiment C, may be explained by mass-action considerations in which 2 enzymes are competing for the same substrates; thus:

The addition of UMP to this system would be expected to suppress the synthesis of UMP and increase Ψ -UMP formation as was actually observed in this experiment. These results indicate that the following reaction sequence is untenable:

Thus, even in crude preparations, the evidence suggested a direct synthesis of Ψ -UMP in *T. pyri-formis* independent of both uridine and UMP. Conclusive evidence was obtained with the purification and separation of the enzymes responsible for UMP and Ψ -UMP biosynthesis.

The apparent Michaelis constants presented for uracil and ribose-5-P may be questioned on the basis of whether or not initial reaction rates were observed during the determinations with low substrate concentrations. The reaction rate for standard assay mixtures containing higher levels of substrate was shown to be linear over a 2 hour period. The time chosen in obtaining the K_M values was 30 minutes, but it is not certain that initial reaction rates were measured during this time for all determinations. The Lineweaver-Burk plots were linear (Figure 8) and the K_M value obtained for ribose-5-P was approximately one-third of that obtained for uracil. These results suggest a somewhat higher affinity of the enzyme for the former substrate. Demonstration of the stoichiometry of the conversion of uracil and ribose-5-P to Ψ -UMP indicated that the reaction was not coupled to energy-yielding degradative transformations of the substrates. Activation

*J. Jarabak, unpublished observations.

of the C_1 position in ribose-5-P by either P_i or PP_i seems unlikely in consideration of experiments in which ribose-5-P proved to be a more efficient precursor of Ψ -UMP than did ribose-1, 5-diP or PP-ribose-P. The observed reaction with these latter derivatives is explained by the fact that the labile C_1 phosphate and pyrophosphate bonds are readily hydrolyzed to yield ribose-5-P. In addition, the commercial PP-ribose-P used contains significant amounts of ribose-5-P.⁴⁰ The presence or absence of P_i had no effect on either the rate of formation of hydrolysis of Ψ -UMP and no release of P_i was detected during Ψ -UMP synthesis. Hence, it would seem that if activation of the ribose-5-P is a necessary step prior to condensation with uracil, this activation is not mediated by P_i or PP_i . It is however conceivable that a covalently-bound enzyme phosphate might combine with the carbonyl position of ribose-5-P to form a transitory activated intermediate that would then react with uracil to yield Ψ -UMP without rupture of the enzyme-phosphate bond. Because purified enzyme preparations were active after extensive dialysis, any activator would have to be present as a very tightly bound cofactor.

Other enzymes have been demonstrated to catalyze the formation of carbon-carbon bonds without activating steps. Thus delta-aminolevulinic dehydrase³⁶ has been shown to catalyze the synthesis of porphobilinogen via the condensation of 2 molecules of delta-aminolevulinic acid. The formation of thymidylic acid from deoxy-UMP, a formyl donor, and tetrahydrofolic acid in the presence of calf thymus thymidylate synthetase³⁷ represents a reaction similar to that proposed in this paper for Ψ -UMP biosynthesis.

A consideration of the possible electronic configurations of uracil and ribose-5-P obviates the necessity (on theoretical grounds) of an intermediate activating step in Ψ -UMP formation. The properties of pyrimidines are governed mainly by the electronegativity of the 2 nitrogen atoms. Since they are separated by 1 carbon atom, each reinforces the electronic effect of the other in the 2-, 4- and 6-positions. Nucleophilic attack is therefore facilitated at these positions and electrophilic displacement, if it occurs at all, would tend to take place at position 5. In other words, position C_5 is least deactivated toward electrophilic reagents.

In uracil, position C_5 is both ortho and para to the strongly ortho-para-orienting hydroxyl groups, and hence represents a favorable site for electrophilic substitution. In agreement with this concept, the electrophilic bromination and nitration of uracil lead to the production of the corresponding derivative substituted at position C_5 . Furthermore, this position in deoxyuridylic acid is the site of enzymatic methylation in the synthesis of thymidylic acid.

In addition to several resonance hybrids, uracil may occur in either the lactam or lactim tautomeric forms, and although the former is highly predominant, interconversion of the tautomers would be favored at the pH used in these experiments. Thus, uracil may display the carbanion configuration as shown in Figure 9, II. The furanose form of ribose-5-P is in equilibrium

Figure 9. Hypothetical reaction sequence for the synthesis of Ψ -UMP.

with the free aldehyde, the C_1 position of which is partially positively charged due to the inductive effect of the carbonyl oxygen. The formation of Ψ -UMP via the electrophilic attack of ribose-5-P on the uracil carbanion might then occur as shown in Figure 9. This hypothetical reaction scheme may be presented in two parts: 1) formation of the carbon-carbon bond via an aldol-type condensation and 2) the elimination of water to form Ψ -UMP. These are separated for clarity and do not imply 2 separate reactions.

Nucleotide formation catalyzed by Ψ -UMP-synthetase was shown to occur with uracil analogs in which the C_2 hydroxyl was replaced by an SH group (2-thiouracil) or by an NH_2 substituent (isocytosine), but not when the C_6 hydroxyl was replaced by an NH_2 group (cytosine). Since the amine group is also strongly ortho-para directing, and would be expected to favor the existence of the uracil carbanion, these findings may reflect the requirement of the C_6 hydroxyl in the formation of an enzyme-uracil complex.

The concept that Ψ -UMP might be formed at the polynucleotide level is attractive in that it conforms to current ideas concerning the coding mechanism. A presumptive s-RNA consisting of the 4 "normal" bases adenine, guanine, cytosine and uracil would be formed on the DNA template and then be transformed into s-RNA by rearrangement of appropriate uridine residues to Ψ -uridine and by methylation of specific bases. Alternatively, Ψ -UMP may be phosphorylated to

the triphosphate and incorporated in some specific manner into s-RNA. This would imply a site of s-RNA synthesis in which a distinction between UTP and Ψ -UTP would be made. Goldberg and Rabinowitz⁴¹ observed that incorporation of Ψ -UTP into RNA was not entirely analogous to UTP and suggested that the presence of 2 sites, differing in their relative selectivity for UTP and Ψ -UTP, in the RNA synthesized by RNA-polymerase systems. If the results of this investigation are of general significance and are not limited to the organism Tetrahymena pyriformis, then they provide support for the latter hypothesis, i.e., that the carbon-carbon ribosyl linkage is formed prior to the incorporation of the Ψ -uridine residue into RNA. Then it would seem that the transmittance of information from DNA (or RNA) which specifies the site of incorporation of Ψ -UTP into RNA might be coded either by more than one nucleotide determinant, or by some as yet undiscovered minor constituent of DNA.

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ENZYMATIC SYNTHESIS OF RIBONUCLEIC ACID. I. PREPARATION OF RNA POLYMERASE FROM EXTRACTS OF *M. LYSODEIKTICUS**

By

T. Nakamoto,[†] C. F. Fox[‡] and S. B. Weiss

The problem of complex polynucleotide synthesis has received considerable attention from numerous investigators.¹⁻³ Grunberg-Manago and Ochoa⁴ and Kornberg, Lehman, and Simms⁵ first successfully demonstrated the *in vitro* polymerization of ribo- and deoxynucleotides with cell-free extracts from microorganisms. Subsequent studies on RNA and DNA synthesis in other laboratories, including our own, were guided primarily by the experience of these two groups. The different mechanisms and substrate specificities demonstrated for polynucleotide phosphorylase⁶ and DNA polymerase⁷ first directed our attention to the possible existence of yet other enzymes which catalyzed nucleotide polymerization. At that time, no adequate mechanism was known for the synthesis of RNA molecules whose base composition resembled that of DNA.⁸

A preliminary report from this laboratory⁹ first described the presence of an enzyme in rat liver nuclei which carried out the incorporation of ribonucleotides into RNA and required the participation of all four ribonucleoside triphosphates. The properties of this mammalian cell-free particulate system for ribonucleotide incorporation were distinctly different from previously reported reactions.¹⁰ However, some investigators had described the stimulation of RNA synthesis by ribonucleoside di- and triphosphates in cell membrane fractions prepared from *E. coli* protoplasts.¹¹ A more detailed analysis of the product formed by the liver nuclei system showed that ribonucleotide incorporation occurred throughout the entire RNA molecule and was dependent upon intact DNA.¹²

Subsequent reports have now confirmed the presence of similar reactions in extracts from other mammalian tissues,¹³ from plants¹⁴ and from microorganisms.¹⁵⁻¹⁸ In all these cases, the reaction mechanism for the DNA-dependent synthesis of RNA (Figure 1) appeared similar to

Figure 1.

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[†] Present address: Rockefeller Institute, New York City, N.Y.

[‡] Predoctoral fellow of the National Science Foundation.

the DNA-dependent synthesis of DNA.⁷ For this reason, we initially referred to this enzyme as RNA polymerase. More recently, we have found that bacterial RNA polymerase preparations also catalyze an RNA-dependent synthesis of RNA in a manner similar to the DNA primed reaction.¹⁹ Originally we were unable to determine whether one or two enzymes were involved. However, closer examination of the reaction properties of both types of primer suggested that a single enzyme was responsible for both activities.²⁰ The ability of RNA polymerase to utilize either RNA or DNA templates to assemble complementary ribopolynucleotide molecules is an important distinction from DNA polymerase. The results on RNA priming will be described in a separate publication.

Whereas the extensive purification of mammalian RNA polymerase from nuclei has not been successful, the preparation of highly purified enzyme from microbial extracts has been possible.^{18,21,22} This report describes in detail the methods for the extensive purification of RNA polymerase from *M. lysodeikticus*, and some of the contaminants associated with this preparation.

EXPERIMENTAL PROCEDURE

Preparation of P³²-labeled ribonucleotides. Ribonucleoside triphosphates labeled with P³² in the ester phosphate were prepared by the procedure of Tener.²³ The 2',3'-benzylidene derivatives of cytidine and uridine, prepared by the method of Baddiley et al.²⁴ and the 2',3'-isopropylidene derivatives of adenosine and guanosine, obtained from the Nutritional Biochemical Corporation, were used to prepare P³²-labeled nucleoside monophosphates. The labeled mononucleotides were converted to the triphosphates by a crude brewers yeast extract which had been purified (2-fold) with respect to its cytidylate kinase activity. The labeled ribonucleoside triphosphates were isolated from the ribonucleotide kinase reaction mixture by chromatography on Dowex 1 (Cl⁻) as previously described.¹⁰ The eluted triphosphates were concentrated by precipitation with BaBr₂ and ethanol, and then converted to the potassium salt with Dowex 50 (K⁺). The degree of contamination of the labeled triphosphates with both mono- and diphosphates was determined by paper electrophoresis and was usually less than a few per cent.¹⁰ Each synthetic ribonucleotide was routinely examined for its characteristic absorption in the ultraviolet region of the spectrum.

Assay of ribonucleotide kinase. The reaction mixture for the enzyme assay contained 5 μ moles of MgCl₂, 20 μ moles of Tris (pH 7.4), 2 μ moles of ATP, 0.05 μ moles of CMP³², and the yeast fraction, in a final volume of 0.20 ml. Various quantities of yeast enzyme were assayed for each preparation. After 15 minutes at 37°, the reaction was stopped by the addition of 0.05 ml of 0.5 M citrate buffer of pH 4.6, and placed in ice. The precipitate which appeared was removed by centrifugation. Aliquots of the clear supernatant fluid (0.03 ml) were subjected to paper electrophoresis in 0.025 M citrate buffer of pH 4.6, at 250 volts, for 16 hours. The enzyme activities of the various fractions were calculated from the amount of P³² in the CTP area located under ultraviolet light. One μ mole of CMP³² converted to CTP³² in 1 hour represents 1 unit of activity. Protein was determined by the method of Lowry.²⁵

Preparation of ribonucleotide kinase. The preparation of this crude kinase system is based on the procedure reported by Lieberman et al.²⁶ Four 500-gm portions of dried brewers yeast (Anheuser-Busch) were blended, each for 1 minute at room temperature, with a total of 8 liters of 0.10 M NaHCO₃ which had been warmed to 45° immediately before use. The mixture was auto-

lyzed at 37° with slow but constant stirring and after 4.5 hours was chilled in an ice bath, centrifuged at 15,000 x g for 15 minutes, and the supernatant fluid saved (Fraction I). Table 1 summarizes the purification steps for the yeast kinase preparation. All further operations were performed at 2 to 4° and all centrifugations were at 15,000 x g for 15 minutes.

Table 1
PURIFICATION OF RIBONUCLEOTIDE KINASE

Fractions	Protein mg/ml	Units total	Specific activity units/mg protein
Autolysate (Fraction I)	21.60	101,000	1.18
First ammonium sulfate (Fraction II)	18.40	90,000	1.22
Second ammonium sulfate (Fraction III)	10.30	98,500	2.40

To 3,970 ml of Fraction I was added 1,050 gm of solid ammonium sulfate, with stirring, and after 20 minutes the precipitate was removed by centrifugation. An additional 1015 gm of ammonium sulfate was added to the supernatant fluid; after 20 minutes the precipitate was collected by centrifugation, suspended in 1 liter of 0.05 M Tris, pH 7.4, and then stored at -20° (Fraction II). The next day, 220 ml of Fraction II was thawed; 3 volumes of cold water were added, followed by 232 gm of ammonium sulfate. After 20 minutes at 0°, the precipitate was removed by centrifugation; 88 gm of ammonium sulfate was added to the supernatant fluid, and after 20 minutes at 0°, the precipitate was collected by centrifugation. The pellet was dissolved in 135 ml of cold water and stored at -20° (Fraction III). Portions of this preparation were thawed, dialyzed against 0.02 M Tris at pH 7.4, and used when needed. This preparation catalyzes the phosphorylation of CMP, AMP, UMP and GMP in the presence of ATP, to their respective triphosphates. It is extremely stable at -20°, and after 4 years still retains most of its activity. The reaction mixture for the conversion of the mononucleotides to the triphosphates with the yeast preparation has been described elsewhere.¹⁰

Assay of RNA polymerase. This assay measures the conversion of a P³²-labeled ribonucleoside triphosphate into an acid-insoluble product. The incubation mixture contained 50 μmoles of Tris-HCl buffer (pH 7.5), 0.80 μmoles of spermidine phosphate, 1.25 μmoles of MnCl₂, 0.40 μmoles each of ATP, CTP, GTP and UTP (one of the triphosphates being labeled with P³² and having a specific activity ranging from 0.50 x 10⁶ to 2 x 10⁶ cpm per μmole), 100 μg of calf thymus DNA, and enzyme, in a final volume of 0.50 ml. The mixture was incubated for 10 minutes at 30°, and the reaction was stopped with 0.10 ml of 50 per cent cold trichloroacetic acid. After 3 minutes at 0°, 2 ml of cold 5 per cent trichloroacetic acid was added; the acid-insoluble material was collected by filtration through membrane filters ("Millipore," Type HA, 0.45 μ pore diameter), and washed 3 times with successive 5 ml portions of cold 5 per cent acid. The filters were then dried in planchets, and the radioactivity determined in a gas flow counter.

Two controls, one omitting Mn⁺⁺, and the other DNA, were run with each assay. A unit of enzyme activity was defined as the amount catalyzing the incorporation of 1 μmole of labeled CTP or GTP into the acid-insoluble material during 10 minutes of incubation at 30°. The specific activity was expressed as units per mg of protein.

The acid-insoluble counts could be extracted with hot salt at neutral pH, precipitated with ethanol, and were non-dialyzable. Treatment with RNase or alkali, but not DNase, converted the labile material into an acid-soluble form.

Calf thymus DNA (Sigma Chemical Company) was dissolved in 0.01 M NaCl-0.01 M Tris at pH 7.5 and adjusted to 40 optical density units per ml at 260 m μ . A solution containing 1.0 mg of DNA per ml was assumed to have an extinction value of 20 at this wavelength. Calf thymus DNA solutions which had stood for a few weeks at 4° were routinely discarded since after 4 weeks their priming activity became progressively poorer. Little or no loss of priming activity was observed for bacterial and bacterial phage DNA over a period of several months. The "inactivation" of thymus DNA varied with each new commercial preparation.

Purification of RNA polymerase. All centrifugations were carried out at 2° to 4° in a Lourdes refrigerated centrifuge, Model LRA, unless otherwise stated. The centrifugal forces indicated below for each spin represent the maximal gravitational force obtained as listed by the Lourdes Speed set table. Except for the washing and lysing of cells, all operations were conducted as close to 0° as possible.

Protein concentrations were estimated by determining the absorbancies at 280 and 260 m μ and calculating the protein concentration by the approximate formula, $1.54 \times A_{280} - 0.76 \times A_{260}$ = protein concentration in mg per ml.²⁷

Where the 280 to 260 m μ ratio was lower than 1.0 (Fractions I and II), protein concentrations were determined by the method of Lowry *et al.*,²⁵ with crystalline bovine serum albumin as standard.

Procedure A

Washing and lysis of cells. Spray-dried cells of *M. lysodeikticus* were purchased from the Miles Chemical Company, Clifton, New Jersey. Sixty g of dried bacteria was suspended in 600 ml of 0.01 M Tris at pH 8.0 in a Waring Blendor for 1 minute at room temperature. The cells were collected by centrifugation at 20,000 x g for 10 minutes, and resuspended in a Waring Blendor in a solution prewarmed to 30° containing 0.01 M Tris at pH 8.0, and 0.20 M sucrose, to a final volume of 1200 ml. For convenient handling, the cell suspension was divided into 2 equal portions, and 150 mg of crystalline lysozyme (Worthington Biochemical Corporation) dissolved in 10 ml of the Tris-sucrose solution, was added to each portion, stirred and incubated at 30°.

At 15 minutes after the lysozyme addition, 1.5 ml of 0.10 M MgCl₂ was added to each portion and stirred vigorously. At 45 minutes, 4.5 ml of 0.10 M MgCl₂ was added to each portion, followed by 600 ml of ice cold water. The lysates were chilled by packing in ice, and stirred vigorously to a uniform viscous gel. The lysed preparation was allowed to stand for 10 minutes to assure complete lysis of protoplasts (Fraction I). Table 2 summarizes the progress of the purification and the enzyme yields.

Streptomycin treatment. 120 ml of 10 per cent streptomycin sulfate was added dropwise with stirring to each portion over a period of about 10 minutes. Initially, the viscous solution was stirred vigorously by hand, but after half the streptomycin had been added, magnetic stirring was possible. After 10 minutes, the precipitate was collected by centrifugation for 10 minutes at 20,000 x g. The supernatant was decanted and the pellets rinsed with 10 ml of cold water. The combined pellets from both portions were washed by resuspending, with the aid of a glass rod, in 400 ml of a solution containing 0.001 M Tris at pH 8.0, 0.20 M sucrose, 0.1 per cent streptomy-

cin sulfate, and 0.00025 M MgCl_2 . This suspension was mixed for 10 minutes and centrifuged at 20,000 x g for 10 minutes. The supernatant fluid was discarded and the pellets were rinsed with 10 ml of cold water.

Table 2
PURIFICATION OF RNA POLYMERASE

Fraction no.	Purification step	Volume ml	Total activity units	280/260	Total protein mg	Specific activity units/mg
I	Lysozyme lysate*	2400	30,000	0.54	20,000	1.5
II	Streptomycin	350	39,300	0.52	4,970	7.9
III	First protamine	90	37,400	1.13	287	130
IV	Second protamine	15	36,200	1.45	128	283
V	Ammonium sulfate	6	36,150	1.57	65	556
VI	Diethylaminoethyl cellulose	6	22,400	1.73	32	700

* Activity measurements were made on the supernatant obtained after 5 minutes sonication and centrifugation at 105,000 x g for 2 hours.

The precipitate was suspended using a loose fitting Teflon homogenizer at high speed, with 360 ml of a solution containing 4 ml of 1.0 M potassium phosphate at pH 7.5, 4 ml of 0.10 M MgCl_2 , and 40 ml of 2.0 M sucrose. After suspension, 16 ml of 1.0 M potassium phosphate at pH 7.5 was added and stirred vigorously. The viscous gel which formed was allowed to stand for 10 minutes before 20 ml of 10 per cent streptomycin sulfate was added dropwise with constant stirring. After mixing for 10 minutes, the supernatant fluid (400 ml) was collected by centrifugation for 30 minutes at 30,000 x g and then centrifuged at 30,000 rpm for 2 hours in a Spinco Model L centrifuge using the Spinco number 30 rotor. The supernatant fluid was carefully decanted to avoid collection of any loosely packed sediment and the volume carefully measured (Fraction II).

First protamine sulfate treatment. From a 2.5 per cent unneutralized protamine sulfate solution (room temperature), an amount equivalent to one-fifth of the total Spinco supernatant was measured and set aside. Assuming that the phosphate concentration of the Spinco supernatant fluid was exactly 0.05 M, sufficient 1.0 M potassium phosphate at pH 7.5, was added so that the final concentration of phosphate buffer after addition of the protamine sulfate solution would be 0.14 M. The measured portion of protamine sulfate was then added dropwise with continuous mixing, and after 10 minutes the precipitate was collected by centrifugation at 20,000 x g for 10 minutes, and the supernatant fluid discarded. The pellets were homogenized in 60 ml of 0.20 M sucrose - 0.20 M phosphate buffer at pH 7.5, and mixed for 10 minutes. The extract was centrifuged at 30,000 x g for 10 minutes and the supernatant fraction saved. The precipitate was extracted once more with 30 ml of the same sucrose-phosphate solution, and the centrifuged supernatant fraction combined with the previous extract (Fraction III).

Second protamine sulfate treatment. To the combined 0.20 M phosphate extracts, 2 volumes of cold 0.10 per cent protamine sulfate were added dropwise with stirring. After 10 minutes, the precipitate was collected by centrifugation at 30,000 x g for 10 minutes, and the supernatant dis-

carded. The precipitate was extracted with 20 ml of 0.20 M sucrose - 0.14 M phosphate buffer at pH 7.5 as described before, and the supernatant discarded after centrifugation. The pellet was then extracted with 10 ml of 0.20 M sucrose - 0.20 M phosphate buffer (same pH), and the supernatant saved after centrifugation for 15 minutes at 30,000 x g. The pellet was extracted once more with 5 ml of the same solution, centrifuged, and the supernatant was combined with the previous extract (Fraction IV).

Ammonium sulfate fractionation. The protein concentration of Fraction IV was adjusted to approximately 6 mg per ml with the extracting medium. A sufficient amount of cold ammonium sulfate solution (saturated at 0°, and previously neutralized to pH 7.0 with NH_4OH) was added dropwise with mixing to 40 per cent of saturation. After mixing for 20 minutes, the precipitate which formed was collected by centrifugation at 30,000 x g for 10 minutes. The pellet was suspended in 3 ml of 0.10 M Tris of pH 7.5, and an equal volume of glycerol was added. This preparation was stored at -20° (Fraction V).

Diethylaminoethyl cellulose treatment. Fraction V was diluted to 80 ml with a solution containing 0.05 M $(\text{NH}_4)_2\text{SO}_4$, pH 7, 0.001 M mercaptoethanol and 20 per cent glycerol (V/V). DEAE (30 mg per mg protein), which had been previously washed with 1 N NaOH, 1 N HCl and equilibrated with 0.01 M Tris HCl buffer, pH 7.5, was added and mixed for 10 minutes. The DEAE-protein slurry was transferred to a conical Buchner funnel, and the solution removed by applying a mild vacuum. The filtrate was discarded. The DEAE was washed on the funnel by successive treatment with 50 ml of a solution containing 0.05 M $(\text{NH}_4)_2\text{SO}_4$, 0.001 M mercaptoethanol and 0.2 M sucrose (pH 7) and then 15 ml of a similar solution which was 0.10 M in $(\text{NH}_4)_2\text{SO}_4$. The enzyme was then eluted with successive 3 ml portions of a similar solution which was 0.30 M in $(\text{NH}_4)_2\text{SO}_4$. Seven fractions were collected (excessive foaming was avoided) and assayed for "polymerase" activity. Although all fractions, except the first, showed activity, only the third and fourth fractions were combined (Fraction VI).

The protein in Fraction VI was concentrated by the addition of solid ammonium sulfate to 40 per cent of saturation at 0°. The precipitate was collected and suspended in 0.01 M Tris (pH 7.5) to a protein concentration of 6 mg per ml. An equal volume of glycerol was added and the preparation stored at -20°. Ammonium sulfate treatment of Fraction VI usually results in 10 - 15 per cent loss of activity.

The specific activities and total units shown in Table 2 for the purification steps, represent the values in a typical preparation. Higher specific activities have been obtained with some preparations. Assays of Fraction I are only approximate since linearity with the addition of enzyme was difficult to achieve. Proportionality with respect to enzyme addition occurs up to 40 μg of protein with Fraction V or VI. Various batches of commercial *M. lysodeikticus* cells may behave slightly differently, e.g., it may be necessary to add more lysozyme per g of bacteria to obtain comparable lysis in the stated time, or longer times of lysing may be required. Although cells stored at -20° for as long as 2 years may be used for the preparation of active polymerase, spray-dried bacteria kept in cold storage for not more than 6 months were preferred. At pH values above 7.8, and in the presence of MnCl_2 , highly colored solutions formed due to various states of manganese oxidation. In the presence of MgCl_2 , a broad pH optimum was found between 7 and 8.5. Since Mn^{++} was more stimulatory for RNA synthesis than Mg^{++} , the reaction was buffered at pH 7.4 and MnCl_2 was used. Mixtures of MnCl_2 and MgCl_2 were nearly as effective as MnCl_2 alone. The addition of 0.1 M NaCl or KCl to the reaction mixture had no effect.

Procedure B

Prior to the more elaborate purification described in Procedure A, a slightly different method was used for the partial purification of "polymerase." Although enzyme obtained in this way has a lower specific activity, the method is easy, relatively rapid, and provides active material.

Lysis, protamine sulfate, gel and DEAE treatment. Forty mg of lysozyme was added to a suspension of *M. lysodeikticus* containing 8 g of sprayed-dried cells in 200 ml of 0.10 M sucrose - 0.01 M Tris (pH 7.5) at room temperature. After 15 minutes, 0.25 ml of 0.10 M MgCl_2 was added, mixed and allowed to stand further. Another 4.0 ml of MgCl_2 was added and stirred after 60 minutes, when the lysis was complete. The viscous lysate was sonicated for 4 minutes (until completely liquid) in a 10 KC Raytheon sonic oscillator, and centrifuged for 20 minutes at top speed. The supernatant fluid was collected and centrifuged for 2 hours at 30,000 rpm in a Spinco, Model L centrifuge. To the Spinco supernatant, which was carefully decanted to leave a loose sediment behind, a 2 per cent solution of protamine sulfate was added dropwise with stirring, in the proportion of 3.0 mg of protamine for each ml of supernatant. The mixture was stirred for 10 minutes and the supernatant fraction obtained after centrifugation discarded. The precipitate was extracted first with 50 ml of 0.20 M sucrose - 0.05 M potassium phosphate buffer (pH 7.5) by homogenization and stirring for 10 minutes. After centrifugation the supernatant was discarded and the precipitate extracted with 30 ml of 0.20 M sucrose - 0.20 M phosphate (same pH), in the same manner, and the supernatant saved after centrifugation.

To 30 ml of the 0.20 M phosphate extract (7.1 mg of protein per ml) was added 20 ml of a calcium phosphate gel suspension (27.5 mg per ml) and then 150 ml of cold water, dropwise with stirring. After 15 minutes the gel was collected by centrifugation for 1 minute and extracted by stirring with 10 to 12 ml of 0.20 M potassium phosphate buffer (pH 7.5) for 20 minutes. The suspension was centrifuged for 5 minutes and the clear solution carefully removed. An equal volume of glycerol was added to the gel extract and stored at -20° . This preparation had a specific activity of about 80. By treating the gel extract with DEAE cellulose as described under Procedure A, the specific activity of the "polymerase" was increased almost 2-fold.

Assay for inorganic pyrophosphatase. RNA polymerase preparations (200 μg of Fraction V and 100 μg of Fraction VI) catalyzed no release of inorganic phosphate when incubated for 60 minutes at 30° in the presence of inorganic pyrophosphate.

Assay for the hydrolysis of nucleoside triphosphates. The purified fractions (200 μg of either V or VI) when incubated in the presence of all four nucleoside triphosphates for 120 minutes at 30° , with either Mg^{++} or Mn^{++} , showed no detectable release of inorganic phosphate or inorganic pyrophosphate.

Assay for ribonuclease. The enzyme preparation was tested for the presence of ribonuclease by the conversion of P^{32} -labeled RNA to an acid-soluble product. P^{32} -RNA was synthesized with a DNA primer and labeled triphosphates by RNA polymerase, and isolated after phenol treatment and CaCl_2 gradient centrifugation as previously reported.²⁸ One optical density unit of the labeled RNA at 260 $\text{m}\mu$ contained approximately 0.20×10^6 to 0.22×10^6 cpm. The partially purified fractions were incubated with 0.05 O.D. units of the P^{32} -RNA, under conditions used for RNA synthesis, except that no DNA was present. Prior to its use, the labeled RNA was heat denatured at 100° for 10 minutes and cooled rapidly in ice to disorder any RNA-RNA hybrids possibly present.²⁹ After incubation for 120 minutes at 30° , the acid-insoluble material was precipitated with acid

and processed for counting as described above. Table 3 indicates that as little as 0.01 μg of pancreatic ribonuclease rendered 84 per cent of the isotopic RNA acid-soluble. Incubation with relatively large quantities of the *M. lysodeikticus* preparation, Fraction VI, showed no apparent hydrolysis of the P^{32} -RNA under these conditions. The sensitivity of this assay for the presence of ribonuclease must be carefully examined. It is possible that some of the P^{32} -RNA was digested but that the "pieces" formed were sufficiently large to remain acid-insoluble. The presence of small quantities of ribonuclease in these preparations (less than 0.01 μg) cannot be satisfactorily determined by this technique.

Table 3
ASSAY FOR RIBONUCLEASE*

Enzyme preparations	Protein	Acid-insoluble P^{32} -RNA remaining after incubation
	μg	cpm
Zero control	0.10	11,158
Pancreatic ribonuclease	0.10	390
	0.05	610
	0.01	1,789
	80.00	11,659
Fraction VI		

* Conditions of assay are described in the text.

A more sensitive assay for the presence of nuclease activity appears to be one involving the molecular integrity of a biologically active RNA polymer. Incubation of TMV RNA with various quantities of Fraction VI (10, 20 and 50 μg) showed no significant decrease in the infectivity of the plant viral RNA when compared with controls which contained no enzyme. Other experiments have shown a marked affinity of the enzyme to complex with nucleic acid (unpublished results). This assay may therefore be subject to criticism if such complexes rendered the viral RNA less susceptible to nuclease digestion. We are grateful to Dr. R. Haselkorn for the plant viral assays reported here.

Assay for deoxyribonuclease. This assay depends on the change in viscosity of DNA solutions during various periods of time by the addition of "polymerase" preparations. The assay was conducted at 25° in suspended level viscometers by Dr. E. P. Geiduschek to whom we are deeply grateful. Figure 2 shows that rather small quantities of pancreatic DNase rapidly lower the specific viscosity of salmon DNA. However, after 8 hours of incubation of Fraction VI, with a similar solution of DNA, only a 25 per cent decrease had occurred. On a weight basis, one may calculate from the slopes of the two curves that 1 gm of Fraction VI protein contains no more than 2.6×10^{-6} g of DNase.

Assay for DNA polymerase. The conditions of Lehman et al.³⁰ were followed for DNA polymerase assay. The labeled substrate used was dTTP^{32} with a specific activity of 0.40×10^6 cpm per μmole , and calf thymus DNA served as the primer. At the end of the reaction, carrier DNA was added and the acid-insoluble material was precipitated by the addition of 50 per cent TCA. The precipitate was processed for counting as described for RNA polymerase. The addition of

Figure 2. Two separate assays were run in suspended level viscometers. One assay mixture contained in 1 ml, 127 μg of Fraction VI, 8.2×10^{-2} M Tris-HCl buffer (pH 7.5), 9×10^{-3} M MgCl_2 , 8×10^{-4} M each of ATP, GTP, CTP and UTP, 8×10^{-4} M spermidine and 100 μg of salmon DNA. The second assay mixture contained in 1 ml, 12×10^{-3} μg of crystalline pancreatic DNase, 9×10^{-2} M Tris-HCl buffer (pH 7.5), 1×10^{-2} M MgCl_2 , and 123 μg of salmon DNA. The specific viscosities were calculated from the outflow times which were measured at the time intervals indicated above, after the addition of enzyme.

200 μg of Fraction VI showed no detectable incorporation of label into the acid-insoluble precipitate. A partially purified preparation of DNA polymerase was kindly supplied by Dr. B. Zimmerman as a test for the above assay system.

Assay for polynucleotide phosphorylase. Beers³¹ has demonstrated that *M. lysodeikticus* can serve as an excellent source for the isolation and partial purification of polynucleotide phosphorylase. The purified fractions of RNA polymerase were assayed for the presence of polynucleotide phosphorylase by the P^{32} -orthophosphate "exchange" procedure reported by Brummond et al.³² The results indicated that Fraction V catalyzed some P^{32} -ADP exchange; however, no appreciable "exchange" occurred with Fraction VI.

Ultracentrifugal analysis. The ammonium sulfate precipitate in Fraction V was suspended in 0.4 M potassium phosphate buffer (pH 7.5) and then subjected to ultracentrifugation in a Beckman Model E analytical centrifuge. The conditions for this run are given in Figure 3. The movement of the protein was followed by Schlieren optics, and indicated the presence of a minor component moving quite rapidly, and a major component moving more slowly. Although the slower moving peak appears to represent the bulk of the protein, it is apparent that Fraction V is not a completely homogenous preparation.

Electrophoretic analysis. Electrophoresis in a density gradient of glycerol was conducted

Table 4
 P_i^{32} -ADP "EXCHANGE" WITH RNA
 POLYMERASE PREPARATIONS*

Preparation	Protein mg	Norit absorbed counts cpm
A.E. V (zero time)	0.30	4,429
	0.20	12,500
	0.40	11,775
DEAE VI (zero time)	0.16	4,890
	0.10	5,393
	0.20	5,869

* The reaction mixture contained 100 μ moles of Tris, pH 8.1, 2 μ moles of $MgCl_2$, 2.5 μ moles of potassium phosphate (S.A. = 2.1×10^6 cpm per μ mole), 2.5 μ moles of ADP and protein from either Fraction V or Fraction VI in a final volume of 1.0 ml. The mixture was incubated for 30 minutes at 30°. The reaction was stopped by the addition of 0.10 ml of 50% trichloroacetic acid after the addition of 1 mg of carrier bovine serum albumin. The mixture was iced for 5 minutes, the precipitate was removed by centrifugation and a 0.50 ml aliquot of the supernatant removed and mixed with 30 mg of washed Norit A. The Norit suspension was shaken for 10 minutes and then collected by passing over membrane filters. After washing with acid, the filters were dried in planchets and the P^{32} content determined in a gas flow counter.

in the LKB column (Figure 4). Of the 6,200 units applied to the column, over 90 per cent were recovered. The protein was distributed over more than 30 fractions, the peak of enzymatic activity occurring between fractions 49 and 54. Enzyme activity occurs in the same region as the 280 $m\mu$ absorbing material, but the two are not coincident (Figure 4B). It can readily be seen that the counts incorporated per unit of optical density material are not uniform throughout the protein band (Figure 4A). The more rapidly moving material (anode side) has a higher specific activity than the trailing side of the band. Similar results were obtained when the electrophoresis was conducted at pH 6.5 and 8.4.

Density gradient centrifugation. Centrifugation of Fraction VI in a density gradient of glycerol was carried out in the SW-39 rotor in a Spinco Model L centrifuge (Figure 5). At the end of the run, the tube was removed and fractions were collected from the bottom by puncture. The fractions were analyzed for 280 and 260 $m\mu$ absorbing material and for RNA polymerase activity. Figure 5B shows that after 10 hours the protein material had moved more than one-third the length of the tube, and that the enzymatic activity was located in the same region as the 280 $m\mu$ absorbing material. The data also show that the two curves are not identical, and that the activity per unit of O.D. absorbing material is not uniform throughout the band (Figure 5A).

Stability of enzyme. RNA polymerase fractions were routinely stored in 50 per cent glycerol at -20°. Under these conditions, Fraction V was stable for a few days, losing 40 - 50 per cent of its activity in 2 weeks, and Fraction VI was usually stable for more than a few months. The enzyme was stored at concentrations ranging from 3 to 6 mg per ml. Since small quantities of en-

Figure 3. Ultracentrifugal analysis of Fraction V. The enzyme concentration for the above run was 6 mg per ml in 0.4 M potassium phosphate buffer at pH 7.5. Centrifugation was carried out at 50,740 rpm in a 15.95 mm cell at 25.15°. The above photograph was taken after 96 minutes from the start of the run at a bar angle of 35°. Schlieren optics were used.

zyme were routinely used (5 to 30 μ g), the amount of glycerol in the reaction mixture did not exceed 1 per cent. Glycerol up to 10 per cent does not interfere with the polymerization reaction. The LKB Fractions containing an average of 40 - 45 per cent glycerol were kept at 0° for over 6 weeks without any loss of activity. Slow freezing of the enzyme in the absence of glycerol, usually resulted in more than a 50 per cent loss of activity. However, the enzyme may be lyophilized with only small losses of activity if frozen rapidly in a thin layer. The lyophilized preparation can be stored in the dry state for many weeks at -20°, if extreme care is taken to exclude water. This method of storage was not favored since the preparations had a tendency to absorb atmospheric water during weighing out of the dry powder causing partial inactivation. Dialysis of the enzyme invariably led to marked losses of activity. However, on occasion, we have observed

Figure 4. Electrophoresis of Fraction V in a density gradient of glycerol by means of the LKB apparatus. The entire apparatus, including the electrode vessels, contained 0.01 M potassium phosphate buffer at pH 7.5. The upper part of the column contained 10% glycerol in the phosphate buffer. The lower part of the column was filled with a linear gradient extending from 30% to 60% glycerol in phosphate buffer. The ammonium sulfate precipitate in Fraction V was suspended in 20% glycerol in 0.01 M potassium phosphate at pH 7.5. A 4.2 ml sample of enzyme (11.9 mg of protein, S.A. = 522) was introduced from above into the "density shelf" between 10 and 30% of glycerol. The enzyme formed a 5-mm band width which was located 285 mm above the lower end of the electrophoresis tube. After electrophoresis for 38 hours at 750 volts (the entire operation was conducted in a cold room set at 4°) fractions of 3.0 ml were collected from the bottom of the column.

Figure 4B presents the 280 mμ absorbing material (●—●) and the cpm incorporated in the acid-insoluble fraction with ATP³² as the labeled substrate (○--○) with 0.05 ml from each fraction as assayed with calf thymus DNA as primer.

Figure 4A shows the relative activities of the fractions per unit of 280 mμ absorbing material.

Figure 5. Density gradient centrifugation of Fraction VI. The DEAE ammonium sulfate precipitate was suspended in a solution containing 10% glycerol and 0.25 M potassium phosphate of pH 7.5. Approximately 300 μ g of protein (S.A. of 400) in 0.20 ml was layered over 4.1 ml of a linear gradient of 10 to 35% glycerol in phosphate buffer of the same concentration and pH. The mixture was subjected to centrifugation in a SW-39 rotor at 35,000 rpm for 620 minutes at 4°.

Figure 5B shows the absorbancy at 280 $m\mu$ (●—●) and the amount of AMP³² incorporated for 0.04 ml of each fraction collected (■—■).

Figure 5A shows the relative activities of the fractions collected per unit of 280 $m\mu$ absorbing material.

little or no loss of activity when dialyzed against high glycerol (30 - 50 per cent) or high sucrose (1 M to 2 M) concentrations.

The enzyme is stable at 30° for a number of hours, although some loss of activity may be incurred with time. At 50° for 20 minutes, approximately 80 per cent of the enzyme activity is retained. After 5, 10 and 20 minutes at 54°, the activity remaining is 71, 51, and 32 per cent respectively.

DISCUSSION

In mammalian, plant and bacterial cells, RNA polymerase activity appears intimately associated with the cellular DNA. For the purification of *M. lysodeikticus* "polymerase," we have taken advantage of this enzyme-DNA association in the crude lysate by first precipitating the DNA with streptomycin (along with other nucleoproteins), and then extracting the DNA-protein complex with phosphate buffer. The high degree of enzyme purification reported here has not yet resulted in a homogeneous preparation. Physical, chemical and biological assays indicate the presence of contaminating protein, trace contaminants of nucleic acid (less than 0.02 per cent), and small quantities of nucleases. Fractionation of the RNA product by methylated albumin columns,³³ and sucrose density gradient studies, indicate the presence of ribopolynucleotides with a wide variety of molecular sizes ranging from 6 to 12s. The various chain lengths of RNA molecules may be due in part to some nuclease action during the reaction, but also to the transcription of specific DNA regions.

In the early stages of purification (Fraction II) priming by polycytidylate for the synthesis of polyguanylate was detected. However, priming by poly A and poly U separately was detected only with the more highly purified preparations.

In general, 120 g of bacteria were processed through the ammonium sulfate stage in 1 day and then stored in glycerol. Although the enzyme at the protamine sulfate stage of purification may be stored safely in glycerol overnight, this was avoided since it altered the behavior of the enzyme in the succeeding steps. Treatment of Fraction VI by a variety of techniques, e.g., chromatography on phosphocellulose, various gel and apatite preparations, sephadexes of different pore sizes, has not proved successful in further purification.

The purified preparations of RNA polymerase are dependent on the addition of a non-specific polyamine (spermidine, spermine, putrescine, etc.) for optimal synthesis. This phenomenon will be discussed more fully in the succeeding paper. By making appropriate adjustments for the slight differences in the method of assay for the RNA polymerases isolated from *E. coli*^{18,21} and *M. lysodeikticus* (e.g., different temperatures of incubation, DNA primers, labeled nucleotides employed and times of incubation), a comparison of the partially purified enzymes from these two sources indicates that they have similar specific activities. The yield of enzyme reported here appears to be similar to that reported by Chamberlin and Berg¹⁸ but considerably higher than that obtained by Furth et al.²¹ Although it is difficult to compare the degree of contaminating enzymatic activities of these different preparations, the *M. lysodeikticus* preparation appears to be free of polynucleotide phosphorylase, DNA polymerase, and relatively free of RNase and DNase.

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ENZYMATIC SYNTHESIS OF RIBONUCLEIC ACID. II. PROPERTIES OF THE DNA PRIMED REACTION WITH M. LYSODEIKTICUS RNA POLYMERASE*

By

C. F. Fox[†] and S. B. Weiss

The discovery of enzymes which catalyze the DNA-dependent synthesis of ribopolynucleotides¹⁻⁶ stimulated an immediate inquiry into the role played by DNA in this unique reaction. The speculation that DNA might serve as a template for the polymerization of ribonucleotides into a complementary RNA molecule was confirmed by studies from a number of different groups.⁷⁻¹⁵ The evidence for this may be summarized as follows: a) using different DNA's (either natural or synthetic) which varied widely in their respective guanine, adenine, cytosine and thymine content, the base composition of the RNA product always resembled that of the particular primer used; b) the nearest-neighbor frequencies for the 16 possible base pairs in the RNA formed were remarkably similar to the frequencies of the same base pairs in the DNA primer; and, c) specific RNA-DNA hybrids could be formed only by annealing the purified RNA product with the DNA which served to direct its synthesis.

Evidence has now been provided, from three independent sources, that the in vitro reaction results in the formation of RNA molecules which are complementary to both DNA strands.¹²⁻¹⁵ Furthermore, the synthesized RNA is self-complementary, and is unique among ribopolynucleotides in that it may form a DNA-like highly ordered secondary structure.¹³ This information is included in the scheme for RNA synthesis (Figure 1). At the present, the question of whether one or both strands of DNA may serve to prime RNA synthesis in vivo is not resolved.

Although the in vitro studies on RNA polymerase have elucidated the function of the primer and some of the physico-chemical properties of the product, very little is known about the polymerization mechanism itself. A study of the properties of the reaction is important as an initial step in understanding the nature of this complex problem

Two separate DNA-primed reactions have been detected with RNA polymerase: 1) in the presence of all four ribonucleoside triphosphates, an RNA product is formed which is complementary in base composition and sequence to its DNA primer (4 NTP reaction), and 2) in the presence of a single triphosphate, a ribopolynucleotide forms which contains only a single base (single NTP reaction). The formation of RNA by the 4 NTP reaction has been studied by several groups¹⁻¹⁵ and the single NTP reaction has also been observed by several investigators.¹⁵⁻¹⁷ We have confirmed and extended the observation of the DNA primed synthesis of an RNA homopolymer. In this paper we shall describe some of the properties associated with the two types of DNA primed reactions when catalyzed by the partially purified RNA polymerase prepared from M. lysodeikticus.

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[†] Predoctoral Fellow of the National Science Foundation.

Figure 1. Reaction scheme for complementary RNA synthesis.

EXPERIMENTAL PROCEDURE

Materials and methods. The enzyme preparations were prepared from lysed extracts of *Micrococcus lysodeikticus* by Procedure A as detailed in the previous communication.¹⁸ The enzyme preparation corresponding to Fraction VI was used in all experiments described here. The assay methods for RNA synthesis and the experimental procedures were as in the previous report¹⁸ unless otherwise stated.

All non-labeled ribonucleotides were of commercial origin. Calf thymus DNA was purchased from the Sigma Chemical Company. Rat liver DNA was prepared by the method of Kay *et al.*¹⁹ Phage T4 DNA was a gift from Dr. L. Grossman. Phage T2 and T7 DNA were isolated by phenol extraction²⁰ from the respective phages. *M. lysodeikticus* DNA was prepared by the procedure of Marmur²¹ as modified by Grossman.* DNA from crab testis was a gift from Dr. M. Rabinowitz. All other DNA's used were generously supplied by Dr. E. P. Geiduschek. DNA samples were dissolved in a solution of 0.01 M NaCl - 0.01 M Tris buffer, pH 7.5. Heat denatured DNA was prepared by heating for 10 minutes at 100° and rapidly quenching in an ice bath.

Ultraviolet-treated DNA was prepared by irradiation with a G15T8 Westinghouse sterilamp by the procedure of Grossman²² except that no Vycor glass filter was employed.

Crystalline pancreatic RNase and DNase were purchased from Worthington Biochemical Corporation. The DNase was further purified by chromatography on DEAE-cellulose as suggested by Dr. S. Spiegelman. Exposure of TYMV RNA to the cellulose-treated DNase resulted in no apparent alteration of the sedimentation rate during sucrose gradient centrifugation. We are grateful to Dr. R. Haselkorn for performing these assays and for providing the viral RNA.

Spermine-HCl and spermidine phosphate were purchased from Mann Research Laboratories, Inc.; Cadaverine-HCl and putrescine-HCl from California Corporation for Biochemical Research. Poly-L-lysine-HBr, molecular weight 110,000 was purchased from Pilot Chemical, Inc.; Protamine sulphate from Nutritional Biochemicals Corporation and calf thymus histone from Worthington Biochemicals Corporation. The polyamines were made up in water and neutralized with NaHCO₃.

Inorganic phosphate was determined by the method of Gomori.²³ Inorganic pyrophosphate

* Personal communication.

was estimated by the increase in Pi released after hydrolysis in 1 N HCl for 20 minutes at 100°. Labeled PPi³² was prepared by the method of Jones and co-workers.²⁴

RESULTS

Requirements for the 4 NTP reaction. The formation of RNA complementary to its DNA primer by RNA polymerase requires all four ribonucleoside triphosphates, a divalent metal ion, and DNA (Table 1). Maximal synthesis requires the presence of spermidine, but other polyamines also stimulate the reaction. The addition of either RNase or DNase results in no appreciable product formation. In the presence of all four deoxynucleoside triphosphates no reaction occurs, although substitution of dTTP³² for UTP³² in a complete system results in the incorporation of measurable quantities of label into the acid-insoluble fraction. The ribonucleoside diphosphates cannot substitute for the triphosphates in this system.

Table 1
REQUIREMENTS FOR THE FOUR NUCLEOTIDE
DNA PRIMED REACTION*

Additions	CMP ³² incorporated mμmoles
Complete	10.3
Omit ATP	< 0.2
Omit UTP	< 0.2
Omit GTP	0.5
Omit Mn ⁺⁺	< 0.1
Omit DNA	< 0.1
Omit spermidine	7.6
Ribonuclease	0.4
Deoxyribonuclease	0.2

* The complete reaction mixture, 0.50 ml, contained 0.10 M Tris-HCl, pH 7.5, 0.4 mM each of ATP, UTP and GTP, 0.4 mM of CMP³² (2.8×10^6 cpm per μmole), 2.5 mM MnCl₂, 2.0 mM spermidine phosphate, 100 μg of calf thymus DNA and 6 units of enzyme. The reaction was incubated for 20 minutes at 30° and then assayed for RNA formation.¹⁸

In the absence of a single ribonucleotide, little or no reaction takes place. Figure 2 shows the dependence of RNA synthesis on each ribonucleotide and the concentrations required to achieve saturation. The calculated Km's for the respective triphosphates range from $3 - 6 \times 10^{-5}$ M and are similar but slightly higher than the values reported for *E. coli* RNA polymerase.²⁴

pH optimum. The reaction is optimal between pH 7 and 8.5, and falls sharply above or below these values.

Metal ion requirement. As mentioned previously, a divalent metal ion is required for RNA synthesis. For *M. lysodeikticus* "polymerase," Mn⁺⁺ is the most effective metal ion tested, although Co⁺⁺ and Mg⁺⁺ also stimulate synthesis (Figure 3). At concentrations higher than 3×10^{-3}

Figure 2. The reaction mixture, 0.50 ml, contained 0.10 M Tris-HCl, pH 7.5, 2.5 mM MnCl₂, 0.8 mM spermidine phosphate, 100 μg of calf thymus DNA, 3 units of enzyme, 0.80 mM each of ATP, GTP, CTP and UTP, and either 0.80 mM GTP³² (6.60×10^{-5} cpm per μmole) or 0.80 mM CTP³² (1.21×10^6 cpm per μmole) were used as the labeled substrate. For demonstrating the dependence of the reaction on ATP a combination of CTP, UTP and GTP³² was used; for CTP, a combination of ATP, UTP and CTP³²; and for UTP, a combination of ATP, GTP and CTP³². The ribonucleotides under study were added at various concentrations and the mixtures were incubated for 10 minutes at 30°. The reactions were stopped with acid and the acid-insoluble material analyzed for radioactivity.

M, both manganese and cobalt inhibit polymerization, while inhibition with magnesium occurs at concentrations higher than 10^{-2} M. Other metal ions such as Ni⁺⁺, Al⁺⁺⁺, Fe⁺⁺, Zn⁺⁺ and Ca⁺⁺ are not active. The metal ion requirement for this enzyme and the enzyme prepared from *E. coli*^{15,25} are similar except for the activation by Co⁺⁺ observed with the *M. lysodeikticus* preparation.

Linearity and temperature. The polymerization reaction is linear only for a relatively short time (Figure 4). After the initial reaction, polymerization proceeds at a slower rate and may continue for several hours. The time at which the reaction levels off varies with different DNA's or with different preparations of the same DNA. In this regard, RNA synthesis with T7 DNA as primer levels off significantly later than incubation mixtures containing calf thymus DNA. The rate of RNA formation is slightly more rapid at 40° than at lower temperatures. With T7 DNA, the rates observed when assayed at various temperatures were not as different as one might expect. However, greater differences have been observed with other DNA primers (calf

Figure 3. The reaction conditions were as for Figure 2 except that MnCl₂, CoCl₂ and MgCl₂ were tested at the concentrations indicated above and 5 units of enzyme were used.

Figure 4. The reaction conditions as for Figure 2 except that GTP³² served as the label and 50 μg of T7 DNA was used as primer. The vessels were incubated at the temperatures and time periods indicated.

thymus) as well as RNA primers (polycytidylate). The effect of temperature on the reaction kinetics with RNA primers is presented in the succeeding paper.

The addition of ribonucleotides and/or DNA after the reaction has reached maximal synthesis does not further stimulate polymerization. The addition of more enzyme, however, results in another burst of RNA synthesis. The exact meaning of this phenomenon is unclear.

Net synthesis and stoichiometry of the 4 NTP reaction. The net synthesis of complementary RNA may be readily demonstrated with *M. lysodeikticus* RNA polymerase (Table 2).⁴ The amount of alkali-sensitive material released after hydrolysis of the acid-insoluble ribopolynucleotide formed during the reaction compares rather favorably with the amount of ribonucleotide polymerized as calculated from the P^{32} incorporation data. Analysis of the acid-soluble fraction after synthesis indicates that the quantity of inorganic pyrophosphate released is nearly equivalent to the total number of micromoles of substrate incorporated. Our criterion for the presence of PPi is based on the increased amount of Pi which appears after acid hydrolysis. The Pi which is present before acid hydrolysis arises from the small amount of Pi normally found in commercial preparations of the nucleotides employed. The relatively high quantity of Pi released after acid hydrolysis is probably due to incomplete adsorption of the acid-soluble nucleotides onto charcoal. Because of this, the analytical error for our PPi determination may be relatively high.

Native and heat denatured DNA priming (4 NTP reaction). In the 4 NTP reaction, both native and heat denatured DNA can serve as primers for RNA formation (Figure 5). Saturation with respect to primer is achieved with denatured DNA at approximately one-tenth the concentration observed for native DNA. Furthermore, under saturation conditions for both primers, native DNA is considerably more efficient in stimulating RNA synthesis than heat denatured DNA. We have found similar results with different DNA primers.

Requirement for polyamine (4 NTP reaction). Optimal rates and extension of RNA synthesis with *M. lysodeikticus* RNA polymerase require the presence of polyamines. The stimulation observed with added polyamines has ranged from 1.2 to 2.0 times that obtained in the absence of any organic cations. The formation of RNA in the presence and absence of spermidine with different DNA primers is presented in Table 3. In each case, the addition of spermidine results in a significant enhancement of polynucleotide synthesis although the absolute amount of RNA formed varies slightly for the different DNA's used.

Spermine and putrescine are equally effective as spermidine for satisfying the polyamine requirement in this system (Table 4). Optimal synthesis with each of these organic cations occurs at different molar concentrations. Table 4 also indicates that calf thymus histone, protamine, and polylysine do not stimulate synthesis. At concentrations higher than those indicated in Table 4, most polyamines inhibit the reaction since they induce precipitation of the DNA primer.

So far, the requirement for polyamines has only been observed in the 4 NTP reaction where helical DNA serves as primer. With native DNA and in the absence of spermine, RNA synthesis appears to level off earlier than when spermine is present (Figure 6). If after 60 minutes of incubation in the absence of any polyamine spermine is added, the rate and extension of RNA synthesis is increased. The exact interpretation of this phenomenon is not clear. With heat denatured DNA, the addition of spermine results in slightly elevated levels of label incorporation. This stimulation is smaller than that obtained with native DNA. With native DNA, the amount of polyamine required for optimal RNA synthesis varies with the concentration of DNA employed.

Table 2
STOICHIOMETRY OF DNA PRIMED RNA SYNTHESIS*
(4 NTP reaction)

System	Acid-insoluble fraction				Acid-soluble fraction			
	Nucleotides incorporated		KOH hydrolysis nucleotides released		Total Pi before HCl hydrolysis	Total Pi after HCl hydrolysis	Total Pi released by hydrolysis	Net Pi/2
	Total μ moles	Net μ moles	Total μ moles	Net μ moles	μ moles	μ moles	μ moles	μ moles
Complete	1.178	1.177	1.50	1.29	1.65	13.30	11.65	1.26
Omit Mn^{++}	0.001		0.21		1.22	10.35	9.13	

* The reaction mixtures, 6.5 ml each, contained 0.10 M Tris-HCl, pH 7.5, 0.4 mM each of GTP, UTP and CTP, 0.4 mM of ATP³² (1.09×10^5 cpm per μ mole), 0.4 mM spermine, 600 μ g of *M. lysodeikticus* DNA and 50 units of enzyme. In addition, one vessel contained 2.5 mM $MnCl_2$. The reaction was stopped after 60 minutes by the addition of 100 μ moles of versene and the volume adjusted to 7.0 ml. A 1-ml aliquot was removed from the determination of AMP³² incorporation into RNA. The total RNA formed was calculated from the AMP³² incorporation data by assuming that the thymidine content of *M. lysodeikticus* DNA was 16 mole %.

A 5-ml aliquot was treated with $HClO_4$ and the acid-insoluble material collected and washed 3 times by solution in 0.2 M NaOH and precipitation with acid. This material was then hydrolyzed in 0.5 M KOH, at 37° for 18 hours, and the release of ultraviolet absorbing material was determined at 260 $m\mu$ after acidification with $HClO_4$ and removal of the precipitate. An E_m of 10.3×10^{-3} mM was used to calculate the number of μ moles released from the acid-insoluble fraction by alkaline hydrolysis. 200 mg of Norit was added to the acid-soluble fraction, mixed and removed by centrifugation. This procedure was repeated once more. One aliquot was taken for Pi determination directly, and another aliquot was adjusted to 1 N HCl with concentrated acid, hydrolyzed for 20 minutes in a boiling water bath, cooled, and then the Pi content determined.

Figure 5. The reaction conditions were as for Figure 2 except that native and heat denatured T7 DNA, at the concentrations indicated above, were used as primers. The reaction mixtures were incubated at 30° for 10 minutes.

Inhibition of the DNA-dependent synthesis by RNA. The DNA primed synthesis of RNA can be markedly inhibited by the addition of various RNA's to the polymerizing system. Synthetic RNA's as well as natural ribopolymers can inhibit the reaction. An example of this type of inhibition may be readily demonstrated by the addition of polyribocytidylate to a DNA primed reaction in which ATP³² serves as the labeled substrate (Figure 7). As the polycytidylate concentration is increased, the incorporation of AMP³² diminishes for each concentration of DNA used (Figure 7A). A Lineweaver-Burke plot of the inhibition data indicates that for each level of polycytidylate tested, the extrapolated curves intersect at the same point on the ordinate, suggesting that the inhibition is of a competitive nature (Figure 7B). This implies that polycytidylate can complex with the enzyme at sites along the enzyme surface where DNA normally binds. We can also demonstrate the reverse situation, that is, the DNA inhibition of the RNA-directed synthesis of RNA by the same enzyme. These findings will be reported in a succeeding paper. Krakow and Ochoa have reported similar results with *Azotobacter* RNA polymerase.²⁶

Effect of ultraviolet irradiation of DNA priming. Geiduschek has reported that under appropriate conditions of nitrous acid treatment, DNA's can be made which are fully reversible after heat denaturation.²⁷ He has concluded that such treatment, in part, results in cross-linking between the double strands of DNA. DNA's so prepared are relatively poor primers.²⁸ Ultraviolet irradiation of DNA has also been reported to cause partial denaturation and cross-linking,^{29,30} and therefore, one might expect to find an impaired priming ability of DNA exposed to this treat-

Table 3
THE EFFECT OF SPERMIDINE WITH VARIOUS DNA PRIMERS*
(4 NTP reaction)

Primer DNA	Spermidine	Polynucleotide synthesized mμmoles
Calf thymus	+	31.5
	-	21.5
Rat liver	+	33.3
	-	24.2
Bacteriophage T2	+	35.4
	-	22.3
Bacteriophage T4	+	16.0
	-	10.9
Salmon sperm	+	42.9
	-	32.8
Sea urchin sperm	+	28.1
	-	21.7
<u>E. coli</u>	+	34.6
	-	25.0
<u>S. marcescens</u>	+	33.0
	-	24.2
<u>P. fluorescens</u>	+	42.9
	-	30.2
<u>A. aerogenes</u>	+	37.2
	-	27.4
<u>M. lysodeikticus</u>	+	31.1
	-	22.1

* The conditions for the reaction were similar to those described in Table 1 except that 50 μg of each DNA and 3.5 units of enzyme were used. Spermidine (2.0 mM) was added where indicated and the reaction time was 60 minutes. The total polynucleotide formed was derived by dividing the mμmoles of labeled substrate incorporated by the mole % of the substrate reported for each DNA.⁴⁰⁻⁴²

ment as well as to nitrous acid. Figure 8 demonstrates that exposure of DNA to ultraviolet irradiation rapidly reduces its ability to prime for RNA synthesis. Furthermore, native DNA is considerably more sensitive to this treatment than heat denatured DNA. The effects of ultraviolet irradiation on nucleic acids are complex.²⁹ An extensive study of irradiated primers and the products formed in enzymatic systems of this type may help to clarify the complex changes which occur.

Requirements for the single NTP reaction. Chamberlin and Berg first described the DNA primed synthesis of polyadenylate catalyzed by E. coli RNA polymerase.¹⁵ M. lysodeikticus "polymerase" also catalyzes a reaction of this nature, and some of the requirements for the formation of two different homopolymers are given by Table 5. In the presence of ATP, manganese ion, and calf thymus DNA, RNA polymerase catalyzes the vigorous synthesis of polyadenylate. Other homopolymers may be formed with different nucleotides or with different DNA's, e.g., the formation of polycytidylate in the presence of P. fluorescens DNA. The extent of homopolymer

Table 4
THE EFFECT OF VARIOUS POLYAMINES
ON RNA SYNTHESIS*

Additions	GMP ³² incorporated mμmoles
None	7.80
Spermidine (0.4 μmoles)	10.35
Spermidine (1.0 μmoles)	11.30
Spermine (0.1 μmoles)	9.78
Spermine (0.2 μmoles)	14.30
Putrescine (2.0 μmoles)	8.96
Putrescine (10.0 μmoles)	13.50
Cadaverine (2.0 μmoles)	8.75
Cadaverine (10.0 μmoles)	9.56
Calf thymus histone (20 μg)	7.98
Protamine (20 μg)	7.84
Polylysine (10 μg)	7.34

* The reaction mixture and conditions used were similar to those described for Table 3 except that 100 μg of calf thymus DNA was used as the primer.

formation for these two separate reactions is not the same. Moreover, the addition of other nucleotides markedly inhibits this synthesis. In the presence of four ribonucleotides, the amount of RNA formed is considerably lower than the ribopolymer assembled when only ATP is present. The relatively poor 4 NTP reaction is due primarily to the absence of polyamine from the reaction mixture. With P. fluorescens DNA, the 4 NTP reaction is less extensive than usual due to the omission of polyamines, but it is still more active than ribopolymer formation with CTP alone.

In the single NTP reaction, the addition of spermidine does not stimulate homopolymer formation. In fact, we have usually observed some inhibition when polyamines are present. DNase addition partially inhibits polyadenylate and polycytidylate synthesis, whereas RNase inhibits only polycytidylate formation. The synthesis of significant amounts of homopolymer even in the presence of added DNase is probably due to DNA fragments which are sufficiently large to serve as primers.

In the absence of manganese or DNA, little or no product forms. Figure 9 illustrates the dependence of RNA formation on the concentration of DNA. The K_m for DNA in the 4 NTP and single NTP reaction in the absence of spermidine, has been determined and is approximately 5.9×10^{-6} , and 3.8×10^{-6} gm per ml of reaction respectively (Figures 9A and 9B). The similarity in the values suggests that in both cases the affinity of the enzyme for the primer is approximately the same.

Metal ion requirement (single NTP reaction). The single NTP DNA-primed reaction, with M. lysodeikticus RNA polymerase, requires manganese ion for synthesis (Figure 10). Optimum

Figure 6. The reaction conditions were similar to those described in Table 1 except that 0.2 mM spermine, 50 μ g of native or heat denatured calf thymus DNA and 4.3 units of enzyme were used. ATP³² (S.A. = 0.30×10^6 cpm per μ mole) was used as the labeled substrate.

synthesis occurs at approximately 2×10^{-3} M MnCl₂, and is similar to the optimum concentration required for the 4 NTP reaction. Although Mg⁺⁺ can partially substitute for Mn⁺⁺ in the 4 NTP reaction, the single NTP reaction is almost completely inactive in the presence of magnesium ion.

Stoichiometry of the single NTP reaction. The polymerization of labeled AMP into an acid-insoluble polymer is accompanied by the release of an equivalent amount of inorganic pyrophosphate (Table 6). Alkaline hydrolysis of the acid-insoluble fraction results in the release of acid-soluble material which has an absorption spectrum identical to AMP, and is approximately equivalent to the amount of labeled substrate incorporated.

The single NTP reaction with various DNA's. The RNA polymerase catalyzed synthesis of ribohomopolymers with various DNA primers is shown in Table 7. With all DNA's tested, except for M. lysodeikticus DNA, a significant incorporation of AMP³² and UMP³² was found for the single NTP reaction. In general, DNA's which are rich in adenine and thymine were somewhat better primers for polyadenylate and polyuridylylate synthesis than those with a high content of guanine and cytosine. For polycytidylylate synthesis, DNA's rich in guanine and cytosine appeared

Figure 7. The reaction conditions were as in Table 1 except that no spermidine was present and ATP³² was the labeled substrate. Four sets of vessels were prepared to which were added 0, 5, 40 and 100 μg of polyribocytidylate. To each set was added a different amount of calf thymus DNA as indicated. the vessels were incubated for 10 minutes at 30° and the amount of AMP³² incorporated into RNA determined. The reciprocal values of the DNA concentrations and the mμmoles of AMP³² incorporated were used for the plots in Figure 7B.

Figure 8. The reaction conditions were as in Table 1 except that 55 μ g of native and heat denatured calf thymus DNA were used. The method for ultraviolet inactivation is described under Methods. 100% GMP³² incorporation with native DNA and heat denatured DNA represents 4.0 and 1.9 $m\mu$ moles respectively.

more efficient than those high in adenine and thymine. Significant GMP³² incorporation could only be demonstrated with T2 DNA. On the whole, polyadenylate synthesis was more vigorous with each DNA tested than the formation of any other homopolymer. The inability of *M. lysodeikticus* DNA to prime for homopolymer synthesis is not understood. In addition, Table 7 shows that each DNA functioned as an efficient primer for complementary RNA synthesis in the 4 NTP reaction. These results differ from those reported by Goldberg *et al.*¹⁶ with regard to the synthesis of polyuridylylate and polycytidylylate in the presence of *M. lysodeikticus* and calf thymus DNA with *E. coli* "polymerase."

Linearity and DNA secondary structure (single NTP reaction). The DNA-primed synthesis of polyadenylate with RNA polymerase is linear for periods exceeding 1 hour (Figure 11). Furthermore, heat denatured calf thymus DNA functions as a better primer for AMP polymerization than native DNA. This agrees with the report by Stevens.¹⁷ Both these properties for the single NTP reaction are distinctly different from those described for the 4 NTP reaction. Heat denaturation of all DNA's tested has given similar increases in RNA synthesis for the single NTP reaction, except for polyuridylylate formation with *P. fluorescens* DNA (Table 8). Significant quantities of polyguanylylate may be formed with heat denatured *M. lysodeikticus* or T2 DNA.

It should be mentioned that the K_m 's for native and heat denatured calf thymus DNA in the single NTP reaction were the same. Therefore, it appears unlikely that the activity observed with native DNA as primer for polyadenylate formation was due to the presence of contaminating denatured DNA.

Table 5

REQUIREMENTS FOR THE SINGLE NUCLEOTIDE DNA PRIMED REACTION*

Primer: calf thymus DNA Substrate: ATP ³² Experiment 1		Primer: <i>P. fluorescens</i> DNA Substrate: CTP ³² Experiment 2	
Additions	AMP ³² incorporated mμmoles	Additions	CMP ³² incorporated mμmoles
Basal mixture	63.7	Basal mixture	5.2
GTP	32.3	ATP	1.3
GTP, CTP	1.6	ATP, GTP	0.6
GTP, CTP, UTP	24.5	ATP, GTP, UTP	27.6
Spermidine	44.8	Spermidine	3.9
Omit Mn ⁺⁺	1.3	Omit Mn ⁺⁺	< 0.1
Omit DNA	0	Omit DNA	0
Ribonuclease	74.8	Ribonuclease	< 0.1
Deoxyribonuclease	20.7	Deoxyribonuclease	0.7

* The basal mixture, 0.50 ml, contained 0.10 M Tris·HCl, pH 7.5, 2.0 mM MnCl₂, 0.8 mM ATP³² (1.4 x 10⁵ cpm per μmole) or 0.8 mM CTP³² (7.14 x 10⁵ cpm per μmole), 100 μg of calf thymus or *P. fluorescens* DNA and 18 units of enzyme. 0.8 mM each of GTP, UTP, CTP and ATP, 2 mM spermidine phosphate, 10 μg of RNase or DNase were added to the complete system where indicated. After 60 minutes at 30°, the reaction was stopped with acid and the insoluble fraction assayed for radioactivity.

Pyrophosphorolysis. Furth *et al.*²⁵ reported that *E. coli* RNA polymerase catalyzed the pyrophosphorolysis of RNA when the latter was prepared *in vitro* with T2 DNA as primer. These workers also report that the reverse reaction is very slow, requires large quantities of enzyme and that relatively small amounts of PPi³² were converted to a Norit-adsorbable form. So far, we have not been able to demonstrate the pyrophosphorolysis of any ribopolynucleotide with *M. lysodeikticus* RNA polymerase. Incubation of mixtures containing highly radioactive inorganic pyrophosphate, RNA polymerase, Mg⁺⁺, synthetic or natural RNA's and in the presence of DNA have resulted in insignificant amounts of Norit-adsorbable counts.

Pyrophosphate exchange. The exchange of PPi³² with ribonucleoside triphosphates is catalyzed by RNA polymerase (Table 9). The exchange reaction requires DNA for optimum activity, a divalent metal ion, ribonucleoside triphosphates and enzyme. The reaction is linear with respect to enzyme at concentrations up to 120 μg per ml, and with respect to time for periods exceeding 1 hour. The Km for ATP and GTP was 3 x 10⁻⁴ M. PPi saturation occurred at approximately 1 x 10⁻³ M. Optimum exchange occurs with 3 to 6 x 10⁻³ M Mg⁺⁺; higher levels result in inhibition. No exchange was detected when Pi³² was substituted for PPi³².

Table 9 shows that the exchange reaction occurred with a variety of DNA's. In the presence of all four ribonucleotides, no significant differences were observed between native and heat denatured DNA. Isolation of the ribonucleoside triphosphates by chromatography on Dowex-1-Cl after the reaction, showed that labeled PPi³² had exchanged with each nucleotide. If one compares the exchange data in Table 9 with the incorporation of ribonucleotides into RNA (e.g.,

Figure 9A. The conditions for the reaction were as in Figure 2, except that various quantities of calf thymus DNA were tested at the levels indicated and no spermidine was added. All four ribonucleoside triphosphates were present at 0.8 mM each. The labeled substrate was GTP³².

Figure 9B. The conditions were identical to Figure 3A except that ATP³² was the only nucleotide present.

Table 3) it is apparent that the PPi exchange reaction occurs at a much slower rate than the forward reaction. In the absence of added DNA, the relatively small conversion of PPi³² to Norit-adsorbable counts was significant and reproducible. This exchange is probably related to the "unprimed" synthesis of RNA which we have detected with *M. lysodeikticus* "polymerase."³¹ Other studies suggest that in the presence of DNA, the "unprimed" reaction does not occur.

When various ribonucleoside triphosphates are tested singly, only the purine nucleotides actively participate in the PPi exchange. This striking phenomenon was found for each of the DNA's tested, and occurred with either native or heat denatured deoxypolynucleotides. With *E. coli* RNA polymerase, Furth *et al.*²⁵ reported a similar observation. With heat denatured *M. lysodeikticus* DNA we noted a slight exchange with CTP alone.

Figure 10. The reaction mixture, 0.50 ml, contained 0.10 M Tris-HCl, pH 7.5, 0.8 mM ATP³² (1.56×10^6 cpm per μ mole), 100 μ g of calf thymus DNA, 4 units of enzyme, and various concentrations of $MnCl_2$ or $MgCl_2$. The mixtures were incubated for 10 minutes at 30° and then analyzed for AMP³² incorporation into the acid-insoluble fraction.

Qualitatively, another pattern appears to develop from these experiments. Exchange reactions carried out with DNA's rich in adenine and thymine exchange better with ATP than with GTP. With DNA's of high guanine and cytosine content, the reverse appears to follow. The exchange reaction with crab dAT-copolymer occurred in the presence of all four ribonucleotides, and with ATP alone. No other single triphosphate gave a significant exchange. This finding is contrary to a report by Goldberg *et al.*³² which demonstrated a marked exchange with UTP alone and a negligible reaction with ATP. It should be mentioned that the exchange reaction shows different quantitative results by the Norit-adsorption assay method, when different mixtures of each ribonucleotide are present. The experiments reported here for the single nucleotide exchange reactions were performed with commercial preparations of ribonucleoside triphosphates which had been purified by chromatography on Dowex-1-Cl before their use.

Table 6
STOICHIOMETRY OF DNA PRIMED POLY A SYNTHESIS*
(Single NTP reaction)

System	Acid-insoluble fraction				Acid-soluble fraction			
	AMP ³² incorporated		KOH released AMP		Total Pi before HCl hydrolysis μ moles	Total Pi after HCl hydrolysis μ moles	Total Pi released by hydrolysis μ moles	Net Pi/2 μ moles
	Total μ moles	Net μ moles	Total μ moles	Net μ moles				
Complete	0.50	0.50	0.87	0.47	1.01	2.48	1.47	0.55
Omit Mn ⁺⁺	0		0.40		0.83	1.20	0.37	

* The reaction mixtures, 6.5 ml and conditions of incubation were as indicated in Table 2 except that GTP, UTP and CTP were omitted and 600 μ g of calf thymus DNA was used as primer. The procedure was identical to that described in Table 2.

Table 7

THE SINGLE AND 4 NTP REACTION WITH VARIOUS DNA's*

Source of primer DNA	DNA base ratio [†] $\frac{A+T}{G+C}$	Total ribopolynucleotide formed				
		4 NTP reaction 10 minutes m μ moles	Single NTP reaction 60 minutes			
			ATP32 m μ moles	UTP32 m μ moles	CTP32 m μ moles	GTP32 m μ moles
Bacteriophage T4	1.90	5.2	33.90	8.32	0.47	0
Bacteriophage T2	1.85	13.2	25.10	5.64	0.32	0.32
Sea urchin sperm	1.70	19.2	23.10	6.62	0.29	0.04
Salmon sperm	1.43	26.0	16.55	3.19	0.26	0.01
<u>B. subtilis</u>	1.36	14.1	39.40	8.10	0.89	0
Rat liver	1.35	21.7	14.92	4.56	0.23	0.09
Calf thymus	1.29	24.9	20.70	3.19	0.23	0.10
Bacteriophage T7	1.08	18.2	3.23	1.11	0.46	0.02
<u>E. coli B</u>	0.96	17.0	18.62	3.50	0.98	0.03
<u>A. aerogenes</u>	0.74	20.8	14.78	4.06	1.24	0
<u>S. marcescens</u>	0.70	22.8	13.00	2.69	1.48	0.04
<u>P. fluorescens</u>	0.59	26.6	7.42	2.27	1.59	0
<u>M. lysodeikticus</u>	0.39	28.3	0.21	0	0.20	0

* The conditions for the 4 NTP reaction were as in Table 1 except that 1.2 mM spermidine phosphate was used and the reaction was stopped after 10 minutes. The single NTP reaction mixture, 0.50 ml, contained 0.10 M Tris-HCl, pH 7.5, 2.5 mM MnCl₂, 1.2 mM of either ATP³², UTP³², CTP³² or GTP³² employed individually and with a specific activity of 8.97×10^5 , 9.4×10^5 , 13.2×10^5 and 13.7×10^5 cpm per μ mole respectively, 50 μ g of DNA and 6 units of enzyme. The reaction was incubated at 30° and assayed for the incorporation of label into an acid-insoluble fraction after 60 minutes.

[†] Base ratios as reported by Chargaff,⁴⁰ Belozersky and Spirin⁴¹ and Sinsheimer.⁴²

DISCUSSION

M. lysodeikticus RNA polymerase catalyzes at least two separate DNA-dependent reactions. With respect to the nature of the product formed, one reaction results in the synthesis of complementary RNA, and the other gives rise to an RNA homopolymer. Although these reactions are similar in some respects, they differ in many ways.

Both reactions require ribonucleoside triphosphates as substrates, and synthesis occurs with the elimination of inorganic pyrophosphate. The release of inorganic pyrophosphate, in each case, is stoichiometric with the amount of ribonucleotide polymerized. Native and heat denatured DNA can prime in either reaction.

The synthesis of complementary RNA requires all four ribonucleotides and either Mn⁺⁺, Co⁺⁺ or Mg⁺⁺ may satisfy the divalent metal ion requirement. On the other hand, the formation of a ribohomopolymer requires the presence of only one ribonucleotide, and only Mn⁺⁺ has been found to fulfill the metal ion requirement effectively. In agreement with Chamberlin and Berg¹⁵

Figure 11. The conditions were as in Table 7 for the single NTP reaction except that the reaction mixtures contained only ATP³², 100 μg of native or heat denatured calf thymus DNA and 15 units of enzyme. The mixtures were incubated at 30° for the time intervals shown.

Table 8
THE SINGLE NTP REACTION WITH NATIVE
AND DENATURED DNA *

Source of primer DNA	Labeled substrate	Ribopolynucleotide formed	
		Native mμmoles	Heat denatured mμmoles
Calf thymus	ATP	38.10	52.60
<u>P. fluorescens</u>	ATP	8.24	15.58
<u>M. lysodeikticus</u>	ATP	0.18	2.44
Calf thymus	UTP	3.65	4.10
<u>P. fluorescens</u>	UTP	2.65	0.73
<u>P. fluorescens</u>	CTP	2.04	2.29
<u>M. lysodeikticus</u>	CTP	0.16	2.90
Bacteriophage T2	GTP	0.22	0.28
<u>M. lysodeikticus</u>	GTP	0.03	0.23

* The conditions for the single NTP reaction were as for Table 7 except that 10 units of enzyme were used. Heat denatured DNA; 50 μg, replaced native DNA where indicated and was prepared as described under Methods.

Table 9
PYROPHOSPHATE EXCHANGE REACTION

Experiment no.	Treatment of DNA	Cofactors added	Norit-adsorbable PPi^{32} after reaction with various DNA's						
			Crab [†] dAT	T2	Rat liver	Calf thymus	<u>M. lyso-</u> <u>deikticus</u>	<u>P. fluor-</u> <u>escens</u>	No [†] DNA
			mμmoles	mμmoles	mμmoles	mμmoles	mμmoles	mμmoles	mμmoles
1	Native	ATP, GTP, CTP, UTP	1.04	2.98	1.39	1.32	1.07	1.58	0.15
	Native	ATP, GTP, CTP, UTP Omit Mg^{++}	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	Native	ATP, GTP, CTP, UTP Spermidine		3.04	1.38	1.53	1.20	1.67	
	Native	ATP	0.41	0.86	2.04	1.58	0.10	0.35	0.38
	Native	GTP	<0.01	0.08	0.25	0.20	0.13	0.58	0.02
	Native	CTP	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	Native	UTP	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
2	Native	ATP, GTP, CTP, UTP			1.17	1.17	1.28	1.53	
	Heat denatured	ATP, GTP, CTP, UTP			1.14	0.95	1.54	1.14	
	Heat denatured	ATP			2.10	1.92	0.40	0.50	
	Heat denatured	GTP			0.70	0.73	3.77	0.77	
	Heat denatured	CTP			<0.01	<0.01	0.21	<0.01	
	Heat denatured	UTP			<0.01	<0.01	0.03	<0.01	
	Heat denatured								

* The reaction mixture, 0.50 ml, contained 0.10 M Tris-HCl, pH 7.5, 5.0 mM MgCl_2 , 0.88 mM PPi^{32} (2.0×10^6 cpm per μmole), 50 μg of the respective DNA's except for crab dAT which was 1.72 μg, 0.8 mM each of ATP, GTP, CTP and UTP where indicated, 0.8 mM spermidine phosphate where indicated and 15 units of enzyme. Heat denatured DNA was prepared as described under Methods. The reaction mixture was incubated at 30° for 30 minutes except for the vessels containing no DNA and crab dAT co-polymers (see below). At the end of this time, 1 mg of carrier albumin was added and the reaction stopped with 50% trichloroacetic acid containing 10^{-3} M PPi . The precipitate was removed and 30 mg of Norit was added and mixed with the acid-soluble fraction. The Norit was collected by centrifugation and washed 4 times with the acid- PPi solution. The Norit was subjected to hydrolysis in 1 N HCl for 20 minutes at 100° and cooled. The volumes in each vessel were adjusted to 2.0 ml with water, centrifuged and 1-ml aliquots were dried and assayed for their radioactive content. Control PPi^{32} standards were treated in a similar manner.

[†] Exchange reaction incubated for 80 minutes.

we find that the latter reaction is inhibited by the presence of more than one nucleotide in the system.

Another apparent difference between the 4 NTP and single NTP reaction is observed when denatured DNA replaces native DNA. In most instances heat denatured DNA is more effective for RNA synthesis in the single NTP reaction than helical DNA (Figure 11). With one DNA we observed the reverse situation. Although other instances of this reverse phenomenon may exist, only with heat denatured M. lysodeikticus and T2 DNA could we observe any polyguanylate formation. There was no significant difference between the Km values for helical and denatured DNA primer in the single NTP reaction.

The efficiency of priming with native and denatured DNA was completely reversed in the 4 NTP reaction. When denatured DNA replaced native DNA, both the rate of complementary RNA synthesis, and the concentration required for DNA saturation, were markedly reduced (Figure 5). This finding is in general agreement with other reports^{11,15,25,26} although some investigators have found little or no change in the priming efficiency of certain DNA's subjected to heat denaturation.¹⁵ One possible explanation for the consistent differences in RNA synthesis which we have observed between ordered and disordered DNA primers, may lie in the addition of polyamine to the polymerizing system.

Recently, we found that when denatured DNA serves as primer in the 4 NTP reaction, the RNA which forms is largely resistant to RNase degradation. Furthermore, equilibrium density-gradient centrifugation in CsCl results in the appearance of labeled RNA where DNA usually bands. These observations are quite different from those previously reported by Geiduschek et al.^{12,13} when native DNA primed for ribopolynucleotide synthesis. The assembly of RNA with single stranded DNA as template appears to result in the formation of an RNA-DNA complex which does not readily separate, and could account for the reduced rate of polynucleotide synthesis observed. A complete description of these experiments will be reported elsewhere. A report by Warner et al.³³ on the formation of DNA-RNA hybrids when denatured DNA serves as primer with A. vinelandii RNA polymerase is in agreement with the observations reported here.

One of the most striking and as yet least understood properties of the 4 NTP reaction is the polyamine stimulation obtained when native DNA is used for RNA synthesis. Priming with heat denatured DNA, either in the 4 NTP or single NTP reaction, does not show this marked polyamine stimulation. In fact, inhibition occurs when spermidine is added to the single NTP reaction. The stimulatory effect of polyamines varies slightly with different DNA's, and this variation appears to be associated with the DNA preparation itself, rather than with the enzyme. For this reason, we have only obtained consistent assays with M. lysodeikticus RNA polymerase in the presence of polyamines.

Tabor, Tabor and Rosenthal³⁴ have recently reviewed some of the effects of polyamines in various systems, and their interactions with nucleic acids. Organic cations such as polyamines bind readily with polynucleotides, as do divalent metal cations. These studies suggest that both types of cations facilitate the formation of highly ordered structures, primarily DNA's high in adenine and thymine.^{35,36} In view of the polyamine stimulation with primer M. lysodeikticus DNA (72 mole per cent guanine and cytosine) by spermidine, some other explanation may be required for the polyamine effect observed in this report. Others have reported that spermine and spermidine partially protect nucleic acids from the hydrolytic action of RNase and DNase.³⁴ This protective action could account for the results observed here; however, the enzyme prepa-

rations and DNA's used in this work have been shown to contain only trace quantities of these degradative enzymes. Moreover, the addition of spermidine to RNA-DNA directed reactions, and to the DNA-primed synthesis of polycytidylate shows no stimulatory effect. Although polyamines may protect either the primer or the product from partial degradation it is also possible that organic cations serve another function in RNA synthesis when primed by native DNA. The concentrations required to achieve optimum synthesis with various polyamines are not the same. This difference may be related to the different affinities of various polyamines towards nucleic acids as well as to steric effects imposed on the polymerizing system.

The formation of homopolymers by the single NTP reaction may occur with a variety of different DNA primers. There appears to be some qualitative correlation between the base composition of the primer and the ability to form certain homopolymers. These results support the suggestion of Chamberlin and Berg¹⁵ that repeating sequences of a single base in DNA can prime the synthesis of a complementary homopolymer. One would expect that the probability of consecutive sequences of a single base occurring in DNA's rich in a particular nucleotide(s) would be relatively high. It is noteworthy that even with DNA's relatively poor in thymidylate an active polyadenylate synthesis was found. Repeating sequences of thymidylate may not, therefore, be restricted only to DNA's rich in this deoxynucleotide. In view of the less active homopolymer synthesis found with UTP, CTP and GTP alone, it is important to know whether thymidylate sequences have a universal meaning in terms of biological function.

The results obtained in the single NTP reaction suggest certain inconsistencies with accepted base-pairing theory. Since every run of thymidylate sequences in one DNA strand should be matched by an equivalent run of adenylate sequences in the opposing strand, it is difficult to account for the quantitative differences in polyadenylate and polyuridylylate synthesized for any of the DNA's tested (Table 7). One possible explanation might be that the P^{32} -polyuridylic acid product was incompletely precipitated by cold trichloroacetic acid.³⁷ On the other hand, the priming mechanism could be more complex than our current data suggests. In the single NTP reaction, heat denaturation of the DNA may allow certain consecutive sequences to prime more effectively.

Bessman *et al.*³⁸ have shown that for DNA polymerase, the rate of PPi exchange with deoxynucleoside triphosphates was of the same magnitude as the rate of deoxynucleotide incorporation into DNA. In the absence of all four triphosphates, these workers showed a reduced but measurable pyrophosphorolytic reaction. With the enzyme under examination in this report, the rates of PPi exchange are much slower than the synthetic rates observed, and no significant pyrophosphorolysis could be demonstrated. The observation that all four ribonucleoside triphosphates were labeled after PPi³² exchange, when a full complement of nucleotides was present, suggests that a reversal of the synthetic reaction had occurred. Under the conditions at which PP-exchange reactions were conducted, the forward reaction still occurs. In a review on pyrophosphorolytic reactions,³⁹ Kornberg points out that the free energy associated with the release of PP is relatively small and that the synthetic direction of many PP-releasing reactions is demonstrably reversible. The difficulty encountered in demonstrating a reversible reaction in this system as well as the poor reversal found with the *E. coli* system²⁵ may not be a simple representation of the synthetic nature of RNA polymerase, but rather a reflection of the complex order in which ribonucleotides are polymerized. The exchange reaction described in this report is interesting because virtually no reaction occurs with UTP or CTP alone, but does occur with ATP and GTP

alone. Assays with a variety of DNA's show the same result.

At the present time, the interpretation of the results obtained by the single NTP reaction and the PPI exchange data should be regarded with caution. Because of certain differences in both of these reactions between the enzyme studied here and the enzyme isolated from E. coli,^{16,32} one must consider the possibility that the enzyme itself may impart certain specificities to the polymerization reaction.

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ENZYMATIC SYNTHESIS OF RIBONUCLEIC ACID. III. THE RNA PRIMED
SYNTHESIS OF RNA WITH *M. lysodeikticus* RNA POLYMERASE*

By

C. F. Fox,[†] W. S. Robinson, R. Haselkorn,[‡] and S. B. Weiss

Two preceding papers describe the partial purification and properties of an enzyme prepared from extracts of *Micrococcus lysodeikticus* which catalyzes the DNA-primed synthesis of ribonucleic acid.^{1,2} An earlier publication from this laboratory presented evidence that a similar enzyme preparation, from the same organism, also catalyzed an RNA-directed synthesis of ribonucleic acid.³ Although the products formed in the RNA- and DNA-directed reactions were complementary to their respective primers in base composition, and the reaction mechanism appeared similar in both cases, it was not possible to decide whether one or two enzymes were responsible for both priming activities. In this paper, we shall present in greater detail some of the properties of the RNA-directed reaction catalyzed by the partially purified preparations of RNA polymerase. Evidence will be presented which suggests the involvement of only one enzyme. Recently Krakow and Ochoa⁴ reported on the partial purification of an RNA polymerase from *Azotobacter vinelandii*. These investigators have also observed RNA synthesis with both types of nucleic acid primers and have reached a similar conclusion, namely, that one enzyme is responsible for both priming activities.

EXPERIMENTAL

Methods. RNA polymerase was prepared from extracts of *M. lysodeikticus* by Procedure A as outlined in the previous report.³ Fraction VI was used in all experiments reported here, unless otherwise stated, and had a specific activity ranging from 400 to 550 units per mg of protein.

The preparations of DNA were obtained as previously stated.⁴ Complementary T7 RNA was prepared with T7 DNA as primer in a 4 NTP reaction with RNA polymerase. The T7 RNA was isolated from the reaction mixture as described elsewhere.⁵ *E. coli* ribosomal and soluble RNA were prepared from the extract obtained after exposure of the organism to sonication for 20 minutes in 0.005 M MgCl₂ and 0.01 M Tris, pH 7.5. The 105,000 x g pellet and supernatant, obtained from the supernatant fluid derived by a previous centrifugation at 30,000 x g, were used for the preparation of ribosomal and soluble RNA. RNA was first isolated by phenol extraction^{6,7} modified to contain 0.10 per cent sodium dodecyl sulphate. The RNA preparations were treated with DNase and then isolated again by the phenol method. The final solutions of RNA were made up to 1 M KCl and the precipitate which formed in the case of the ribosomal RNA solution was collected, dialyzed and saved. No KCl precipitate formed with soluble RNA. Both RNA prepara-

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[†] Predoctoral Fellow of the National Science Foundation.

[‡] Committee on Biophysics, University of Chicago.

tions were stored in 0.01 M Tris, pH 7.5. MS ϕ 2 RNA was isolated by the phenol extraction method from the virus.* TYMV RNA was prepared from the virus by the procedure of Boedtker and Simmons⁸ and the RNA isolated by phenol extraction as modified by Singer et al.⁹

Requirements for RNA priming. RNA polymerase preparations catalyze ribonucleotide polymerization utilizing natural or synthetic RNA primers (Tables 1 and 2). Synthesis requires the participation of enzyme, a divalent metal ion, a ribopolynucleotide and the appropriate triphosphate(s). In priming with homopolymers, only a single complementary ribonucleotide is required. Priming with RNA copolymers requires the presence of all the complementary triphosphates.

Tables 1 and 2 demonstrate that the RNA-primed reactions are insensitive to DNase and do not require a polyamine for maximum synthesis. Furthermore, exposure of the primer to heating does not affect RNA synthesis. These properties differ from those described for the 4 NTP reaction primed by DNA.² However, in both reactions, no synthesis occurs with the single omission of manganese, primer or ribonucleoside triphosphate, nor in the presence of heated enzyme. The partial requirement reported previously³ for the ribonucleoside triphosphates with viral RNA primers, was due to slight cross contamination of our nucleotide preparations. To demonstrate a nearly absolute requirement for each triphosphate, it is necessary to purify the substrates prior to use.

As in the 4 NTP DNA-primed reactions, the inclusion of RNase in the RNA-primed reaction almost completely inhibits product formation, except where the primer is resistant to RNase digestion, e.g., polyadenylate. The fact that UMP polymerization occurs in the latter case suggests that the polyuridylyate product is complexed and resists complete enzymatic degradation. The addition of RNase to viral RNA-primed reactions after sufficient synthesis has taken place, results in very little disappearance of the formed product. This RNase resistance appears similar to that observed in reactions primed with heat denatured DNA.[†]

It may be noted that under conditions where substrate and primer are present in saturating quantities, the RNA-primed reaction is linear with respect to enzyme at concentrations exceeding 450 μ g per ml.

Metal ion requirement. The RNA-primed reaction requires a divalent metal ion and this requirement may be satisfied by Mn⁺⁺ or Mg⁺⁺. For priming with different homopolymers, the metal ion requirement is illustrated in Figure 1. In all cases tested, Mn⁺⁺ is more effective, and Mg⁺⁺ is active only with certain primers. Reactions containing TYMV RNA as primer show a metal ion response similar to that demonstrated for priming with polycytidylyate. The optimum Mn⁺⁺ concentration differs with various primers. For example, at Mn⁺⁺ concentrations which are optimal for priming with polycytidylyate, the same metal ion concentration is inhibitory when the reaction is primed by polyadenylate. The differences observed for optimal metal ion concentrations with the various homopolymers tested, as well as the differences between Mg⁺⁺ and Mn⁺⁺ with the same ribopolymer, may be related to differences in the extent of polymer aggregation.

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[†] Unpublished results.

Table 1

REQUIREMENTS FOR RNA SYNTHESIS WITH DNA AND RNA PRIMERS*

Additions	Amount of primer	GMP ³² incorporation into RNA with various DNA and RNA primers							
		T7 DNA	Calf thymus DNA	TYMV RNA	TMV RNA	MS2 RNA	T7-C- RNA	Ribo- somal RNA	Soluble RNA
	μg	$\text{m}\mu\text{moles}$	$\text{m}\mu\text{moles}$	$\text{m}\mu\text{moles}$	$\text{m}\mu\text{moles}$	$\text{m}\mu\text{moles}$	$\text{m}\mu\text{moles}$	$\text{m}\mu\text{moles}$	$\text{m}\mu\text{moles}$
Complete	1	3.08	0.70	0.63	0.45	0.31	0.55	0.23	0.17
Complete	15	12.60	7.90	1.75	1.00	0.66	1.23	0.47	0.33
Omit CTP, UTP	15	0.14	0.15	0.22	0.13	0.09	0.10	0.08	0.09
Omit Primer	0	0.13	0.17	0.13	0.07	0.07	0.07	0.13	0.13
Omit Mn ⁺⁺	15	0.02	0.04	0.03	0.05	0.03	0.03	0.03	0.03
Heat denatured primer	15	4.10	5.36	1.50	0.78	0.70	1.07	0.51	0.39
Spermidine (0.60 μmoles)	15	24.30	12.20	1.39	0.85	0.90	0.37	0.33	0.15
RNase (2 μg)	15	0.56	0.66	0.04	0.05	0.05	0.09	0.04	0.03
DNase (5 μg)	15	0.81	0.65	1.68	0.90	0.51	1.02	0.46	0.34
Heated enzyme	15	0.03	0.05	0.03	0.05	0.05	0.05	0.03	0.03

* The reaction mixture, 0.30 ml, contained 0.10 M Tris HCl buffer, pH 7.5, 0.15 mM each ATP, UTP and CTP, 0.10 mM GTP³² (S.A. = 2.0×10^6 cpm per μmole), 2.5 mM MnCl₂, DNA or RNA in the quantities shown, and 6 units of enzyme. The mixtures containing the primer were pre-incubated with RNase and DNase for 3 minutes just prior to the addition of enzyme. The complete reaction mixture was incubated for 20 minutes at 30° and then assayed for label incorporation into the acid-insoluble fraction.

Table 2

REQUIREMENTS FOR RNA SYNTHESIS WITH RIBOHOMOPOLYMER PRIMERS*

Additions	RNA-P ³² formed with various primers			
	Polycytidylate	Polyadenylate	Polyuridylate	Polyadenylate + Polyuridylate
	mμmoles	mμmoles	mμmoles	mμmoles
GTP ³²	22.55	0.03	0.03	0.10
UTP ³²	0.02	19.00	0.05	7.10
ATP ³²	0.06	0.03	6.50	3.43
CTP ³²	0.13	0.12	0.05	0.08
Heated enzyme	0.08	0.03	0.03	0.05
Omit Mn ⁺⁺	0.06	0.06	0.01	0.03
Omit primer	0.06	0.05	0.11	0.04
Spermidine	19.00	8.00	2.30	2.35
DNase (5 μg)	22.05	20.04	6.80	6.08
RNase (2 μg)	0.03	14.00	0.16	4.07

*The reaction mixture, 0.30 ml, contained 0.1 M Tris buffer, pH 7.5, 0.4 mM each of GTP³², UTP³², ATP³² and CTP³² (S.A. ranging from 0.5 - 1 x 10⁶ cpm per μmole), 15 μg each of polycytidylate, polyadenylate, and polyuridylate, 2 mM spermidine, 13.5 units of enzyme and optimal concentrations of MnCl₂ for each RNA homopolymer as indicated in Figure 1. The labeled substrates used with the primers polycytidylate, polyadenylate, polyuridylate and the 1:1 complex of polyadenylate plus polyuridylate were GTP³², UTP³², ATP³² and UTP³² respectively. The reaction mixtures were incubated for 20 minutes at 30°, stopped with acid and assayed for label incorporation.

Stoichiometry and net synthesis. The polycytidylate primed polymerization of GMP by RNA polymerase preparations occurs with elimination of inorganic pyrophosphate (Table 3). Alkaline hydrolysis of the acid-insoluble fraction results in the digestion of both the polycytidylate primer and the newly formed product. The high amount of ultraviolet absorbing material in the control is derived from the primer. The net increase of 256 mμ absorbing material rendered acid-soluble after alkaline hydrolysis corresponds favorably with the estimated quantity of GMP³² incorporated.

Isolation of intact polyguanylate, free of its primer, may be accomplished by phenol extraction of the reaction mixture and treatment with RNase under appropriate conditions. Figure 2A shows that the product recovered after phenol extraction has an absorption spectrum in the ultraviolet region (curve 3) unlike that of either free GMP or polycytidylate (curves 1 and 2). After treatment with relatively large quantities of RNase at 63°, the undigested product shows an altered spectrum which does not resemble that seen for GMP (Figure 2B, curve 4). However, alkaline hydrolysis now results in the concomitant appearance of an acid-soluble material which exhibits a typical guanine spectrum and a marked hyperchromicity (curve 5). These findings strongly suggest that polyguanylate is the product of the polycytidylate-primed reaction. Furthermore, it appears that the monoesterified base loses its characteristic absorption spectrum when converted to a polyesterified macromolecule.

Figure 1. The reaction mixtures, 0.50 ml, contained 0.1 M Tris buffer, pH 7.5, 10 units of enzyme and the following combinations of substrates and RNA primers: 1.2 mM GTP³² (S.A. = 1.38×10^6 cpm per μ mole) and 20 μ g of polycytidylate; 1.2 mM UTP³² (S.A. = 1.04×10^6 cpm per μ mole) and 20 μ g of polyadenylate; 1.2 mM ATP³² (S.A. = 1.04×10^6 cpm per μ mole) and 20 μ g of polyuridylate; 1.2 mM UTP³² (same S.A.), and 20 μ g each of polyadenylate and polyuridylate. MnCl₂ and MgCl₂ were added separately at the concentrations indicated for each set of reactions. The mixtures were incubated for 10 minutes at 30°, stopped with acid and assayed for label incorporation.

Substrate affinity. The formation of polyguanylate is dependent upon the concentration of GTP (Figure 3). Saturation with respect to substrate is achieved at relatively low concentrations and the K_m has been calculated as approximately 2×10^{-5} M. This K_m value is very similar to those found for the nucleotides required by the DNA-primed synthesis of RNA with similar preparations of *M. lysodeikticus* RNA polymerase.²

Table 3
STOICHIOMETRY FOR POLYGUANIDYLATE SYNTHESIS*

Additions	Acid-insoluble fraction					Acid-soluble fraction			
	Nucleotide incorporation		KOH released GMP			Total Pi before hydrolysis	Total Pi after HCl hydrolysis	HCl released Pi	Δ Pi/2
	Total GMP ³²	Δ GMP ³²	Absorbancy at 256 m μ		Δ GMP				
			Total material	Δ material					
	μ moles	μ moles	units	units	μ moles	μ moles	μ moles	μ moles	
Complete	0.62	0.62	15.80	8.88	0.73	1.10	6.16	5.06	0.69
Omit Mn ⁺⁺	0		6.92			0.73	4.41	3.68	

*The reaction mixture, 6.0 ml, contained 0.1 M Tris buffer, pH 7.5, 3 mM MnCl₂, 0.8 mM GTP³² (S.A. = 2.8×10^5 cpm per μ mole), 200 μ g of polycytidylic acid and 180 units of enzyme. The reaction mixture was incubated for 1 hour at 30°, stopped by the addition of versene and appropriate aliquots taken for determining label incorporation into the acid-insoluble fraction, alkaline hydrolysis and release of acid-soluble material, and PPi determination of the acid-soluble fraction. The procedure and method of analysis were identical to those described for the stoichiometric analysis of the DNA-primed reaction.⁴

Figure 2. The reaction mixture, 5.0 ml, was similar to that described in Table 3. After 1 hour at 30°, an equal volume of phenol was added, shaken for 30 minutes and the aqueous phase removed, extracted with ether 3 times and then dialyzed against 0.001 M versene - 0.005 M Tris buffer, pH 7.5. Figure 2A gives the absorption spectrum for polycytidylate, GMP and the dialyzed reaction product at pH 7.5. To the dialyzed material was added 200 µg of pancreatic RNase and the mixture was heated at 63° for 45 minutes. A precipitate formed and this was removed by centrifugation. Two volumes of ethanol was added to the supernatant and the precipitate collected, dissolved in 0.10 M NaCl - 0.01 M Tris buffer, pH 9 with slight heating, and dialyzed against 0.01 M Tris buffer, pH 7.5. Figure 2B shows the spectrum of the dialyzed RNase treated product before and after 17 hours of digestion in 0.3 M NaOH and then adjusted to pH 7.5. The absorbancy values obtained after alkaline hydrolysis were adjusted to represent the same quantity of product as before hydrolysis. The estimated recovery of the GMP polymerized was 40%.

Primer concentration. The extent and rate of RNA synthesis are dependent upon the primer concentration (Figure 4). For the natural RNA primers, the K_m ranges between $0.5 - 3 \times 10^{-6}$ gm per ml. Saturating primer concentrations are similar for the plant viral RNA's, but MSφ2, ribosomal, and soluble RNA saturate the system at slightly lower levels. The inhibition of synthesis observed with increasing amounts of soluble RNA is not understood. The relatively crude soluble RNA preparation used here is not homogeneous and probably contains a variety of nucleic acids including transfer RNA's. Synthetic homopolymers exhibit a higher K_m than the natural RNA primers and will be discussed in another section.

Actinomycin D. At Actinomycin D concentrations (1-4 µg per ml) which inhibit the DNA-

Figure 3. The reaction mixture, 0.50 ml, was similar to the polycytidylate mixture described in Figure 1, except that 3 mM MnCl_2 and 4.5 enzyme units were used. GTP^{32} was added in the concentrations shown in the diagram and the reaction was incubated at 30° for 10 minutes.

primed synthesis of RNA,^{10,11} no inhibition was observed for the RNA-primed reactions. At concentrations of Actinomycin D exceeding 70 to 80 μg per ml, approximately 20 per cent inhibition of the RNA-primed reaction was seen.

Product analysis. It was previously reported that different synthetic RNA copolymers primed the synthesis of various RNA's whose base compositions were complementary to their respective primers.³ These results may now be extended with the demonstration that natural RNA's also prime the synthesis of a complementary product. The method of nearest neighbor frequency analysis, first described by Josse *et al.*,¹² has been used to determine RNA product composition in the DNA and RNA primed reaction.^{3,13} When the same viral RNA is used as a primer in 4 separate limited reactions (5 minutes), each with a different labeled substrate, alkaline digestion of the P^{32} -RNA formed and subsequent isolation of the hydrolyzed products indicate that all four 2',3'-mononucleotides are labeled. An example of this using TYMV RNA as primer is shown in Table 4. These data may be treated in the manner previously reported,^{3,12} from which the base composition of the synthesized RNA is derived. The base proportions of the TYMV, TMV and MS ϕ 2 RNA-primed products have been determined in this manner (Table 5). Column (A) in Table 5 lists the reported base compositions for the viral RNA's^{14,15} and column (B) lists the mole per cent as determined by the alkaline hydrolysis method used for product composition analysis.

The compositions of the synthetic ribopolymers obtained with the viral RNA primers are

Figure 4. The reaction mixture, 0.30 ml, was similar to that described in Table 1 except that the labeled substrate was UTP³² (S.A. = 7.65×10^6 cpm per μ mole), 8 units of enzyme were used, and the concentration of each RNA was varied as indicated in the figure. The mixtures were incubated for 30 minutes at 30°.

Table 4
DISTRIBUTION OF P³² IN NUCLEOTIDES ISOLATED AFTER
ALKALINE HYDROLYSIS OF P³²-PRODUCT
PRIMER: TYMV RNA (LIMITED REACTION)*

Isolated 2'(3')- ribonucleotides	Distribution of P ³² after KOH hydrolysis of P ³² -RNA's formed with different P ³² -substrates			
	Reaction no. 1 ATP ³²	Reaction no. 2 UTP ³²	Reaction no. 3 GTP ³²	Reaction no. 4 CTP ³²
	cpm	cpm	cpm	cpm
Up	657	1,682	6,264	6,430
Ap	1,456	1,067	7,370	3,927
Cp	1,125	1,448	11,666	4,418
Gp	3,001	3,126	13,294	7,946

* The reaction mixture, 2.0 ml, was similar to that described for Table 1. Four separate reactions were incubated in a complete system containing 48 μ g of TYMV RNA each, 160 units of enzyme each, and a different labeled triphosphate ranging in S.A. from 4 - 10×10^6 cpm per μ mole. After 5 minutes at 30°, the reaction was stopped with acid and the acid-insoluble fraction washed and extracted with hot NaCl as previously described.¹³ The hydrolysis of the labeled products, paper electrophoresis and isotope determination for the individual mononucleotides were the same as previously reported.¹³

Table 5
BASE COMPOSITION OF VIRAL RNA PRIMED PRODUCT*
(limited reaction: 5 minutes)

Base	Base proportions: mole %							
	TYMV RNA			TMV RNA			MS ϕ 2 RNA	
	(A)	(B)	Primed product	(A)	(B)	Primed product	(B)	Primed product
G	17	19	39	25	27	18	27	27
A	23	20	19	30	26	30	22	27
C	38	39	23	19	18	25	25	24
U	22	22	19	26	29	27	26	22

* Column (A) lists the reported values for the composition of TYMV and TMV RNA.^{14,15} Column (B) lists the composition of the viral RNA's indicated as determined in this laboratory by the method of alkaline hydrolysis.¹³ The composition of the TYMV RNA primed product was calculated from the data in Table 5 by the method of Josse et al.¹² The composition of the TMV and MS ϕ 2 RNA primed products were calculated from similar data (not shown here) by the same method.

distinctly different. TYMV RNA has a low guanine but high cytosine content whereas in TMV RNA the relative proportions of these two bases are reversed. Within the limits of experimental error, the products appear to represent complementary copies, in composition, to the plant viral RNA primers. In this regard, the RNA-primed reaction is similar to the DNA-primed synthesis of RNA. The composition of the product formed with MS ϕ 2 RNA primer reflects the nondistinctive base proportions of MS ϕ 2 RNA itself. However, it is significant that the composition of the product obtained with this primer does not resemble either of the products formed with the plant viral RNA primers.

In an extended reaction (several hours) with TYMV RNA as primer, the guanine to cytosine ratio of the product was distinctly lower than observed in the limited reaction. This would be expected if the newly synthesized RNA in turn served as template for additional synthesis. Ultimately, we should expect the guanine to cytosine ratio of the product to approach unity. However, complete base analysis of the product of extended reactions is not possible at this time since an "un-primed" synthesis of various homopolymers occurs after several hours.

Linearity and temperature. The rate of RNA synthesis with various RNA primers depends on the temperature of incubation (Figure 5). The primers polycytidylate and TYMV RNA are similar in that increased rates are observed with increasing temperature (Figure 5A and 5B). For polycytidylate, the reaction eventually reaches a maximum, between 80 and 120 minutes, and only slight further synthesis occurs. For TYMV RNA, the initial rate of reaction decreases sharply after the first 5 to 10 minutes, although label incorporation continues for 8 hours.

With polyuridylate and polyadenylate as primers, a decreased rate of synthesis is found at 40° compared with the initial reaction rates at 20° and 30° (Figure 5C and 5D). When polyuridylate and polyadenylate are mixed, a complex is formed which appears to have a DNA-like secondary structure.¹⁶ Such complexes also prime the synthesis of RNA and the anomalously low rate of synthesis observed for the single polymers at 40° is no longer found (Figure 6). The rates of polymerization in reactions containing the 1:1 complex of polyuridylate plus polyadenylate show

Figure 5. The reaction mixture for the RNA homopolymers was 0.50 ml and the same as in Figure 1 except that 6.5 units of enzyme and optimal MnCl_2 for the different primers were used (see Figure 1). For TYMV RNA the reaction mixture was the same as in Table 1 except that 24 μg of RNA was used. The various temperatures and times of incubation are indicated in the diagram.

a dependence upon temperature similar to that found when polycytidylate, viral RNA and DNA are used as primers. The reaction mechanism is complex and the rates observed with different primers may be related, in part, to the secondary structure characteristics as well as enzyme affinity for the various primers.

Polyadenylic and polyuridylic complexes. Polyadenylic acid, polyuridylic acid and the 1:1 complex of these two homopolymers may prime the synthesis of complementary polymers. Other investigators have described the formation of multistranded complexes, as well as the 1:1 complex, when these homopolymers are present in solution in different proportions.¹⁷ We have investigated the priming nature of various polyadenylate and polyuridylylate mixtures and the results are summarized in Table 6.

At 40°, the rates of UMP³² and AMP³² polymerization are most rapid when the adenine and uridine containing homopolymers exist in a complex form. Table 6 indicates that UMP³² polymerization (primer polyadenylate), at 40°, is most efficient when polyadenylic and polyuridylic

Effect of Temperature
on 1:1 Poly A + Poly U Priming

Figure 6. The reaction mixture, 0.50 ml, was as in Figure 1 for the polyuridylyte and polyadenylyte complex reaction except that 2 mM $MnCl_2$, 8 units of enzyme and the labeled substrates indicated above were present. Each set of reactions was incubated at the temperatures and for the times shown in the figure.

acids are present at nearly equivalent concentrations. On the other hand, the rate of AMP^{32} polymerization (primer polyuridylyte) appears to reach a maximum when the concentration of polyuridylyte and polyadenylyte approaches a ratio of 2:1.

Inhibition by RNA. The polymerization reaction in the presence of one RNA primer may be readily inhibited by the addition of a second polynucleotide. Figures 7 and 8 demonstrate the inhibition of the polycytidylyte primed formation of polyguanylyte when either polyadenylyte or polyuridylyte is added to the reaction mixture.

Maximal rate of UMP polymerization with polyadenylic acid as primer was found to occur between 1 and 1.5×10^{-3} M Mn^{++} . Above or below this metal ion concentration, polyuridylyte synthesis falls sharply (see Figure 1). Priming with polycytidylic acid is maximal at 4×10^{-3} M Mn^{++} , and at 1.5×10^{-3} M Mn^{++} synthesis is reduced but the reaction is still fairly active. Figure 7A illustrates that at optimal manganese ion concentration for polycytidylyte priming, polyadenylyte inhibits poorly, while at metal ion concentrations where polyadenylyte functions as an excellent

Table 6

THE PRIMING EFFICIENCY OF VARIOUS POLYURIDYLATE
AND POLYADENYLATE MIXTURES*

Primer: polyadenylic acid (20 μ g)			Primer: polyuridylic acid (20 μ g)		
Polyuridylylate added μ g	Ratio Poly U/Poly A	UMP ³² Incorporated m μ moles	Polyadenylate added μ g	Ratio Poly U/Poly A	AMP ³² Incorporated m μ moles
0		0.78	0		0.32
10	0.50	1.35	10	2.00	1.22
15	0.75	1.41	15	1.33	0.73
20	1.00	1.39	20	1.00	0.36
30	1.50	1.00	30	0.67	0.32
40	2.00	0.27	40	0.50	0.34
100	5.00	0.14	100	0.20	0.20

* The reaction mixture, 0.50 ml, was as in Figure 1 except that 5 units of enzyme were employed and the mixtures were incubated at 40° for 10 minutes. For the reaction mixtures containing UTP³² or ATP³² as substrates, the concentration of MnCl₂ was 2 mM and 3mM respectively.

primer, it also serves as a good inhibitor. The same data may be represented as the per cent inhibition of GMP³² incorporated (Figure 7B). This information suggests that one possible function of the metal ion is to promote the formation of an enzyme-primer complex. The situation is, however, somewhat more complex since in the presence of Mg⁺⁺, neither polyadenylic nor polyuridylic acid function as primer although they can inhibit the polycytidylate-primed reaction very effectively. It is possible that the extent of polymer aggregation in the presence of these metal ions accounts, in part, for the results observed.

Figure 8A shows that at 30° and 40° polyuridylylate inhibits the polycytidylate-primed reaction. The inhibition curves appear similar. Yet, if one examines Figure 8B, it may be seen that at the linear portion of inhibitor concentrations (5 μ g per ml) inhibition by polyuridylylate at 30° is nearly twice that observed at 40°. Once again, the data suggest that under conditions where a ribopolynucleotide is a good primer it will also be a more effective inhibitor.

Inhibition by DNA. The polymerization of GMP or AMP with polycytidylate and polyuridylylate primers respectively, is dependent upon the primer concentration (Figure 9A and 9C). The Km's for these synthetic homopolymers range between $1 - 2 \times 10^{-5}$ gm per ml of reaction and are slightly higher than the Km's for the natural RNA primers. In the preceding paper,² we showed that the DNA-primed synthesis of RNA, with the same enzyme fraction, was competitively inhibited by the addition of polycytidylate. Figure 9A and 9C show that the RNA-primed reactions, with two separate homopolymers, are inhibited by the addition of DNA. Figure 9B and 9D show that the inhibition is of a competitive nature.

Heat inactivation of enzyme. Preparations of RNA polymerase are stable for several hours at temperatures below 50°. Above 50°, the enzyme is rapidly inactivated over relatively short periods of time. If the enzyme is preincubated above 50° for different times, and then assayed at 30° in a complete reaction mixture containing either an RNA or a DNA primer, the apparent rates

Figure 7. The reaction mixture and conditions were the same as for polycytidylate primer in Figure 1 except that 25 units of enzyme were used. Two sets of reaction mixtures were run, one with 4 mM $MnCl_2$ and the other with 1.5 mM $MnCl_2$. Polyadenylate was added to each set as indicated in the diagram.

of enzyme inactivation are not the same (Figure 10). Figure 10A shows the difference in rates of heat inactivation with DNA and TYMV RNA as primers when the enzyme was preincubated at 54° . Figure 10B is a similar experiment with DNA and polycytidylate primers when the enzyme was preincubated at 52° . Similar results are found in both cases; the inactivation of the enzymatic activity appears to occur at a slightly slower rate when measured with a DNA primer than with an RNA primer. The greatest difference in activity for the separate primers, in either experiment, did not exceed 15 per cent. Alterations in the secondary structure of the enzyme by heating might result in altered DNA and RNA affinities for the enzyme. Affinity changes of this kind might account for the observed differences in priming after partial heat inactivation of the enzyme.

Electrophoresis. Under conditions previously outlined,¹ RNA polymerase preparations may be subjected to a glycerol-gradient electrophoresis in the LKB apparatus without loss of activity. The enzyme migrates at least half of the electrophoretic distance as a fairly uniform material and fractions may be collected for enzymatic assays. Figure 11 illustrates an experiment of this kind in which the fractions collected after electrophoresis were assayed separately for RNA syn-

Inhibition of Poly C Priming by Poly U Effect of Temperature

Figure 8. The reaction mixture and conditions were similar to those in Figure 7 except that 3 mM $MnCl_2$ was used. Two sets of reaction mixtures were incubated, one at 30° and the other at 40°. Polyuridylic acid was added to each set at the concentrations indicated in the diagram.

thesis with DNA and polycytidylic acid as primers. The results show that one peak of 280 m μ absorbing material was found, and that both priming activities were associated with this peak. Of the 3600 enzyme units subjected to this treatment, more than 80 per cent were recovered after 33 hours of electrophoresis.

Specific activity ratios. Throughout the purification procedure for *M. lysodeikticus* RNA polymerase, ribopolynucleotide synthesis may be demonstrated with a variety of nucleic acid primers. At the very early stages of purification, the reactions primed with DNA and polycytidylylate are most easily detected. As the purification progresses, priming with polyadenylate, polyuridylylate and the natural RNA's also become apparent. Table 7 illustrates the specific activities found with three different primers at various stages of enzyme purification. The ratios of specific activities, relative to priming with DNA, indicate that very little change occurs between the ability of RNA and DNA primers to direct RNA synthesis over the range of purification examined. So far, no treatment of the enzyme preparation has resolved these activities into two separate entities.

Figure 9. The reaction mixture, 0.50 ml, was as in Figure 1 for the primers polycytidylylate and polyuridylylate except the 3 mM MnCl_2 was used and 0.8 mM each of CTP and GTP was included in the polyuridylylate primed reaction to inhibit the DNA primed synthesis of polyadenylylate. For primer polycytidylylate (Figure 9A), three sets of reactions were incubated; one contained no DNA, and the other two sets contained 20 μg and 100 μg of calf thymus DNA. For primer polyuridylylate (Figure 9C), two sets of reactions were incubated; one set contained no DNA and the other set contained 50 μg of *P. fluorescens* DNA. The amounts of primer used in each set is shown in the diagram. The mixtures were incubated at 30° for 30 minutes. Figures 9B and 9D are reciprocal plots of the data shown in Figures 9A and 9C respectively.

Pyrophosphate exchange. RNA polymerase preparations carry out a conversion of PPi^{32} into a Norit-adsorbable form in the presence of RNA homopolymers as well as with DNA (Table 8). The exchange reaction is dependent upon a divalent metal ion and the addition of the appropriate ribonucleotides. In one case, with polyadenylylate but in the absence of any triphosphate, the slight but significant incorporation of PPi^{32} into a Norit-adsorbable form suggested that pyrophosphorolysis had occurred. It is interesting to note that in agreement with a similar study in the preceding paper, only the purine nucleotides function in this exchange reaction.² Except for polyadenylylate, each homopolymer requires the presence of its complementary ribonucleotide for exchange to occur. In this instance, ATP is required for a demonstrable exchange reaction,

Figure 10. Enzyme, in 0.1 M Tris, was preincubated at the temperatures shown and then an aliquot was removed at the time periods indicated and incubated in a complete reaction mixture, 0.50 ml, with calf thymus DNA, TYMV RNA or polycytidylate as primer at 30° for 10 minutes. The labeled substrate was GTP³² (S.A. = 2.0×10^6 cpm per μ mole). For primer DNA, TYMV RNA and polycytidylate, the amounts of GMP³² incorporated before preincubation at the elevated temperatures were 24, 3.1 and 19 μ moles respectively.

and UTP, which is normally polymerized with this primer, is almost completely inactive. This phenomenon occurs again when the 1:1 polyadenylate-polyuridylylate complex is tested. In the presence of ATP and the 1:1 complex, one observes significant PPi^{32} exchange, but not when UTP is present alone.

DISCUSSION

In the preceding report,² highly purified preparations of RNA polymerase were shown to catalyze the synthesis of RNA in the presence of a DNA primer. Two different reactions, both primed by DNA were described. In this study and in an earlier communication,³ we have shown that RNA polymerase prepared in the same manner, from the same organism, also catalyzes an RNA primed assembly of ribonucleic acid. This finding has been confirmed by Krakow and

Figure 11. Fraction VI, corresponding to the DEAE-cellulose step in the enzyme purification procedure,³ was used in this experiment. Enzyme, 3600 units, was electrophoresed for 33 hours at 800 volts in 0.01 M phosphate buffer, pH 6.7 in an LKB apparatus under conditions outlined in a preceding paper.³ The fractions collected at the end of the electrophoresis (5 ml), were examined for 280 m μ absorbing material and assayed separately with the primers calf thymus DNA and polycytidylic acid using GTP³² as the labeled substrate.

Table 7
SPECIFIC ACTIVITIES WITH DIFFERENT PRIMERS*

Enzyme fraction		Specific activities				
No.	Description	Primers			Ratio	
		DNA units/mg	Poly C units/mg	TMV RNA units/mg	$\frac{\text{DNA}}{\text{Poly C}}$	$\frac{\text{DNA}}{\text{TMV}}$
III	1 st protamine-SO ₄	105	90	10	1.17	10.5
IV	2 nd protamine-SO ₄	262	189	26	1.33	9.7
VI	DEAE cellulose	500	414	37	1.21	13.5

* The enzyme fractions listed were derived during the purification of *M. lysodeikticus* RNA polymerase and assays with DNA and RNA primers were as described in the preceding report.³

Ochoa⁴ working with a similar enzyme isolated from *Azotobacter vinelandii*.

The DNA- and RNA-directed polymerization of ribonucleotides are similar in that both require a divalent metal ion, ribonucleoside triphosphates serve as substrates, polymerization results in the release of PPi, and the products formed appear to be complementary to their respective primers. On the other hand, unlike the DNA-primed reaction, the RNA-directed synthesis of RNA is not inhibited by DNase or heat denaturation of the primer, and is not enhanced by the presence of polyamines. In most instances, the addition of spermidine was inhibitory for RNA

Table 8
PYROPHOSPHATE EXCHANGE*

Cofactors added	Polynucleotide present	PPi ³² converted to Norit-adsorbable counts
		mμmoles
ATP, CTP, UTP, GTP	DNA	3.46
None	DNA	<0.01
GTP	Polycytidylate	1.41
CTP	Polycytidylate	0.06
None	Polycytidylate	0.01
GTP, omit Mg ⁺⁺	Polycytidylate	<0.01
ATP	Polyuridylate	0.56
UTP	Polyuridylate	<0.01
None	Polyuridylate	<0.01
ATP, omit Mg ⁺⁺	Polyuridylate	<0.01
UTP	Polyadenylate	0.07
ATP	Polyadenylate	1.17
None	Polyadenylate	0.19
UTP, omit Mg ⁺⁺	Polyadenylate	<0.01
ATP + UTP	1:1 Polyadenylate + Polyuridylate	0.59
ATP	1:1 Polyadenylate + Polyuridylate	1.31
UTP	1:1 Polyadenylate + Polyuridylate	<0.01
None	1:1 Polyadenylate + Polyuridylate	0.06

* The reaction mixture, 0.50 ml, contained 0.10 M Tris buffer, pH 7.5, 2.5 mM MgCl₂, 0.8 mM each of ATP, CTP, UTP and GTP singly or in combination as indicated above, 0.88 mM PPi³² (S.A. = 1.34 x 10⁶ cpm per μmole), 20 μg each of polycytidylate, polyadenylate, and polyuridylate singly or in combination as indicated above, 50 μg of DNA where indicated, and 3.5 units of enzyme. The reaction was incubated at 30° for 90 minutes, stopped with acid, and the acid-soluble fraction treated with Norit as described in the preceding communication.⁴

formation. Also, Mg⁺⁺ can partially replace Mn⁺⁺ in reactions with some RNA primers but is completely inactive with others (Figure 1).

Some of the experiments reported here suggest that RNA secondary structure may influence the priming efficiency similar to the 4 NTP DNA-directed reaction. With polyuridylate or polyadenylate primers, incubation reactions at temperatures above 30° show a decreased rate of RNA synthesis. This anomaly can be abolished by complexing polyuridylate with polyadenylate. With the appropriate mixtures of these two homopolymers, polymerization of either AMP or UMP proceeds at a faster rate than with either homopolymer alone at 40° (Table 6), again suggesting that ordered primers are preferred. However, we have not been able to demonstrate

priming with polycytidylate-polyguanylate complex or any increased efficiency of polycytidylate priming by the addition of polyinosinic acid. Perhaps it is important to note that polynucleotide secondary structure may not only be important for the various "polymerases" which utilize RNA and DNA primers but also in systems which utilize RNA templates for protein synthesis.¹⁸

In terms of total polynucleotide material formed in this system, the relative efficiency of the primers tested in decreasing order is DNA, RNA homopolymers, complementary, and natural RNA's. Although the natural RNA's are relatively poor primers, incorporation of labeled substrates may be readily detected. Analysis of the products formed by viral RNA priming indicates that the polynucleotide assembled is complementary in base composition. These results simply suggest that in this system the RNA and DNA primed reactions appear to function in a similar template-like fashion, and yield no information concerning the mechanism of viral replication in vivo.

The RNA-directed synthesis of RNA appears to result in the formation of a primer-product complex, similar to the priming of RNA by heat-denatured DNA.* The priming by polycytidylate for GMP polymerization results in the formation of a primer-product complex from which the product may be isolated by selectively degrading the primer. The addition of RNase to polyadenylate-primed reactions only partially reduces the amount of polyuridylate formed. The addition of RNase, before enzyme addition, to reactions primed by polyuridylate, polycytidylate, and the viral RNA's, results in no product formation. However, we have observed that the addition of RNase to similar reactions containing natural RNA primers after sufficient polymer synthesis has occurred, results in very little disappearance of the acid-insoluble product. These experiments are consistent with the idea that a primer-product complex forms during reaction but further work is required to demonstrate the formation of highly ordered DNA-like structures.

The purification procedures used for RNA polymerase and electrophoresis studies have not resulted in the resolution of two separate enzymes. On the contrary, each step of enzyme purification results in no appreciable change between specific activity ratios when the preparation is assayed with DNA and RNA primers. The most convincing argument in favor of one enzyme for both activities arises from the observation that either type of nucleic acid primer appears to competitively inhibit the other. No complete explanation can be given for the different rates of heat inactivation of the enzyme when assayed with the different primers. However, the rates are not markedly different and these discrepancies might arise from different affinities between enzyme and primer especially since the molecular sizes of the primers differ considerably.

The rate and extent of PPi exchange reactions with RNA homopolymers are orders of magnitude lower than the synthetic reactions. This same observation was made with the DNA dependent reaction. The differences in extent of the forward reaction between these two primers is similarly reflected when the extent of PPi exchange is compared in reactions containing either of these nucleic acids (Table 8). Once again, the fact that only the purine nucleotides participate in the exchange reaction, when the triphosphates are added singly, occurs in the presence of RNA homopolymers as well as with DNA. No adequate explanation for these results can be offered at this time.

* Unpublished results.

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STUDIES ON THE INTERACTION BETWEEN BOVINE SERUM ALBUMIN AND
NATURAL AND SYNTHETIC POLYRIBONUCLEOTIDES. III. IMMUNOLOGIC
STUDIES ON HEATED MIXTURES OF POLYRIBONUCLEOTIDES
AND BOVINE SERUM ALBUMIN*

By

S. Yachnin†

Attempts in this laboratory to produce antibodies to synthetic polynucleotides and to highly purified mammalian RNA, DNA, and apurinic acid have proven fruitless.¹ In the hope that combination of polynucleotide with a potent protein antigen would permit the production of antibodies having some specificity for the polynucleotide moiety, methods of preparing such a product were sought. Greenstein has noted that the thermal coagulation of bovine serum albumin (BSA) could be prevented by DNA.² Studies on the interaction between DNA and native BSA have revealed limited complex formation under restricted conditions of ionic strength and pH.^{3,4} Zubay and Doty⁵ later demonstrated that protection against the heat coagulation of BSA afforded by DNA depends upon the formation of a large complex between the two macromolecules. According to their observations this complex could be dissociated by the presence of 8 M urea. From this they concluded that complex formation was not due to simple electrostatic interaction, as had been suggested for native BSA-DNA complexes,³ but rather was dependent upon hydrogen bonding.

Subsequent studies have shown that natural and synthetic polyribonucleotides are capable of preventing the thermal coagulation of BSA.⁶ On the presumption that their mode of interaction with BSA was similar to that of DNA in that a relatively stable complex was formed, a study of the immunologic properties of such heated mixtures was undertaken. The results to be reported in this paper† indicate that antibodies formed to such heated polyribonucleotide-BSA mixtures show no specificity for the polynucleotide moiety. However, antigenic differences between simple heat denatured BSA, and BSA denatured in the presence of polynucleotide, have been detected.

MATERIALS AND METHODS

Crystalline BSA was purchased from the Armour Pharmaceutical Corporation. Polyadenylic acid (Poly A) and polyinosinic acid (Poly I) were synthesized by means of polynucleotide phosphorylase isolated from Micrococcus lysodeikticus.⁷ The ribonucleoside diphosphates used in this synthesis were purchased from the California Corporation for Biochemical Research. Highly polymerized calf thymus DNA was a product of the Sigma Chemical Company. RNA was prepared from rat and guinea pig liver by the method of Laskov et al.⁸ and contained 2.4 and 2.8 per cent protein respectively. Complete Freund's adjuvant was obtained from the Difco Corporation. 0.0015 M citrate buffer pH 5.5 (C - Buffer), and 0.145 M NaCl in 0.015 M phosphate buffer pH 7.4 (P - S Buffer) were used as solvent and diluent in many of the experiments.

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† John and Mary R. Markle Scholar in the Medical Sciences.

‡ A brief summary of some of these findings has already been published in abstract form, Yachnin, S., J. Clin. Invest., 41:1414, 1962.

Preparation of antigen solutions and immunization procedure

Native BSA. A solution containing 3 mg protein/ml P-S buffer was prepared. Two adult rabbits weighing between 2 and 4 kg were given an initial intramuscular injection of antigen in Freund's adjuvant followed by 3 weekly series of 3 injections in P-S buffer. The animals were bled by cardiac puncture 7 days after the last injection and the antisera were pooled. Each animal received a total of 30 mg of protein.

Heated BSA. The immunization procedure was similar to that for native BSA. The heated BSA was prepared by heating a P-S buffer solution of BSA (3 mg/ml) in a boiling water bath (100° C) for 10 min. Three rabbits were immunized, and the antisera were pooled.

Heated BSA - polynucleotide mixtures. Polynucleotide and BSA were dissolved in C buffer in a ratio of 1:3 to 1:2.5 (wt:wt). The solution was then heated at 100° C for 10 min. Under these conditions BSA alone is completely coagulated, but due to the presence of polynucleotide these solutions remained clear.⁶ Two animals were immunized with each of the following heated mixtures: BSA-DNA, BSA-rat liver RNA, BSA-guinea pig liver RNA, BSA-Poly A. Each animal received a total of approximately 10 mg polynucleotide and 30 mg BSA. These sera were stored separately.

Sera were either frozen at -20° C until used, or were preserved by means of merthiolate and phenol and kept at 4° C.⁹ Prior to analytical procedures all sera were heated at 56° C for 30 min and centrifuged at 10,000 x g for 15 min to remove any sediment.

The following techniques of immunologic analysis were employed: complement fixation, quantitative precipitin techniques, single and double diffusion in agar gel. In general the procedures corresponded to those described by Kabat and Mayer.^{9a} Fresh antigen solutions were prepared for each analytical procedure. Prior to use heated polynucleotide-BSA mixtures prepared in C-Buffer, 1/10 volume of 10X concentrated P-S buffer was added in order to eliminate the possibility of nonspecific precipitation of serum protein by polynucleotide which occurs below pH 6.0.¹⁰ Heated polynucleotide-BSA mixtures were also prepared in P-S buffer and will be so identified. The extent of cross reaction between a given antiserum and a heterologous antigen, when utilizing quantitative precipitin techniques, is defined as

$$\frac{\text{Maximum antibody precipitated by heterologous antigen}}{\text{Maximum antibody precipitated by homologous antigen}} \times 100$$

All protein determinations were done by a modified Folin technique¹¹ utilizing BSA as a protein standard. RNA determinations were performed by the orcinol reaction and DNA concentrations were determined by the diphenylamine procedure.¹² Ultraviolet spectrophotometry was also used to determine polynucleotide concentrations and whenever necessary appropriate correction was made for protein. A Zeiss PMQ II spectrophotometer and cuvettes with a 1 cm light path were used throughout.

RESULTS

Search for antibody to polynucleotides alone. Antisera prepared against heated polynucleotide-BSA mixtures were analyzed for antibody to their homologous polynucleotide alone by the following techniques: complement fixation, Oudin and Ouchterlony techniques, and quantitative precipitin analysis. No evidence of antibody to polynucleotide was encountered.

Quantitative precipitin studies. Quantitative precipitin studies of antisera to heated polynucleotide-BSA mixtures revealed that homologous antigen mixtures were capable of precipitating a greater quantity of antibody than either heated BSA or native BSA alone (Table 1). The extent of cross reaction of the latter two antigens with these antisera varied; however, the majority of antisera cross reacted to a greater extent with heated BSA than with native BSA.

While antisera to heated BSA-polynucleotide mixtures do not react with polynucleotide alone, the possibility remained that the polynucleotide acting as hapten was responsible for the greater amount of antibody precipitable from such antisera by the heated mixture as opposed to heated BSA alone. Homologous polynucleotide dissolved in P-S buffer was added to antiserum and the mixture was incubated for 30 min at 37° C. Control serum was equally diluted and incubated. Both antisera were centrifuged at 10,000 x g for 15 min. No sediment was obtained. The two antisera were then subjected to quantitative precipitin analysis with the same homologous heated BSA-polynucleotide mixture. No significant differences were noted. Therefore it seemed reasonable to conclude that the polynucleotides were not behaving as haptens.

Analysis of immune precipitates formed by homologous antigen with heated BSA-polynucleotide antisera employing ultraviolet spectrophotometry failed to reveal the presence of detectable amounts of polynucleotide in these precipitates. Conversely, within the limits of experimental error, all polynucleotide could be found in the supernatant.

From the results so far described, it seemed likely that the greater amount of antibody precipitable from heated BSA-polynucleotide antisera by homologous antigen when compared with simple heated BSA was not the result of direct polynucleotide effect. If so, this difference should be accountable for by changes in the BSA moiety brought about by differences in the conditions of ionic strength and pH under which the BSA was heat denatured. Thus, BSA heat denatured in P-S buffer and subsequently mixed with polynucleotides heated separately in the same buffer, should show no increase in the amount of antibody precipitated from these antisera when compared with heated BSA alone. This concept was supported by the results shown in Table 2. In addition, BSA-RNA heated together in P-S buffer behaved very much like BSA heated alone in P-S buffer in a quantitative precipitin experiment using Anti BSA-RNA. The polynucleotide used in preparing the heated BSA-polynucleotide antigen in C Buffer was non-specific in the immunologic sense, since Poly I-BSA resembled closely the homologous RNA-BSA antigen when tested against RNA-BSA antiserum.

Studies on heated BSA-absorbed antisera. When antisera to BSA-polynucleotide complexes were absorbed with heated BSA in the region of slight antigen excess for this heterologous antigen, the resultant absorbed antiserum, incapable of further reaction with heated or native BSA, was nevertheless capable of further antibody precipitation by homologous heated BSA-polynucleotide mixtures. The amount of precipitate was surprisingly small (Figure 1) but was reproducible on repeat determinations. The maximum amount of protein precipitable from the absorbed antiserum, ranged from 6 to 21 per cent of the amount precipitated from the unabsorbed antiserum at equivalent antigen concentration, but equalled only 2 to 5 per cent of the maximum amount of protein precipitable from the original antisera. This precipitation was not inhibited by preincubation of the absorbed serum with its homologous polynucleotide, and as expected, no antibody was precipitated from this absorbed antiserum by polynucleotide alone.

Precipitin curves of pooled antisera to native and heated BSA were determined using both these antigens as well as heated polynucleotide-BSA mixtures. As expected, heated BSA was

Table 1

Rabbit no.	Immunized with	μ g antibody precipitated with			Cross reaction with	
		Homologous antigen	Heated BSA	Native BSA	Heated BSA %	Native BSA %
8374	Heated rat RNA-BSA	4762	1454	Not done	30.6	Not done
8349	Heated rat RNA-BSA	2294	968	1211	42.3	53
8020	Heated DNA-BSA	683	585	383	85.6	56
8401	Heated guinea pig RNA-BSA	987	542	230	54.8	23.4
8373	Heated guinea pig RNA-BSA	1314	974	610	74.1	46.4
8377	Heated Poly A-BSA	1515	602	661	39.7	43.6
8376	Heated Poly A-BSA	2332	1056	880	45.3	37.8
Mean (excluding No. 8374)					57	43.3

One ml of antiserum, or a saline dilution thereof, was mixed with 1 ml of homologous antigen in P-S buffer (the ratio of BSA:polynucleotide was approximately 2.5-3:1 and was kept constant at every protein concentration). Similar mixtures were made at each protein concentration with native BSA and heat denatured BSA. After an appropriate period of incubation (usually 1 hr at 37° C, followed by 24 hr at 4° C), the precipitates were centrifuged, washed, dissolved in 0.5 ml 0.1 N NaOH, diluted with distilled water, and subjected to Folin analysis.

Table 2

Rabbit serum no.	Immunized with	μ g antibody precipitated by			Cross reaction with	
		Homologous antigen	BSA heated in P-S buffer	BSA plus polynucleotide heated separately in P-S buffer and then mixed	Heated BSA %	Polynucleotide-BSA mixture heated separately %
8349	Heated rat RNA-BSA	832	503	435	60.5	52.0
8376	Heated Poly A-BSA	1333	1005	900	75.4	67.5
8401	Heated guinea pig RNA-BSA	339	142	209	42	62
Mean					59.3	60.5

The relative amounts of BSA and polynucleotide in the antigen solutions were equal at every dilution used in determining the quantitative precipitin curves. Homologous BSA-polynucleotide mixtures were heated together in C-buffer, and subsequently brought to isotonicity with 10X concentrated P-S buffer (see Text for discussion).

inefficient in precipitating antibody from anti-native BSA. The converse was also true.¹³ Surprising, however, was the relative efficiency of heated polynucleotide-BSA mixtures in precipitating antibody from both these antisera. Such heated mixtures prepared in C buffer precipitated slightly more antibody from heated BSA antiserum than did the homologous antigen. In addition, these heated mixtures prepared in C buffer displayed a much greater immunologic identity with

Figure 1. Precipitin curves of heated BSA-polynucleotide antisera absorbed with heated BSA, when subsequently tested with homologous antigen. Numbers in parentheses refer to amount of precipitate obtained in unabsorbed serum at the equivalent antigen concentration. ●—● Serum #8376 anti Poly A-BSA (BSA:Poly A in antigen used = 2.5:1); ○—○ serum #8349 Rat RNA-BSA (BSA:RNA in antigen used = 2.9:1); x—x serum #8373 guinea pig RNA-BSA (BSA:RNA in antigen used = 2.8:1).

native BSA than did heated BSA alone. If, however, polynucleotide-BSA were heated together in P-S buffer and tested against anti-native BSA, the immunologic resemblance to native BSA was lost, and such a mixture now behaved like heated BSA alone. Thus, in this phenomenon as well, the role of polynucleotide was an indirect one, and the changes in immunologic behavior of BSA when heated together with polynucleotide depended very strictly upon the conditions of ionic strength and pH under which the heated mixtures were prepared (Table 3).

Agar gel diffusion studies. Analysis of the various antisera and antigens by means of the Ouchterlony technique confirmed and extended the observations made by means of quantitative immunoprecipitation. When heated BSA-polynucleotide mixtures were allowed to react with homologous antisera it was found that at least 2 and usually 3 precipitin lines were formed. The most rapidly diffusing band gave a reaction of identity with native BSA and a reaction of partial identity with heated BSA. The middle band gave a reaction of identity with heated BSA. The major,

Table 3

Antiserum	μ g antibody precipitated by					Cross section (%) with				
	Native BSA	Heated BSA (P-S buffer)	Heated Poly A-BSA (C-buffer)	Heated RNA-BSA (C-buffer)	RNA-BSA Heated together (P-S buffer)	Native BSA	Heated BSA (P-S buffer)	Heated Poly A-BSA (C-buffer)	Heated RNA-BSA (C-buffer)	Heated RNA-BSA (P-S buffer)
Anti native BSA	1090	277	878	803	217		25.4	80.5	73.7	20
Anti heated BSA	52	342	368	392	Not done	15		108	114	Not done

Heated polynucleotide-BSA mixtures prepared in C-buffer retain a strong immunologic relationship to native BSA. This property is lost if the polynucleotide-BSA mixtures are prepared in P-S buffer (see Text for discussion).

most slowly diffusing band did not cross react with either native or heated BSA. However, if Poly A-BSA and RNA-BSA were tested simultaneously against their respective antisera, complete identity was seen between both major and minor bands, again demonstrating the lack of polynucleotide specificity in the new antigenic determinants induced by their interaction with BSA during heat denaturation in C buffer (Figure 2).

Figure 2. A. 1, Anti RNA-BSA (#8349); 2, Anti native BSA; 3, RNA-BSA; 4, Native BSA. B. 1, Anti RNA-BSA (#8349); 2, Anti heated BSA; 3, RNA-BSA; 4, Heated BSA. C. 1, Anti Poly A-BSA (#8376); 2, Anti native BSA; 3, Poly A-BSA; 4, Native BSA. D. 1, Anti Poly A-BSA (#8376); 2, Anti heated BSA; 3, Poly A-BSA; 4, Heated BSA. E. 1, RNA-BSA; 2, Poly A-BSA; 3, Anti RNA-BSA (#8373); 4, Anti Poly A-BSA (#8376); 5, Anti RNA-BSA (#8401).

As noted previously, polynucleotide alone gave no precipitin lines when tested against polynucleotide-BSA antisera. As expected, preaddition of polynucleotide to polynucleotide-BSA antisera in no way affected the precipitin lines formed between these antisera and their homologous antigens.

Antisera to heated polynucleotide-BSA mixtures preabsorbed with heated BSA were no longer capable of cross reacting with native or heated BSA. Such an absorbed antiserum was still capable of forming a single precipitin line with both homologous and heterologous polynucleotide-BSA antisera, the line being identical with the major slowly diffusing antigen. Other similarly absorbed

antisera were capable of forming only a much fainter precipitin line with homologous antigen, and some absorbed antisera formed no lines at all (Figure 3).

When tested against anti-native BSA, the heated polynucleotide-BSA antigens showed a strong pattern of identity with native BSA, this pattern being formed between the most rapidly diffusing band of the complex antigen and the single band between anti-native BSA and the native protein. The convexity toward the antigen well of the precipitin line formed between native BSA

Figure 3. A. Native BSA. 1, Anti Poly A-BSA (#8377); 2, Anti Poly A-BSA (#8376); 3, Anti RNA-BSA (#8349); 4, Anti RNA-BSA absorbed with heated BSA (#8374). B. Heated BSA. 1, Anti Poly A-BSA (#8377); 2, Anti Poly A-BSA (#8376); 3, Anti RNA-BSA (#8349); 4, Anti RNA-BSA absorbed with heated BSA (#8374). C. RNA-BSA. 1, Anti Poly A-BSA (#8377); 2, Anti Poly A-BSA (#8376); 3, Anti RNA-BSA (#8349); 4, Anti RNA-BSA absorbed with heated BSA (#8374). D. Poly A-BSA. 1, Anti Poly A-BSA (#8377); 2, Anti Poly A-BSA (#8376); 3, Anti RNA-BSA (#8349); 4, Anti RNA-BSA absorbed with heated BSA (#8374). E. RNA-BSA. 1, Anti RNA-BSA absorbed with heated BSA (#8349); 2, Anti Poly A-BSA absorbed with heated BSA (#8377); 3, Anti RNA-BSA absorbed with heated BSA (#8401); 4, Anti RNA-BSA absorbed with heated BSA (#8373). F. Poly A-BSA. 1, Anti RNA-BSA absorbed with heated BSA (#8349); 2, Anti Poly A-BSA absorbed with heated BSA (#8377); 3, Anti RNA-BSA absorbed with heated BSA (#8401); 4, Anti RNA-BSA absorbed with heated BSA (#8373).

and homologous or heterologous antiserum, and the rate of diffusion of the native protein were always greater when compared with the precipitin line identical to it formed between polynucleotide-BSA and antiserum, suggesting that the antigen involved in the latter precipitin line was of larger molecular size. Anti-native BSA reacted poorly, if at all, with the slowly diffusing antigen of the BSA-polynucleotide mixture.

The pattern of precipitin lines formed by antiheated BSA with its homologous antigen and BSA-polynucleotide mixtures was of interest because this antiserum reacted strongly with the slowly diffusing antigen of the BSA-polynucleotide mixture, and this precipitin line merged with that formed between anti-heated BSA and its homologous antigen. This was in accord with the efficacy with which polynucleotide-BSA mixtures reacted with heated BSA antiserum in the quantitative immunoprecipitation experiments (Figure 2).

DISCUSSION

Although there are marked antigenic differences between heated polynucleotide-BSA mixtures and BSA heated in the absence of polynucleotide, the experimental evidence presented in the preceding section suggests that polynucleotide does not contribute in any direct way to these differences, that is, it acts neither as hapten nor as antigen. The possibility remains that these antigenic differences might involve "link" antibodies requiring portions of both polynucleotide and carrier protein, such as have been described for polypeptidyl proteins.¹⁴ The strongest evidence against this possibility is the absence of detectable polynucleotide in the specific precipitates. It would appear then that these antigenic differences are in all probability a reflection of some change in the nature of BSA when heated together with polynucleotide, and further, that the conditions under which heating of the mixture occurs are crucial, since polynucleotide-BSA mixtures heated either separately or together in P-S buffer, behave very much like BSA heated alone in this buffer, whereas if the pH and ionic strength are lowered, and the mixture is heated together in C buffer, new antigenic properties, presumably of the protein, emerge. The use of BSA heated alone in C buffer was precluded by the fact that BSA coagulates under these conditions (*supra vide*) and is therefore not suitable for studies of this type. The emergence of these new antigenic properties of BSA may be viewed as a secondary effect of its interaction with polynucleotide. On the basis of the antigenic properties of mixtures of BSA and polynucleotide heated in P-S buffer, it would seem that no interaction occurs between the two substances under these conditions of ionic strength and pH. However, verification of this conclusion for the heated mixture requires other methodology.¹⁵

Even if, on the basis of the immunologic evidence accumulated, one were to regard the polynucleotide moiety as completely inert, it is surprising that antisera to the complex BSA-polynucleotide antigens failed to precipitate any polynucleotide joined to immunologically reactive BSA. One explanation for this might be that BSA combined with polynucleotide is also rendered immunologically inert, but since this seemed unlikely in view of the heightened antigenicity of these heated mixtures when compared with heated BSA alone, other explanations were sought. Such a result would be expected, if in fact, the interaction between heated BSA and polynucleotide were not a stable one, and were dissociated by simple alterations in pH and ionic strength. A study of this interaction by physico-chemical methods was therefore undertaken.¹⁵ The results of this study indicate that in fact electrostatic forces play a predominant role in stabilizing heated BSA-polynucleotide complexes, and that physiological conditions of ionic strength and pH are sufficient to dissociate them without the addition of such a reagent as urea as postulated by Zubay and Doty.⁵ Thus the secondary role of polynucleotide in the antigenic studies described is thrown into a clearer light. Still unanswered however, is the original question posed, namely, will combination of polynucleotide with an effective protein antigen allow them to participate in immunologic reactions? Since this work was begun others have shown that such an approach is fruitful, for the

combination of a purine analog with BSA induced the formation of antibodies with specificity for the purine moiety which could cross react with DNA.¹⁶

Since the heated BSA-polynucleotide mixtures are antigenically heterogeneous, one might superficially attribute the differences in the immunological behavior of heated polynucleotide-BSA mixtures when compared with heated BSA or native BSA, to the fact that the former preparations are in fact composed of a simple mixture of heated and native BSA. Closer analysis, however, reveals that this hypothesis is not sufficient to explain the observed phenomena. Thus, absorption of BSA-polynucleotide antisera with heated BSA removes all antibody capable of cross reacting with native and heated BSA, but leaves antibody still capable of reacting with homologous antigen; this antigen is found on Ouchterlony plate analysis to be the slowly diffusing new antigen band. BSA heated in the presence of polynucleotide in C buffer may be presumed to be denatured by virtue of the fact that enzymatic digestion of polynucleotide results in coagulation and precipitation of the protein.^{2,6} When separated from polynucleotide under conditions which do not result in precipitation, all the BSA is found to be aggregated, since no protein with the sedimentation properties of native BSA can be detected.¹⁵ In addition, the rate of diffusion and shape of even the fastest migrating band of the heated polynucleotide-BSA mixtures in agar suggest aggregation when compared with native BSA.¹⁷ Thus even the protein band of the heated polynucleotide-BSA mixture which gives a reaction of identity with native BSA on Ouchterlony analysis, and which probably accounts for the efficiency with which polynucleotide-BSA mixtures precipitate antibody from native BSA antisera, is aggregated. The retention by this protein component of a close immunologic relationship to native BSA, despite aggregation, supports the concept of heat denaturation as being a multi-step process involving changes in the molecular structure of BSA not attributable to aggregation per se.¹³ The effect of polynucleotide in preserving immunologic features of native BSA during the heat denaturation of BSA-polynucleotide complexes is reminiscent of the action of small molecular weight anions in protecting BSA against heat denaturation.¹⁸ The failure of polynucleotide to perform a similar protective effect on BSA when the two are heated together in P-S buffer is due to the lack of association between them under these conditions.¹⁵

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BIOLOGIC PROPERTIES OF POLYNUCLEOTIDES. I. THE ANTICOMPLEMENTARY ACTIVITY OF POLYNUCLEOTIDES*

By

S. Yachnin[†]

The biologic role of nucleic acids is, under normal circumstances, concerned with protein synthesis and cell replication, although there are instances where polynucleotides may participate in biological phenomena not directly concerned with such synthetic processes. For example, certain deoxyribonucleic acids (DNA) have the capacity to elicit specific antibody formation in experimental animals;¹ in the human, the sera of patients suffering from lupus erythematosus have been shown to contain antibodies specific for DNA;² and under conditions of rapid cellular destruction, the release of large amounts of DNA into the circulation has been thought to cause small vessel occlusion and death from widespread pulmonary infarction.³

Recent evidence suggests⁴⁻⁶ that it is possible to effect transformation of mammalian cells by exogenous nucleic acids; thus it is possible that these materials may become useful as therapeutic agents in genetic disease, and so entail the administration of large amounts of nucleic acids to humans. Therefore a systematic investigation of the effects of polynucleotides on various biological systems has been undertaken. In addition to studies with naturally occurring nucleic acids, synthetic ribopolynucleotides have been examined because these substances offer the opportunity to observe the effects of variations in base composition and secondary structure on the participation of nucleic acids in such phenomena.

During the course of previous work on the potential antigenicity of homoribopolynucleotides in experimental animals,⁷ it was noted that polyinosinic acid (poly I)[‡] exhibited strong anticomplementary (Anti-C') activity. The present report describes this phenomenon in detail, and deals with quantitative as well as qualitative aspects of the poly I-complement (C') interaction. In addition, evidence is presented that will define some of the structural requirements necessary for the endowment of ribopolynucleotides in general with Anti-C' properties.

MATERIALS AND METHODS

Synthetic polynucleotides were synthesized by means of polynucleotide phosphorylase isolated from Micrococcus lysodeikticus. Purification of the enzyme was carried through an acetone fractionation, and the protein precipitating between 40-65 per cent acetone (v/v) was used.⁸ The 280 m μ /260 m μ absorbancy ratio of this enzyme preparation was on one occasion as high as

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[†] John and Mary R. Markle Scholar in the Medical Sciences.

[‡] Abbreviations used in this paper: biosynthetic homopolymers of adenylic, guanylic, uridylic, cytidylic and inosinic acid: poly A, poly G, poly U, poly C, and poly I respectively; copolymer of inosinic and uridylic acids: poly IU; copolymer of guanylic and uridylic acids: poly GU; 5'-monophosphates of guanosine, uridine, or inosine: GMP, UMP, or IMP. Oligonucleotides are indicated as follows: a phosphate is designated by p; when placed to the right of a nucleoside symbol, the phosphate is esterified at C-3' of the ribose moiety; when placed to the left of the nucleoside symbol, the phosphate is esterified at C-5' of the ribose moiety.

1.77, indicating a low degree of contamination by nucleic acid material; this paucity of potential primer might explain the difficulty encountered in synthesizing not only poly G but also those mixed GU copolymers containing more than 50 per cent G. In synthesizing the GU copolymers it was necessary to add a small amount of pApApA primer.^{9*} Even then the synthesis of copolymer no. 2 (66 per cent G, 34 per cent U) required 72 hours to approach equilibrium, and copolymer no. 1 (85 per cent G, 15 per cent U) required 215 hours, with intermittent replenishment of enzyme, for synthesis of polymer in relatively low yield. The ribonucleoside diphosphates used as substrate in these syntheses were purchased from the Pabst Laboratories.[†] The 5' monophosphates used in certain of the experiments were obtained from the same firm.

Calf thymus DNA was a commercial product.[‡] Various mammalian ribonucleic acids (RNA) were prepared by the method of Laskov, Margoliash, Littauer, and Eisenberg.¹⁰ In general, following the phenol denaturation step, the RNA was precipitated by ethanol and consisted of total cellular RNA. In one instance, however (rabbit liver RNA), precipitation of RNA was accomplished by 1 M NaCl, a procedure which favors the isolation of high mol wt material. *Escherichia coli* soluble (S) and microsomal (M) RNAs were prepared by ultrasonication, differential centrifugation in a sucrose medium, phenol extraction, DNase digestion and a second phenol treatment. The M RNA was then precipitated with 1 M KCl and the S RNA with ethanol.[§] Apurinic acid was prepared from calf thymus DNA by the method of Tamm, Hodes, and Chargaff.¹¹

The synthetic polymers were virtually protein-free, while the naturally occurring nucleic acids contained a maximum of 2 per cent protein. Two batches of synthetic homoribopolynucleotides were used and no essential differences were found between them in their effects on the complement system. A list of all the polynucleotides used together with some of their properties is given in Table 1. Stock solutions of polynucleotides containing 4 μ moles P/ml were prepared in 0.15 M NaCl, 0.01 M Tris buffer, pH 7.4, and were stored at -20° C. Because of the difficulty of dissolving poly I at this pH it was usually dissolved in Tris-saline, pH 9.2, and subsequently titrated to pH 7.4 with small amounts of HCl.

Protein determinations were done by the method of Hewitt,¹² and phosphorous determinations by the method of Fiske and SubbaRow.¹³ Base compositions of ribopolynucleotides were determined by alkaline hydrolysis followed by paper electrophoresis in 0.025 M citrate buffer, pH 3.5, for 20 hours at 400 v.¹⁴ Because separation of IMP and UMP could not be accomplished by electrophoresis, the alkaline hydrolysates of poly IU were subjected to descending paper chromatography in an isopropanol:concentrated HCl:water solvent system (680:132:188 v/v). In this system the R_f values of IMP and UMP are 0.39 and 0.77 respectively. While the base composition of the IU and GU copolymers were roughly proportional to the concentrations of the respective nucleoside diphosphates used in the synthetic step, significant differences were noted.

All C' studies were carried out in barbital buffered saline, pH 7.4, containing optimal (Ca^{++}) and (Mg^{++}) at $37 \pm 0.1^\circ \text{C}$. C' titrations were carried out by the 50 per cent hemolytic unit ($\text{C}'\text{H}_{50}$) assay technique as outlined in Kabat and Mayer's text.¹⁵ Single batches of pooled human (45 $\text{C}'\text{H}_{50}/\text{ml}$) and guinea pig (295 $\text{C}'\text{H}_{50}/\text{ml}$) serum stored in aliquots at -80° C were used

* Kindly supplied by Dr. Maxine Singer, National Institutes of Health, Bethesda, Md.

† Milwaukee 5, Wisconsin.

‡ Sigma Chemical Company, St. Louis 18, Missouri.

§ Kindly supplied by Dr. William Robinson, Argonne Cancer Research Hospital.

Table 1
SELECTED PROPERTIES OF POLYNUCLEOTIDES

Polynucleotide	Base composition (%)					$\epsilon_{M(P)}^{*\dagger} (x 10^{-3})$	$S^{\ddagger\ddagger}$
	A	U	G	C	I		
I. RNAs							
Guinea pig liver RNA	18.2	20.5	34.5	26.8		7.61	
Rat liver RNA	18.9	16.3	38.1	26.7		7.85	
<u>E. coli</u> S RNA	19.2	20.3	32.1	28.4		7.69	
<u>E. coli</u> M RNA	26.7	19.0	30.8	23.5		7.82	
II. DNAs							
Calf thymus DNA [§]	27.4	29.1 [¶]	21.4	21.0		6.44	
Calf thymus apurinic acid		58 [¶]		42.0		4.21	
III. Synthetic polyribonucleotides							
Poly A	100					8.78	5.4
Poly U		100				9.18	3.8
Poly C				100		5.64	3.9
Poly I					100	6.13	5.6
Copolymer no. 1 poly GU		15	85			8.22	
Copolymer no. 2 poly GU		34	66			10.1	
Copolymer no. 3 poly GU		68	32			9.68	
Copolymer no. 4 poly IU		17			83	6.73	
Copolymer no. 5 poly IU		44			56	7.71	
Copolymer no. 6 poly IU		69			31	8.26	

* Molar absorbandy expressed in terms of P concentration.

[†] Tris saline buffer, pH 7.4.

[‡] Beckman Model E ultracentrifuge, UV optics.

[§] Base composition from The Nucleic Acids, E. Chargaff and J. N. Davidson, Eds., Vol. 1, Academic Press, New York, 1955, p. 354.

[¶] Thymine.

throughout. The C' titer of these reagents was assayed periodically and remained constant during these studies. The quantitation of Anti-C' activity was a modification of the method described by Taylor.¹⁶ A known amount of C' sufficient to cause 100 per cent lysis was used, usually 3 or 4 C'H₅₀. Following a preliminary titration to define the approximate range of activity of the substance under study, a titration was performed with amounts of the test substance which would span the range of 10 to 90 per cent hemolysis. The concentration of the test material was then plotted against per cent hemolysis on logarithmic probability paper and the amount giving 50 per cent lysis was read from a line fitted to the points by inspection. From this, the amount of material needed to inhibit 1 C'H₅₀ could be calculated. For example, if the test system contained 4 C'H₅₀, and 0.6 μ mole of test substance yielded 50 per cent lysis, then 0.2 μ mole (0.6/3) of the substance would inhibit 1 C'H₅₀. Although the order of addition of reagents was not critical the following sequence was adopted: sensitized cells, buffer, test substance, C'. The standard 7.5 ml test system was used, except where the test material was available in very small amounts when a 1.5 ml system was employed, with all the reagent volumes reduced by 1/5. In these instances the supernatant was diluted 1:10 and read at 413 m μ instead of the usual 540 m μ wavelength. However, the Anti-C' potency of all substances is expressed in terms of the 7.5 ml system.

Reagents lacking each of the four C' components (R₁, R₂, R₃, R₄) were prepared by established techniques.^{15a} Aliquots of a 1:10 dilution of each reagent were stored at -80° C. At this concentration, none of these reagents would support hemolysis in the standard test system, nor did any of them display Anti-C' activity when tested with 4 C'H₅₀. Titration of these reagents for each of the 4 C' components, using 50 per cent hemolysis as the endpoint, were performed.

Sheep cells preserved in Alsever's solution and amboceptor were commercial preparations.* Since the Anti-C' effect of heparin has been extensively studied,^{16,17} several preparations of heparin[†] were assayed for their Anti-C' potency to allow comparison with the polynucleotides under study. The Anti-C' potency of polynucleotides is expressed in terms of μ moles P, and that of heparin in terms of μ moles S, needed to inhibit 1 C'H₅₀.

Pancreatic ribonuclease (5 x crystalline) was a product of Worthington Biochemicals.[‡] Cro-talus adamanteus venom was obtained from Ross Allan Snake Farm.[§] Digestion of poly GU was accomplished by incubating 1 μ mole P poly GU and 50 μ g ribonuclease in 1 ml barbital buffered saline, pH 7.4, at 37° for 2 hours.

pH measurements were made with a Beckman Zeromatic pH meter. All spectrophotometric measurements were made on a Zeiss PMQII spectrophotometer in cuvettes with a 1-cm light path.

RESULTS

The results of a typical poly I titration are shown in Table 2, and are plotted graphically in Figure 1. From the latter it can be seen that 0.022 μ mole P poly I yields 50 per cent lysis. Since

* Markham Laboratories, Chicago, Illinois.

† Heparin HWD (Hynson, Westcott and Dunning, Baltimore, Md., Lot no. 262, 13 per cent S). Heparin U (Upjohn Company, Kalamazoo, Mich., Lot no. MK-449-HL, 13.18 per cent S). Heparin T (Testagar and Company, Inc., Detroit, Mich., Lot no. 12778, 9.5 per cent S).

‡ Freehold, New Jersey.

§ Silver Springs, Florida.

there are 4 C'H₅₀ present in this system, 0.0073 μ mole P poly I inhibited the hemolytic activity of 1 C'H₅₀. Table 3 shows the results of a series of similar titrations using both human and guinea pig C' which were performed throughout the course of this study. The amount of poly I needed to inhibit 1 C'H₅₀ was similar for the human and guinea pig reagent. The following polynucleotides were tested for Anti-C' activity in both human and guinea pig C' systems and were

Table 2
TITRATION OF THE INHIBITION OF C' BY POLY I

Tube	Sensi- tized cells (ml)	C'(4 C'H ₅₀) (ml)	Buffer (ml)	μ mole P poly I	A ₅₄₀	A ₅₄₀ (Av.) -A ₅₄₀ blank	Lysis (%)
1	1.0	1	5.5	0.06	0.024	0.002	0.3
1'	1.0	1	5.5	0.06	0.023		
2	1.0	1	5.5	0.05	0.027	0.008	1.2
2'	1.0	1	5.5	0.05	0.031		
3	1.0	1	5.5	0.04	0.035	0.020	2.9
3'	1.0	1	5.5	0.04	0.047		
4	1.0	1	5.5	0.03	0.122	0.106	15.3
4'	1.0	1	5.5	0.03	0.131		
5	1.0	1	5.5	0.025	0.188	0.167	24.2
5'	1.0	1	5.5	0.025	0.188		
6	1.0	1	5.5	0.020	0.456	0.433	62.8
6'	1.0	1	5.5	0.020	0.452		
7	1.0	1	5.5	0.015	0.661	0.648	94
7'	1.0	1	5.5	0.015	0.675		
C' control	1.0	1	5.5		0.713	0.692	100
C' control	1.0	1*	5.5		0.382	0.361	52.3
Cell blank	1.0		6.5		0.021		
100% lysis	1.0		6.5†		0.711	0.690	

* 1 C'H₅₀.

† 0.1 % Na₂CO₃.

found to be completely inactive in amounts as high as 1 μ mole P polynucleotide: calf thymus DNA, both native and heat denatured, apurinic acid, guinea pig liver RNA, rat liver RNA, *E. coli* S RNA, *E. coli* M RNA, poly A, poly U, and poly C. Oxalate ion in amounts up to 1 μ mole had no effect on C' activity. 5' ribonucleotides, alone or in combination, were also devoid of Anti-C' activity. The Anti-C' activity of the several heparins studied is shown in Table 4. In comparison

Figure 1. Titration of the inhibition of C' by poly I. A graphic representation of the experiment outlined in Table 1. 0.022 μ mole P poly I yields 50% lysis. Since the test system contained 4 C'H₅₀, 0.0073 μ mole P poly I inhibits 1 C'H₅₀.

Table 3

THE AMOUNT OF POLY I (μ MOLE P) NEEDED TO INHIBIT
1 C'H₅₀ OF HUMAN AND GUINEA PIG C'

A series of titrations using single batches of each C' were performed during the course of this study.

Determination no.	Guinea pig C'	Human C'
1	0.0073	0.0086
2	0.0073	0.0110
3	0.0082	0.0065
4	0.0075	0.0068
Mean $\pm$ S.D.	0.0076 $\pm$ 0.0004	0.0082 $\pm$ 0.0018

with heparin, poly I was an extremely potent Anti-C' substance, being approximately 300 times more potent (S:P molar ratio).

The experiment shown in Table 5 was designed to establish whether poly I was capable of preventing the attachment of amboceptor to sheep red cells. 0.08 μ mole P poly I completely in-

Table 4
THE AMOUNT OF HEPARIN (μ MOLE S) NEEDED TO INACTIVATE
1 C'H₅₀ OF HUMAN AND GUINEA PIG C'

Heparin	Guinea pig C'	Human C'
HWD		2.64
U	2.22	2.48
T	2.23	2.32
Mean	2.23	2.48

Table 5
THE EFFECT OF VARYING THE STAGE AT WHICH POLY I
IS ADDED TO THE HEMOLYTIC SYSTEM

When added during the amboceptor-red cell sensitization step, and removed by washing the cells prior to C' addition, poly I will not inhibit red cell lysis

	Constituents		
	Tube no. 1	Tube no. 2	Tube no. 3
Sensitization step			
Sheep cells	0.5 ml	0.5 ml	0.5 ml
Hemolysin	0.5 ml	0.5 ml	0.5 ml
Saline		1.0 ml	1.0 ml
Poly I 0.08 μ mole/ml	1.0 ml		
Incubate 37° 30 minutes. Wash cells 3 times. Add following:			
Hemolysis step			
Saline	6.5 ml	5.5 ml	6.5 ml
Poly I 0.08 μ mole/ml		1.0 ml	
4 C'H ₅₀ /ml	1.0 ml	1.0 ml	1.0 ml
Incubate 37° 60 minutes			
% hemolysis	100	2	100

hibited lysis when added during the second phase of the experiment, but did not interfere with the sensitization of sheep cells to lysis if present only during the first phase. However, in common with other Anti-C' substances,¹⁶ increasing the amount of hemolysin in the test system, even while keeping C' constant, overcame the inhibitory effects of poly I (Figure 2A).

In an attempt to discover which components of C' were involved in the inhibition of C' activity by poly I, the various R reagents were added to a system containing C' and optimal inhibitory amounts of poly I, and the restoration of hemolytic potency was measured (Table 6). All four reagents caused partial restoration of hemolytic activity; the relative degree of restoration being

Figure 2. A. Effect of increasing hemolysin concentration upon Anti-C' activity of poly I. Each tube contained 0.5 ml sheep cells, 0.5 ml hemolysin dilution, 4 C'H₅₀, and 0.04 μ mole P poly I in a total volume of 7.5 ml. B. Effect of increasing hemolysin concentration upon hemolytic potency of a C' limited system. There is no poly I present. Each tube contained 0.5 ml sheep cells, 0.5 ml hemolysin dilution, 0.75 C'H₅₀ and buffer to a total volume of 7.5 ml.

maintained at both poly I concentrations. The order of magnitude of this hemolysis restoring ability was $R_3 > R_4 > R_2 > R_1$.

Under the conditions of ionic strength and pH used in these experiments poly I exists largely as a random coil.¹⁸ However, poly I will react stoichiometrically with both poly C and poly A to form ordered hydrogen bonded helical structures.^{8a} The experiment shown in Figure 3 demonstrates that the formation of such helices destroys the Anti-C' activity of poly I. The amount of poly C necessary to restore 50 per cent hemolysis was 1.7 times the amount of poly A required. This is consistent with the known structures of the respective helices which in the case of poly C is two-stranded (I + C), while poly I + poly A yields a triple-stranded helix (2I + A).^{8a} In this experiment the helices were preformed prior to their addition to the test system. However, even if C' and poly I were first mixed, the inhibitory effect of poly I could be abolished by subsequent immediate addition of poly A and poly C. Poly U, which cannot react with poly I, had no effect on the latter's Anti-C' activity even at poly U:poly I ratios of 6:1. The Anti-C' activity of poly I was destroyed by digestion with snake venom (*Crotalus adamanteus*) phosphodiesterase.

It can be seen (Table 7) that the Anti-C' potency of the IU copolymers is very much less than that of poly I. In addition, it is noteworthy that poly GU (85 per cent G, 15 per cent U) is 5 times more potent than poly IU of similar base ratio. Digestion of the GU copolymer with ribonuclease did not destroy its Anti-C' properties.

Table 6

THE CAPACITY OF R REAGENTS TO RESTORE HEMOLYSIS
IN A POLY I INHIBITED C' SYSTEM

Total reaction volume 7.5 ml. All tubes contained 3 C'H₅₀ human C'. Poly I and R reagents (1.0 ml) were added as indicated.

Amount of poly I present	R reagent added	% hemolysis
none	none	100
0.02 μ mole	none	8.6
	R ₁	24.9
	R ₂	33.8
	R ₃	94.2
	R ₄	45
0.03 μ mole	none	3.2
	R ₁	10.9
	R ₂	14.2
	R ₃	29.8
	R ₄	18

DISCUSSION

These studies demonstrate that certain synthetic polyribonucleotides possess powerful Anti-C' activity, poly I being the most potent. Although the phosphate groups of polynucleotides are known to bind Ca⁺⁺ and Mg⁺⁺ on an equimolar basis,^{8b} it is clear from the data presented that this cannot explain the Anti-C' effect of poly I since, 1) the amounts of poly I used were much too small to bind either of these cations completely, 2) other polynucleotides lacking Anti-C' activity are known to have similar chelating potential, and 3) oxalate ion in 130 times the concentration of poly I had no inhibitory effect.

That poly I is primarily single stranded under the conditions of pH and ionic strength used in these experiments is not unique, for poly U and poly A are also single stranded under these conditions. Molecular size is not the critical factor in explaining the Anti-C' property of poly I since the poly A and poly I had very similar sedimentation coefficients (5.4 and 5.6 respectively). If it is assumed that the Anti-C' activity of poly I depends upon its ability to interact with one or more of the serum proteins which constitute the C' system, such an interaction must depend primarily upon a structural feature of the polymer bestowed by the constituent base. This contrasts with the interaction of DNA and ribopolynucleotides with bovine serum albumin, where all the nucleic acids are capable of combining with the protein in an interaction which is primarily electrostatic in nature, involving the negatively charged phosphate groups.¹⁹ Although we have had no success as yet in synthesizing poly G it seems reasonable to conclude from the data on the Anti-C' potency of the IU and GU copolymers that it may be at least as potent, or indeed even more potent, than poly I* in Anti-C' activity. We may conclude that the Anti-C' activity of polynucleotides depends

* A theoretical objection to this assumption may be found in the recent work of Fresco²⁰ who

Figure 3. Effect of helix formation on the Anti-C' potency of poly I. Each tube contained 1 ml sensitized sheep cells, 4 C'H₅₀, and 0.08 μ mole P poly I, together with the indicated amount of either poly A or poly C. 50% hemolysis occurs when either 0.029 μ mole P poly A or 0.048 μ mole P poly C is added to the test system.

in part upon the presence of a purine ring possessing an OH group at the 6 position. In the case of poly I, involvement of the OH group in hydrogen bond formation in the helical structures (I + C) and (2I + A) stoichiometrically abolishes Anti-C' activity.

Uridylic acid was chosen as the second base in the synthesis of the IU and GU copolymers to eliminate the possibility of intermolecular hydrogen bond formation. Such copolymers probably exist in solution largely as random coils. Since IMP and GMP are lacking in Anti-C' activity, we may conclude that polymerization of these molecules is critical in endowing them with this property. Since poly U was incapable of preventing the Anti-C' activity of poly I even when present in large excess (6:1), the marked drop in Anti-C' potency of the IU copolymers cannot be explained on the basis of active reversal by the presence of polymerized U within these molecules. If all that was necessary to endow an I residue with Anti-C' properties was its presence in a polymer, then the Anti-C' potency of the I molecules in the IU copolymers should remain unchanged. The most reasonable explanation for the precipitous decline in the Anti-C' potency of polymerized I residues in these copolymers is that, in order for I residues to possess Anti-C' activity, they

has found that in 0.15 M NaCl, pH 7.4 poly G exists as a multistranded helix. Ordered structure of polynucleotide may be incompatible with Anti-C' activity.

Table 7

THE ANTI-C' POTENCY OF A SERIES OF IU AND GU COPOLYMERS

	Polymer no.	Base composition (%)			μ mole P needed to inhibit 1 C'H ₅₀	Anti-C' potency compared with poly I (based on % I or G content)
		I	G	U		
Poly IU	4	83		17	1.23	1/134
	5	56		44	*	
	6	31		69	*	
Poly GU	1		85	15	0.25	1/27
	2		66	34	†	
	3		32	68	*	

* No Anti-C' activity with 3 μ mole P polymer.

† Slight Anti-C' activity with 3 μ mole P polymer.

must occur in a repetitive extended sequence uninterrupted by another base.

An estimate of the critical chain length (k) may be deduced from the fact that the I residues in the (83 per cent I + 17 per cent U) copolymer possess only 0.75 per cent the potency of poly I. Statistical methods may be used to estimate k since the incorporation of base into polymer by polynucleotide phosphorylase occurs in a random fashion.²¹ Such an analysis* utilizing the base composition of this copolymer reveals that a k of 43 I residues is necessary for a sequence of I to equal poly I in Anti-C' potency. If it is assumed that poly G = poly I in Anti-C' activity, then the k for G sequences is 28 (Figure 4). Chains of this length are maximum estimates, and it is likely that shorter k 's of I or G possess graded Anti-C' activity. Experiments to test this hypothesis will be reported on subsequently. The requirement for an extended base sequence for Anti-C' activity resembles the antigenic determinants in DNA which react with DNA antibodies in lupus sera, where an extended sequence of thymine base plays a critical role.²²

This concept explains the lack of discernible Anti-C' activity in the synthetic copolymers containing 60 per cent or less of I or G, and also explains the lack of Anti-C' activity in naturally occurring RNA where the G content ranges between 30 and 40 per cent; in addition in the latter case, base distribution is not random and there is a large amount of inter- and intramolecular base pairing.²³

The fact that ribonuclease digestion of the (85 per cent G, 15 per cent U) copolymer did not destroy its Anti-C' properties is compatible with the hypothesis outlined since it means that the products of digestion which consist predominantly of oligonucleotides of the general structure $G(pG)_nU_p$ retain as much Anti-C' activity as was originally present in the intact molecule.

The ability of increasing hemolysin concentrations to reverse the Anti-C' activity of poly I reflects the fact that the hemolysin used is one which, in a limited C' system, increases the relative hemolytic potency of whatever C' is free to act (Figure 2B). The exact nature of poly I-C' interaction, in terms of the 4C' components is by no means clear. The experiments with the R reagents suggest that C'_1 may be the most vulnerable factor but if one examines the relative C'_1

* See appendix.

Figure 4. The probability of various chain lengths (k) of I or G occurring in IU or GU copolymers. See appendix for equations used in deriving these curves.

titers of the C'_1 containing reagents, this concept breaks down, since R_3 , which was most effective in restoring hemolytic potency in a poly I inhibited C' system, had the lowest C'_1 titer. Clearly, as other authors have concluded,²⁴ the use of the R reagents is of limited value in studies of this kind. Investigations presently under way are aimed at pin-pointing the site of poly I- C' inhibition by the use of the kinetic analysis methods of Mayer and others, and by a study of its effects on C'_1 esterase activity.

These experiments define a heretofore unrecognized biologic property of certain polyribonucleotides. Apart from the inherent interest of this phenomenon, the potency of poly I as an Anti- C' substance, surpassing as it does the activity of other Anti- C' substances such as heparin and the dextran sulfates,¹⁶ may warrant its trial in situations where the participation of C' in immune reactions leads to cellular injury or death. It seems reasonable to assume that a potent Anti- C' substance may prove useful in the treatment of certain immunologic diseases of men where C' , in association with other factors, leads to tissue injury.

APPENDIX

Given an infinite molecule of the form ABBABBBAAAB where A and B are incorporated in a random fashion in the proportions p for A and q for B. Let k = any given length of uninterrupted A molecules. Let T_k = fraction of A molecules in chains of length k . Then

$$T_k = k q^2 p^{k-1}$$

Let S_k = fraction of A molecules in chains of length $\geq k$. Then

$$S_k = p^{k-1} (p + kq).$$

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