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MASTER

Semiannual Report to **THE ATOMIC ENERGY COMMISSION**

MARCH 1962

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
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IMMUNE RESPONSE IN IRRADIATED MICE WITH
PEYER'S PATCH SHIELDING*

By

L. O. Jacobson, E. K. Marks, E. L. Simmons, and E. O. Gaston

In 1950¹ we reported that the capacity to produce antibodies to injected particulate antigen was retained in the rabbit if the spleen or appendix were shielded while the rest of the body received an LD₅₀ dose of x-radiation. We were, in fact, able to demonstrate that this capacity was retained if these shielded tissues were removed 24 hours, or even 3 hours, after irradiation.² These observations have been confirmed by Taliaferro and others.^{3,4} We also showed that a more rapid regeneration of the lymphatic tissues occurred in rabbits which had the appendix or spleen shielded during irradiation than in control animals. These studies were conducted using dosages of irradiation in the LD₅₀ range, thus permitting the bone marrow of survivors to regenerate, though not as rapidly as the lymphatic tissues.⁵ Recently Süssdorf⁶ has repeated our original study and made essentially similar observations.

One of our most interesting findings was that no granulocytopoiesis, megakaryocytopoiesis or erythropoiesis was observed in the shielded appendix, suggesting that under the conditions of the experiment these lymphatic cells did not exhibit a multipotential capacity as far as local production of other blood elements was concerned. In the light of present knowledge concerning cellular colonization,^{7,8,9} the rapid proliferation of lymphatic tissues following appendix- or spleen-shielding is presumably the result of migration and subsequent colonization of cells from the protected lymphatic tissues.

It seemed of interest, therefore, to repeat the experiment of protecting a pure source of lymphoid cells during total-body exposure to a supralethal dose of x-radiation, and to study (1) lymphocyte migration, recolonization, and potential for the production of other cell types; (2) the modifications of the immune response that might result; and (3) the value of the technique as an assay of the control of lymphopoiesis.

MATERIALS AND METHODS

A patch of Peyer in the small intestine of the mouse measuring about 2.5 mm x 1 mm and weighing about 5 mg was selected for these studies. Approximately 5 million fixed and free lymphatic cells are contained in such a patch as estimated by geometric calculations.

Adult female CF No. 1 and (C3H x 101)F₁ mice approximately 14 weeks old, were used as recipients in most of these studies. Donor tissues were obtained from female A/Tex mice and from male Holtzman rats.

The shield used to protect the Peyer's patch during irradiation was constructed of 1/4 inch lead, 11 mm wide and had a groove 4 mm in diameter to house the intestine. Under Nembutal anesthesia, the small intestine was brought out through a small incision into the peritoneal cavity and a single Peyer's patch was shielded during total-body irradiation with 950 r. In con-

* Preliminary report presented at the May, 1960, meeting of the Radiation Research Society (Rad. Res., 12:444, 1960).

trol groups, either an equivalent portion of the intestine having no Peyer's patch was shielded, or a sham operation was performed without any shielding. Following irradiation the intestine was returned to the peritoneal cavity and the incision sutured with silk.

Since 100×10^6 rat bone marrow or 10×10^6 mouse bone marrow cells afford good initial protection in lethally irradiated mice, these cell concentrations were used in the experiment. Preparation of the various suspensions has been described previously.¹⁰ The suspensions were made in isotonic saline and 0.5 ml injections were given via the tail vein within 4 hours after irradiation.

Dosimetry. The x-rays were generated in a 250 kv G.E. Maxitron operating at 30 Ma and using 0.25 mm copper and a 1-mm aluminum filter. The half-value layer in copper of the filtered beam was 1.02 mm. The exposures were measured with a Victoreen condenser meter equipped with a 250 r chamber. Measurements were made in air at a position estimated to be the center of the animal's body. The dose rate averaged 120 r per minute at 57 cm.

RESULTS

Survival. As can be seen in Figure 1, 40 per cent (20 of 50) CF No. 1 mice in the control group given rat bone marrow following total-body irradiation without shielding were alive at 14

Figure 1. Survival of CF No. 1 mice receiving 950 r and treatments as shown.

days, whereas only 4 per cent (1 of 25) survived in the group given rat bone marrow following protection of one Peyer's patch. Furthermore, the survival pattern shows that death in such Peyer's patch protected mice occurred significantly earlier ($P < 0.001$) than in sham operated controls that were lethally irradiated without shielding and without cellular injection.

In an additional control group, an 11-mm portion of intestine that contained no Peyer's patch was protected during exposure to 950 r, and these mice were also injected with rat bone marrow cells. Death also occurred significantly earlier in this group ($P < 0.02$) than in irradiation-only controls, although the results were not as dramatic as in the Peyer's patch group. By the 14th day, however, only 9 per cent (2 of 22) were alive.

In a companion series of experiments using homologous strains, when (C3H x 101)F₁ hybrid mice were given bone marrow from Strain A/Tex mice following Peyer's patch shielding during irradiation with 950 r, an early death pattern also resulted (Figure 2). In this strain combination, however, protection of 11-mm of intestine lacking a Peyer's patch did not produce an equally severe reaction, and 48 per cent (14 of 29) were alive at 14 days. This may be interpreted as indicating that fewer lymphoid cells are required to reject a heterologous graft than to reject an homologous graft.

Figure 2. Survival of (C3H x 101)F₁ mice receiving 950 r and treatments as shown.

Furthermore, it must be kept in mind that in addition to the fact that the genetic relationship in this experiment is closer than in the rat-to-mouse combination (thus diminishing the antigenic differences), only 10×10^6 mouse bone marrow cells were injected while 100×10^6 rat cells were used. In a preliminary experiment, (C3H x 101) F_1 mice that received larger numbers of A/Tex bone marrow cells (20 to 40×10^6) following the shielding of 11 mm of the small intestine did not survive.

These results led us to investigate whether the shielding effect could be reproduced by intravenous injection of isologous lymphocytes or spleen cells into mice treated with rat bone marrow following exposure of the entire body to lethal irradiation. Lymphocytes from the mesenteric lymph node and spleen cells from adult CF No. 1 donors were injected into CF No. 1 mice 4 hours after the irradiation bone-marrow treatment. Results agree with those of van Bekkum and Vos¹¹ and indicate that in the CF No. 1 mouse also, fewer than one million cells from lymphatic tissue or spleen of its own strain, given by intravenous injection, hasten the death of the recipient.

Hybrid (C3H x 101) F_1 mice that received A/Tex bone marrow (10×10^6 cells) or rat bone marrow (100×10^6 cells) after total-body x-irradiation and were subsequently injected with 10×10^6 cells from the mesenteric lymph node of their own strain responded in the same manner, and all mice died by the 14th day after treatment.

It requires a supralethal dose of whole-body x-irradiation (about 900 r) to suppress the capacity of the mouse to reject a heterologous (rat) bone marrow graft. The amount of x-irradiation required to suppress the capacity of the Peyer's patch or of 11 mm of intestine to reject a heterologous graft, was determined by removing the lid from the shield after a calculated number of roentgen units had been given to the mouse. Thus the protected Peyer's patch or intestine received dosages of from 200 r to 700 r at the same time as the rest of the body received 950 r (Table 1). These values were found to be 650 r and 400 r respectively, and suggest that

Table 1

COMPARATIVE EFFECT OF VARYING X-RAY DOSAGES DELIVERED TO SHIELDED
PEYER'S PATCH AND INTESTINE IN LETHALLY IRRADIATED (950 r)
CF NO. 1 MICE INJECTED WITH RAT BONE MARROW

X-ray dosage to Peyer's patch (r)	No. of mice	14-day no.	Survival (%)	X-ray dosage to 1.1 mm intestine	No. of mice	14-day no.	Survival (%)
None	25	1	4	None	15	1	6.6
200	10	0	0	200	10	0	0
300	10	0	0	300	10	0	0
400	10	0	0	400	10	2	20
600	10	0	0	600	10	1	10
650	10	3	33.3	650	10	4	40
700	10	6	60.0	700	10	9	90
950	50	20	40.0	950	50	20	40

the smaller the lymphoid population, the smaller is the dose of x-irradiation necessary to suppress the total immune response. In addition, these findings indicate how few immunologically competent cells need remain in the body after irradiation to successfully defeat heterotransplantation.

In another series of experiments, the Peyer's patch was protected initially while the body was given 950 r. Within 10 minutes the protected patches were given 950 r, followed by the injection of 100×10^6 rat bone marrow cells. All mice died and the early death pattern was again manifest, which suggests that following irradiation stress there is immediate migration of a sufficient number of lymphoid cells from the Peyer's patch into the damaged body, where they presumably recolonize and maintain the immune response of the individual.

Histology. Histological examination of mice following Peyer's patch protection shows that before death ensues the shielded patch has accomplished partial or essentially complete repopulation of the depleted lymphatic structures, including the thymus. We have sacrificed such animals at daily intervals from 1 through 12 days after the shielding-irradiation procedure. Celloidin-embedded tissues were cut at 6 microns and sections stained with hematoxylin-eosin and azure. In the shielded Peyer's patch itself we observed increased mitotic activity 24 hours after shielding, and this may be seen in other lymphoid structures as repopulation proceeds. In many animals repopulation of the spleen, lymph nodes, and thymus is well along at 6 days, but essentially complete repopulation is not accomplished before 10 to 12 days (Figure 3). No bone marrow regeneration was observed (Figure 3-D) nor was there evidence of granulocytic, megakaryocytic, or erythrocytic regeneration elsewhere.

One interesting finding in these mice, for which we have as yet no explanation, is the great prominence of plasma (or plasma-like) cells in the regional mesenteric lymph node during repopulation (Figure 3-A). These plasma cells, which by 3 days constitute about 90 per cent of the free cell population at this site, are found principally in association with the blood vessels. They show marked mitotic activity, and under low power light microscopy the lymph node appears to be composed of virtually hundreds of rosettes of them.

An increased number of such plasma (or plasma-like) cells is also found in the mesenteric node of mice given whole-body x-radiation in the LD_{50} range or above, without Peyer's patch shielding, but in this case not until 6 to 8 days postirradiation. Since these cells are morphologically identical in both experimental models, we made an attempt to determine whether they were also functionally identical in both situations, and the following experiment was performed to test the capacity of a mesenteric node transplant to reject a rat bone marrow graft previously given to irradiated mice.

CF No. 1 mice were lethally irradiated and treated with 100×10^6 rat bone marrow cells. About 3 hours later, two mesenteric lymph nodes taken from mice 6 days after exposure to 950 r were transplanted to the peritoneal cavity of one group of recipients (Table 2). Similarly, two mesenteric lymph nodes obtained six days after lethal irradiation from mice having a shielded Peyer's patch were implanted into another group of rat-treated irradiated mice. As a control procedure, one-fourth to one-half of a normal lymph node was transplanted to a third group of irradiated mice treated with rat bone marrow. The average weights of the mesenteric lymph nodes used were as follows: Normal mice, 110 mg; 950 r x-irradiated mice, 42 mg; irradiated, Peyer's patch shielded mice, 53 mg.

The data in Table 2 indicate that immunologically competent cells were present in the mes-

Figure 3. A—Mesenteric lymph node of CF No. 1 mouse six days after 950 r with Peyer's patch shielded, showing plasma-like cells (X 1567). B—Normal mesenteric lymph node of CF No. 1 mouse (X 1567). C—Spleen of CF No. 1 mouse six days after 950 r with Peyer's patch shielded, showing foci of lymphoid recolonization (X 36). D—Aplastic bone marrow in CF No. 1 mouse six days after 950 r with Peyer's patch shielded, showing absence of any recolonization (X 36).

Table 2

EFFECT OF LYMPH NODE TRANSPLANTATION ON SURVIVAL OF 950 r-IRRADIATED CF NO. 1 MICE TREATED WITH RAT BONE MARROW

Group	Donor mesenteric* lymph nodes	Survival (days)	
		8	14
1	2 nodes from mice 6 days after 950 r	17/18	14/18
2	2 nodes from mice with shielded Peyer's patch 6 days after 950 r	3/14	2/14
3	One-half normal node One-fourth normal node	2/24 7/11	0/24 1/11

*Transplantation of spleens instead of lymph nodes from similarly treated mice gave comparable results.

enteric lymph node six days after mice with the Peyer's patch shielded were lethally irradiated. The nodes from irradiated mice without such shielding either contained no immunologically competent cells or contained a number insufficient to reject the heterologous graft. Transplantation of a normal lymph node caused rejection of the rat bone marrow group in the control animals.

These results were not unexpected since it is well known¹¹ that cells from a normal mesenteric lymph node transplant will reject a rat bone marrow graft previously given to lethally irradiated mice. Neither was it unexpected that the mesenteric node from a mouse previously given whole-body x-radiation should fail to cause rejection of a rat bone marrow graft which had been given to an irradiated mouse. However, the mesenteric node from Peyer's patch-shielded mice proved as capable of rejecting a rat bone marrow graft as the normal node. This latter observation indicates that although these two groups of nodes are rich in plasma cells, apparently morphologically identical, they are functionally quite different. To be sure, the mesenteric nodes in both these groups contained perhaps 10 per cent lymphocytes, or lymphocyte-like cells, and so one cannot be sure that the plasma cells were responsible for the immune response. It can be stated categorically, however, that even though the population of these two experimental groups is morphologically similar, their functional capacities are vastly different.

DISCUSSION

It is now well established that lethal irradiation destroys the ability of the mouse to react against foreign bone marrow cells,^{7,8,9} and that such injected cells colonize and substantially reduce acute radiation mortality. In Peyer's patch-shielded animals injected with heterologous or homologous bone marrow cells, the grafted cells are rejected by the mouse's own regenerating lymphatic tissues, and since lymphocytes alone cannot support life at 950 r, the mice succumb. Lymphoid repopulation is well established in the spleen and mesenteric lymph node by the sixth day, and this coincides with the early death pattern observed. Histological evidence shows that lymphocytes normally tend to accumulate in the intestinal wall. Even though it is doubtful that as many as 5×10^6 are present in an 11-mm segment, the supply of lymphatic elements made available by shielding is sufficient to elicit rejection of the foreign rat cells. The results obtained when a Peyer's patch is shielded during lethal irradiation and the host is subsequently injected with heterologous cells agree with those of van Bekkum and Vos.¹¹ These investigators treated irradiated mice with rat bone marrow and subsequently injected them with a small number of isologous lymph node cells, thus preventing the transplant of the rat bone marrow from being successful. Barnes¹² reported similar results in irradiated mice treated with homologous marrow before isologous lymph node cells were injected. We, too, have found that relatively few cells from lymphatic tissues are needed to reject a heterologous or homologous graft.

The lymphocytic tissues in the Peyer's patch of the guinea pig are reportedly at least as active, if not more so, than those in the mesenteric lymph nodes. The germinal centers tend to be larger in the Peyer's patches than in the lymph nodes, while mitoses are especially numerous in the peripheral region of the center.¹³

Cole and Garver¹⁴ report that the intraperitoneal injection of 0.6 ml of mouse blood (either isologous or homologous) annuls the protective effect of rat bone marrow in LAF₁ mice. These authors indicate that when the blood and marrow are of the same strain (C3H) there is no indication that the C3H bone marrow transplant is rejected.

Goodman and Congdon¹⁵ reported the "killing-effect" in irradiated mice of a mixture of

homologous bone marrow and isologous blood. Histologic studies showed evidence of temporary transplantation of bone marrow and then rejection. Irradiation of the blood, or of the donor prevented this reaction. These results support the theory that immunological reactive cells are present in the peripheral blood.

Santos and Cole¹⁶ have also shown that the injection of isologous lymphoid tissue from the spleen or thymus elicits early rejection of rat marrow transplants in irradiated mice.

Under the experimental conditions described, the lymphoid cells of the Peyer's patch, although capable of repopulating other lymph structures, cannot serve as precursors of other cell types of the blood-forming tissues.

It is interesting to note that an x-ray dose of 650 r or more is required before the capacity of the lymphatic tissue in the Peyer's patch or intestine is impaired sufficiently to permit the retention of a heterologous graft.

This model, which involves the survival of tissue foci in vivo provides us with an opportunity of studying the factors involved in the normal control of growth and differentiation of lymphoid tissue, as well as of evaluating the substances which will enhance or inhibit immune responses.

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STUDIES ON THE IMMUNOLOGIC UNRESPONSIVENESS DURING THE
SECONDARY DISEASE PERIOD OF LETHALLY IRRADIATED
MICE PROTECTED BY HOMOLOGOUS BONE MARROW*

By

J. S. Thompson,[†] E. L. Simmons, and D. Hofstra

Following the observations that animals can be protected from acute lethal doses of irradiation by spleen shielding, spleen transplantation, or the injection of spleen or embryo cells,^{1,2} and the subsequent conclusions that this protective effect is associated with the successful transplantation of donor cells,³⁻⁶ many parallels to the broad field of transplantation immunology have become apparent.

In general, the sequence of events which follows the transplantation of hemopoietic elements into lethally irradiated adult animals is similar to that which follows their inoculation into the embryonic or newborn host. A wasting disorder, known as secondary disease⁷⁻⁹ follows the successful transplantation of homologous or heterologous hemopoietic tissues into a lethally irradiated host. Similarly the "runt" syndrome first described in young mice inoculated at birth with homologous spleen cells¹⁰ has been observed in a variety of donor and host combinations.¹¹⁻¹³

Billingham and Brent¹⁴ have called attention to the similarity of these phenomena and conclude that in both instances a chimera of host and donor tissues exists as the consequence of a hostile reaction directed by the donor cells against their genetically different hosts, i.e., a graft vs host reaction.

It still remains undecided whether the state of unresponsiveness to homologous grafts^{15,16} induced by lethal irradiation of the adult host is based on a mechanism similar to that of immunologic tolerance manifested by the animal injected at or before birth with homologous cells. Is a "central failure"¹⁷ operating in both cases?

The initial purpose of this study was to compare radiation-induced unresponsiveness with immunologic tolerance by injecting isologous lymphocytes into adult mice previously exposed to lethal x-rays and protected with homologous marrow. If survival were due to the persistence of homologous hemopoietic elements, and if a central failure were the mechanism operating to perpetuate this survival, it might be expected that isologous lymphocytes would produce death of the transplanted marrow and, as a consequence, more rapid death of the host. In addition, it is important to know whether these passively transferred lymphocytes survive with their functional capabilities intact, and this might be tested by their ability to react against a heterologous tissue genetically distinct from either the donor or host.

MATERIALS AND METHODS

Animals. C3H/Cum female mice, DBA/2 Jax and AKR Jax mice were used in these experiments. In the initial experiments in which the C3H mice were the recipients of DBA/2 Jax bone

* Summary of a paper that will appear in *J. Immunol.*

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marrow, they were not virus-factor free, but all subsequent studies were performed on C3H_f/Cum mice. At the time of irradiation both the donor and host mice were 12-16 weeks of age. Bone marrow, spleen, or lymphocytes, were always taken from donors of the same sex as the host.

Irradiation and bone marrow protection. Mice were irradiated with 900 r in air delivered by a G. E. Maxitron-250, operated at 250 kvp and 30 ma, with 0.25 mm Cu and 1.0 mm Al filtration. Within 4 hours of the completion of irradiation, the animals received intravenous injections of 0.5 ml of Tyrodes solution which contained 10×10^6 nucleated isologous or homologous bone marrow cells, or a control inoculation of 0.5 ml of Tyrodes solution only. The bone marrow cells were withdrawn from the femurs of the donors on the day of use.

Preparation and administration of normal and immune isologous lymphocytes and spleen cells. Isologous lymphocytes or spleen cells were administered intravenously to mice of identical sex, strain, and approximate age, which had been lethally irradiated and protected by either isologous or homologous bone marrow cells 21 days previously. The cellular suspensions were prepared from spleens or mesenteric lymph nodes using sterile techniques designed to reduce the amount of stroma and cytoplasmic debris which are often lethal to the host when injected intravenously, and also to partially remove any preformed antibodies. The volume was adjusted with Tyrodes solution so that 0.5 ml contained approximately 10×10^6 nucleated cells. The viability of the cells was estimated periodically¹⁸ and under these conditions approximately 90-95 per cent of the cells were considered viable.

After the final dilution, 10×10^6 lymphocytes or spleen cells were slowly injected into the recipient host. Control mice received 0.5 ml of Tyrodes solution intravenously. These injections were routinely made 21 days after the x-ray and bone marrow treatment.

Lymphocytes were obtained from C3H mice made immune to AKR spleen cells by injecting C3H mice with approximately 10×10^7 AKR spleen cells in 0.5 ml Freund's Adjuvant* intraperitoneally and 10×10^6 spleen cells in 0.2 ml Tyrodes solution intravenously and subcutaneously the same day. A second intraperitoneal injection of 10×10^7 spleen cells was given 3 weeks later, and one week after this the mesenteric lymph nodes were removed for use on the same day.

Lymphocytes or spleen cells immune to rat erythrocytes were prepared from rat blood obtained by aortic exsanguination of normal adult CFN rats,[†] using heparin as an anticoagulant. The plasma was separated and the erythrocytes were washed 3 times in cold saline. On the day of first injections, C3H mice were given a total of 0.2 ml of washed packed rat erythrocytes intravenously, subcutaneously, and (mixed with 0.2 ml of Freund's Adjuvant) intraperitoneally. Thereafter, at weekly intervals for 4 weeks they received 0.2 ml of washed packed rat erythrocytes subcutaneously and intraperitoneally. Approximately 1-2 weeks after the last routine injection some of the animals were injected intravenously with Cr⁵¹-labeled rat erythrocytes: others were sacrificed and the spleens and mesenteric lymph nodes excised for administration to isologous hosts that day.

Method of chromation and measurement of red cell survival. The methods of chromation

* Difco Laboratories, Detroit, Michigan.

† Wistar stock obtained from Carworth Farms, Inc., New City, New York.

and estimation of the survival of labeled rat erythrocytes were as previously reported,¹⁹ except that the rat erythrocytes were thoroughly washed in cold saline before chromation, and C3H mice were the consistent recipients of 0.15 ml of the Cr⁵¹-labeled rat red cells.* The survival of rat red cells was studied in the following groups of C3H mice: (1) Mice actively immune to rat erythrocytes; (2) mice which had received an intravenous injection of 10×10^6 isologous lymphocytes or spleen cells 7 days previously from mice which were actively immune, and (3) untreated mice. In addition the survival of these cells was determined in irradiated experimental C3H mice as described individually in the text. The first experimental sample was routinely withdrawn 24 hours after the injection of labeled cells, with one exception—an additional 1-hour sample was drawn in the group of mice which had been made actively immune to rat erythrocytes.

RESULTS

The survival of C3H mice receiving 900 r and Tyrode's solution. In preliminary and control studies of a total of 164 C3H and AKR mice receiving 900 r followed by Tyrode's solution only, the longest survival time was 16 days. We therefore assumed that mice which survived for more than 21 days after 900 r and bone marrow therapy did so by virtue of the transplanted bone marrow. In consequence we use the word "saved" to indicate survival beyond this arbitrary time limit, and in the survival data this is designated "day zero." Experimental animals that died within 21 days were considered to have died of "primary disease," and are not included in the data. When early death was predominant, the entire group was discarded.

The effect of isologous lymphocytes on the survival of lethally irradiated mice "saved" by isologous or homologous bone marrow. Figures 1, 2, and 3 demonstrate that the 90-day sur-

Figure 1. Effect of C3H lymphocytes (L) on survival of lethally irradiated C3H mice saved by isologous C3H or homologous DBA/2 bone marrow (BM).

*Cr⁵¹ obtained as Rachromate from the Abbott Laboratories, North Chicago, Illinois.

Figure 2. Effect of AKR lymphocytes (L) on survival of lethally irradiated AKR mice saved by isologous AKR or homologous C3H bone marrow (BM).

Figure 3. Effect of C3H lymphocytes or spleen cells on survival of lethally irradiated C3H mice saved by isologous C3H or homologous AKR bone marrow (BM).

vival of lethally irradiated mice is significantly reduced when homologous rather than isologous bone marrow cells are inoculated within four hours after irradiation. This mortality was especially high when the donor and host differed genetically at the H-2 locus, as between DBA/2 ($H-2^d$) and C3H ($H-2^k$), but it was still noticeable when the donor and host were similar at this locus, as when both C3H and AKR are $H-2^k$. Figures 1, 2, and 3 further demonstrate that the

inoculation of isologous lymphocytes into the host 21 days after lethal irradiation and protection by either homologous or isologous marrow exerted no statistically significant effect on mortality. Perhaps the rapid mortality in C3H mice receiving DBA/2 cells did not permit the expression of a still more accelerated death pattern when isologous lymphocytes were introduced (Figure 1), but certainly there was ample time for such an expression when AKR mice received C3H marrow or when C3H mice received AKR marrow (Figures 2, 3). Similarly, isologous spleen cells did not appear to influence the subsequent survival of irradiated C3H mice protected with homologous AKR marrow (Figure 3). Figure 3 also reveals that isologous spleen cells had no more effect than isologous lymphocytes or Tyrode's solution on the survival of C3H mice that had been x-rayed and "saved" by homologous AKR cells. Finally, Figure 4 demonstrates that isologous lymphocytes from C3H mice immune to AKR did not significantly alter the 90-day mortality ($P > 0.1$).

Figure 4. Effect of normal or pre-immunized C3H lymphocytes on survival of lethally irradiated C3H mice saved by homologous AKR bone marrow (BM).

The survival of passively transferred isologous cells in x-irradiated C3H mice protected by isologous or homologous bone marrow. Isologous lymphocytes and spleen cells from mice previously immunized against washed rat erythrocytes were injected into lethally irradiated C3H mice which had been protected by either isologous C3H or homologous AKR bone marrow 21 days before. Seven days later these animals were given a transfusion of Cr^{51} -labeled rat erythrocytes, and the survival of these heterologous cells was established. If survival time was shorter than in the controls injected only with Tyrode's solution, presumably the donor cells remained immunologically competent after transfer. In other words these cells had conferred "adoptive immunity"²⁰ to the x-rayed hosts because they had survived. It was therefore necessary to establish that our techniques did indeed produce active immunity. Additionally, it was important to determine the effect of this adoptive immunity after 7 days in normal C3H mice as

a baseline for comparison with the irradiated groups. Twenty-six C3H mice previously immunized against rat erythrocytes were challenged intravenously with 0.15 ml of Cr^{51} -labeled rat red cells. Nineteen of these animals died within a few minutes, whereas this same inoculum had no such deleterious effect on C3H mice which were normal, irradiated, or on the normal and irradiated animals that had received tissues from mice identically immunized. In the seven mice which survived, only one-fifth of the expected radioactivity was present in the 1 hour blood sample, and virtually all radioactivity had disappeared 24 hours after the transfusion. We can therefore assume that the tissues obtained from the identically treated donors were activated to rat erythrocytes. Figure 5 reveals that the passive transfer of these cells to normal C3H mice reduces the subsequent survival of Cr^{51} -labeled erythrocytes when transfused 7 days later. Adoptive immunity appears to have been established in these normal mice.

Figure 5. Effect of adoptive immunity on survival of rat erythrocytes in normal C3H mice.

An even more striking effect of the adoptive transfer of these immune tissues was seen in C3H mice that had been irradiated and "saved" by either isologous C3H or homologous AKR marrow (Figures 6, 7). Here, the rat erythrocytes survive much longer than is normal in irradiated hosts injected only with isologous (Figure 6) and particularly with homologous (Figure 7) bone marrow. The data reveal that C3H mice treated 28 days previously with x-ray and C3H marrow, took approximately 3 days longer to eliminate the circulating heterologous cells (Figure 6). When homologous AKR marrow was given after irradiation and the rat cells were transfused 28 days later, the survival of the rat cells was at least twice as long as in normal C3H mice (Figure 7), and detectable activity was still present 10 days after administration of the Cr^{51} -tagged erythrocytes.

Whereas the transfer of normal lymphocytes one week before the labeled transfusion re-

Figure 6. Effect of adoptive immunity on survival of rat erythrocytes in lethally irradiated C3H mice saved by C3H bone marrow.

Figure 7. Effect of adoptive immunity on survival of rat erythrocytes in lethally irradiated C3H mice saved by AKR bone marrow.

sulted in a reduction in red cell survival in both the C3H and AKR marrow groups, it is important to stress that the survival in the C3H mice saved by AKR bone marrow was decreased from 10 to 7 days by the prior injection of normal C3H lymphocytes (Figure 7). Activated tissues produced a still further reduction in both groups, but the survival of heterologous antigen was never shortened beyond that of the normal control mice.

The data therefore reveal that adoptive immunity can be established by isologous tissues in C3H mice that survived 900 r x-irradiation and subsequent administration of either C3H or homologous AKR marrow. Furthermore, it appears that non-activated isologous lymphocytes administered to these same hosts survive, are functional, and are capable of immune response to heterologous antigen.

DISCUSSION

Van Bekkum *et al.*²¹ and Santos and Cole²² have reported that when isologous lymphocytes, thymocytes, or spleen cells are administered within 24 hours to lethally irradiated mice that have been injected with homologous bone marrow, the protective effect of the homologous marrow is lost and the 30-day survival time is greatly reduced. Jacobson and colleagues²³ have similarly shown that lymphoid cells migrating from a single Patch of Peyer protected during irradiation will reject a rat bone marrow graft. Recently, Cole and Garver²⁴ and Goodman and Congdon²⁵ have demonstrated that isologous whole blood will exert a like effect on the survival of irradiated mice injected with foreign bone marrow cells. In these studies the blood was also administered on the same day as the marrow. It may be that the immunologically active isologous tissues destroy the transplanted hemopoietic cells and as a consequence the host, having no source of marrow repopulation, dies of "primary disease." However, since such mice often die sooner than those receiving only lethal irradiation, it may also mean that an immune interaction is taking place.

The interactions involved are even more complicated when isologous lymphoid tissues are inoculated two or more weeks after lethal irradiation and protection by homologous bone marrow. Trentin²⁶ has reported that normal non-immune isologous lymphocytes caused the rejection of long-term BALB/c skin grafts placed on 9 CBA mice irradiated and protected with (BALB/c x CBA)F₁ hybrid marrow. Several of these mice developed anemia and died following the inoculation. The author believed that the isologous lymphocytes destroyed the state of tolerance, thus causing the rejection of both the homografts of skin and bone marrow. Since this result appeared to parallel the classic experiments of Billingham, Brent, and Medawar,²⁷ i.e., the rejection of long-term skin homografts in tolerant mice by the injection of normal or immune lymphocytes, he concluded that the mechanism of a "central failure" was operating in both cases to perpetuate the immunologic unresponsiveness to the foreign tissues.

The data of Barnes *et al.*,²⁸ however, indicate that normal and immune CBA lymphocytes do not produce an accelerated mortality in CBA mice which had received 950 r followed by (CBA x T6)F₁ bone marrow 14 days previously. Furthermore, by using an aberrant chromosome as marker, it could be observed that the T6 positive donor cells, which were predominant at 14 days in the CBA/(CBA x T6)F₁ chimera, became progressively less numerous following the injection of CBA spleen cells. This induced reversion toward a predominantly host-cell population, termed "transpopulation" by these authors, does not occur when normal lymphocytes rather than spleen cells are given. Since the lymphocytes did not appear to decrease the donor

cell population or cause the death of the host, these studies cast doubt on the theory of a common mechanism.

Our studies involving several donor and host combinations indicate that isologous lymphocytes do not influence the subsequent survival of mice "saved" by homologous marrow, nor, when C3H mice are the lethally irradiated hosts, do isologous spleen cells or immune lymphocytes affect the rate of mortality. The systems studied by Trentin,²⁶ Barnes *et al.*,²⁸ and in our laboratory are all somewhat different, and the variables which exist may be critical to the outcome.

In our preliminary studies as many as 200×10^6 normal and immune lymphocytes injected by the intraperitoneal route had no detectable influence on the survival of C3H mice "saved" by AKR bone marrow. Since intravenous injections of spleen cells or lymphocytes containing more than 10×10^6 cells were often lethal and had no greater effect than the lower dosage in the few mice that survived, we routinely injected 10×10^6 cells intravenously in our experiments. Billingham and Brent¹⁴ injected a much larger number of isologous cells (400×10^6 to 800×10^6) intraperitoneally to abolish tolerance, but several investigators (29-31) have demonstrated the superiority of the intravenous route in obtaining colonization of bone marrow or spleen cells. Nevertheless, we cannot absolutely exclude this variable.

When we began these experiments a possible method of abrogating secondary disease seemed possible. Briefly, if immunologically active isologous tissues caused the rejection of the transplanted cells in lethally irradiated animals injected with homologous bone marrow, repopulation of the host might be effected by exogenous isologous hemopoietic tissues and secondary disease would be avoided. This would mean that one could wait until the symptoms of secondary disease were evident before instituting the manipulations. In view of the theoretical and practical reasons favoring the use of homologous marrow in a human host, this technique might offer an "escape valve" should the wasting disease develop. This proposal requires at least three premises: 1) that a chimera exists after irradiation and is responsible for the host's continuing survival; 2) that the perpetuation of the homologous cells is due to immunologic unresponsiveness similar to that in a tolerant animal; and 3) that secondary disease in these animals is the consequence of immunologically competent grafted cells attacking the host.

Our studies clearly demonstrate that isologous lymphocytes (normal or immune) and spleen cells do not alter the rate of mortality of irradiated mice whether "saved" by isologous or homologous marrow.

There may be several reasons for this:

1) Isologous tissues inoculated 21 days after x-ray and bone marrow therapy do not survive for technical reasons, or because the graft actively destroys these cells.

2) The isologous tissues survive, but lose their functional capabilities. The data, which illustrate the persistence of adoptive immunity to rat red cells for at least 7 days in both the groups of mice saved by isologous or homologous bone marrow, indicate that isologous tissues survive and are not destroyed by an active graft *vs* host reaction during the period of 21 to 28 days after irradiation. Furthermore, the transfer of adoptive immunity demonstrates that they retain their activated characteristics.

3) The isologous tissues remain functionally capable but "spontaneous reversion" to a host population has already occurred by 21 days. We cannot exclude this possibility in our sys-

tem, but the data of Barnes et al.²⁵ describe the persistence of a homologous chimera for at least 98 days.

4) The isologous tissues retain their functions and induce "transpopulation" toward a predominantly host population. This may happen when spleen cells are inoculated, but Barnes and coworkers²⁵ were unable to show that lymphocytes were capable of inducing transpopulation. Furthermore, in our studies, if a graft vs host reaction were the sole explanation of the secondary disease, and transpopulation replaced all of the donor tissues, this theory would not explain why the rate of mortality continues unchecked after the donor cells are eliminated. There would be no donor graft remaining to carry on the attack.

5) The isologous tissues functionally survive, and may or may not induce transpopulation of some hemopoietic elements, but exert no influence on the immunologically active cells of the graft because a central failure is not the mechanism responsible for maintenance of these homologous tissues in the unresponsive irradiated host. In addition to the fact that these experimental animals do not die more rapidly after the inoculation of isologous lymphoid cells, our data demonstrate another feature not encountered in a tolerant animal. That is, rather than a localized inability to react only against tissues of the same genetic homologous donor type, the C3H mouse protected by homologous AKR bone marrow demonstrated a definite limitation in rejection of the heterologous rat red cells. This lack of specificity has also been demonstrated to tumor grafts for certain x-ray induced donor-host combinations.¹⁶ Finally, our data indicate that isologous normal lymphocytes passively transferred to x-rayed C3H x AKR hosts were capable of shortening the time of the heterologous antigen. If they partially contributed to re-establishment of the immune defenses of the host in this manner, one might anticipate a further response on their part against whatever foreign tissues were present. When lymphocytes immune to AKR cells were given, an even more potent reaction against the donor tissues might be predicted. The fact that the host did not die sooner suggests that it was not dependent on the transplanted marrow for survival 21 days after lethal irradiation. Thus, whether or not homologous cells persist at this interval, the host is capable of an adequate regeneration of its own hemopoietic tissues for immediate survival.

It is apparent from our studies that mice which have received homologous marrow tend to die earlier than do those receiving isologous marrow, regardless of subsequent manipulations. Is it possible that the transplantation into a lethally irradiated host of hemopoietic tissues which are able to attack a host (which itself is normally able to destroy the graft) sets in motion a chain of events that may be perpetuated by either donor or host cells? Thus, when the host's immunologic tissues become sufficiently numerous and active following irradiation they receive the information to attack not only the graft, but their own cells as well. In this fashion heavy irradiation and the presence of foreign antigen might induce autoimmunization. Under the appropriate circumstance, which may be related to the genetic disparity of donor and host and the dosages of irradiation and transplanted cells, secondary disease might be the consequence of a graft vs host, a host vs graft, a host vs host, or a combination of these reactions.

Makinodan³² and associates consider that the graft vs host postulate does not explain their data on the irradiation-induced rat to mouse chimera. More recently this theory has been questioned by Davies and Doak³³ and by Fowler and West.³⁴

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RADIATION DAMAGE TO THE STEM CELL*

By

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Most investigators attempting to quantitate radiation injury to mammalian cells have heretofore employed in vitro systems, although there is an extensive body of literature dealing with in vivo irradiation of unicellular organisms and the meristem of the bean root. The difficulties encountered in quantitative estimates of radiation damage in mammals arise partly from the scarcity of effective parameters for measurement, and partly from the heterogeneous nature of the cell populations irradiated. The Fe⁵⁹ incorporation into newly-formed red cells or the reticulocyte count afford effective methods for measuring erythropoiesis, but irradiation of normal animals yields data difficult to interpret because all stages of a continuum from stem cells to reticulocytes are irradiated, and the possibility that the red cell exhibits differential sensitivity at various stages of its life cycle cannot be dismissed. Further, with time after irradiation, as production of red cells decreases, the stimulus for erythrocyte production undoubtedly increases, thus confusing interpretation of the recovery phase. In our laboratories, recent inquiries into the physiology of red cell production have yielded results which suggest these variables can to some degree be controlled. As a result it is possible to obtain a clearer picture of damage to one particular type of cell, the stem cell, and observe its recovery from radiation injury.

It is generally conceded that the process of erythropoiesis is regulated by at least one humoral factor, erythropoietin,¹ which acts upon the stem cells of the reticulum directing their development into the red cell line.²⁻⁴ This humoral factor has been isolated from the blood of anemic sheep and its chemical composition is considered to be that of a glycoprotein.⁵

In vivo studies on erythropoiesis carried out by Jacobson and his associates² have shown that the transfusion-induced polycythemic mouse is a most valuable subject for such research. Using these animals we have found it possible to demonstrate a definite quantitative response to a single injection of erythropoietin.⁶ Similarly, we have traced the sequence of events in the reticulum of the spleen in response to a single injection and have noted the cumulative response to multiple injections.⁶ More recently autoradiographic methods have been used to confirm earlier demonstrations that erythrocyte precursors make their appearance within 24 hours after a subcutaneous dose of erythropoietin has been administered to the polycythemic mouse.⁷

The present paper is concerned with studies on damage to the stem cell as reflected by the impairment of this response produced by total-body x-irradiation, a preliminary note on which has already appeared.⁸

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[†] John and Mary Märkle Foundation Scholar.

MATERIALS AND METHODS

The animals utilized were virgin female CF No. 1 mice, 7-8 weeks of age. Polycythemia was induced in these mice by a single intraperitoneal injection of 1 ml of packed red cells from retired breeder mice of the same strain given on two consecutive days. Studies were begun 5 days after the second transfusion. At this time the hematocrit of the test animals was above 70 per cent, and the absence of reticulocytes from the peripheral blood, and of morphologically identifiable red cell precursors from bone marrow and spleen indicated that erythropoiesis had been almost completely suppressed. Hematocrits and reticulocyte counts were estimated on blood drawn from a tail vein, and histologic preparations of the spleen were made as previously described.⁹

These mice were now exposed to single doses of total-body x-irradiation ranging from 0 to 400 r, the physical factors being 250 kv, 130 ma, target distance 79 cm, filter 1/4 mm Cu plus 1 mm Al. Within 30 minutes after irradiation, the animals were given single subcutaneous injections of 6 units (a unit as defined by Goldwasser and White),¹⁰ of erythropoietin dissolved in 0.25 ml saline, a dose selected because of its demonstrated ability to produce a maximal response.⁶ Two days later, the mice received single injections of 1 μ c of Fe⁵⁹-citrate via the tail vein. At this time unirradiated controls demonstrated a rising concentration of normoblasts in the marrow and spleen, and new reticulocytes had begun to appear in the peripheral blood.

Four days after injection of Fe⁵⁹ the percentage of radioiron in the newly-formed reticulocytes was determined in a sample of blood withdrawn from the heart.

Comparison of the data thus obtained at the different dose levels of irradiation served to indicate the extent of the suppression of erythropoiesis and hence of the damage to the stem cell pool.

In order to investigate recovery of the stem cell compartment from irradiation, different groups of mice received their challenging dose of erythropoietin at different times after irradiation. The Fe⁵⁹ administration and subsequent sacrifice of each group bore the same relation to the time of erythropoietin administration as that outlined in the preceding experiments.

RESULTS AND DISCUSSION

Figure 1 shows the percentage of radioiron incorporated into the red cells of control and irradiated animals plotted on a vertical logarithmic scale. The number of animals in each experimental group is indicated in parenthesis and the values are expressed as the mean $\pm$ one standard deviation. While variation within any group is great, the mean values indicate that the response of a given group is predictable. The amount of the tracer dose in newly-formed peripheral red cells of controls, produced as a consequence of stimulation of the stem cell pool by erythropoietin, was 27 per cent. Fifty roentgens decreased the average radioiron incorporation to 19 per cent, and when the log of Fe⁵⁹ incorporation was plotted against roentgens administered, a linear relationship was obtained over the range 0-300 r. At 300 r less than 2 per cent of the tracer dose was incorporated and at 400 r it was only 0.6 per cent, thus indicating the almost complete suppression of erythropoiesis.

These results confirm those of other investigators¹¹ who have previously reported the suppressive effect of radiation upon erythropoiesis. The method affords a simple quantitative technique for evaluating the effect of radiation on the stem cell in vivo. Since erythropoiesis is suspended at the time of irradiation, the impairment of the response to subsequently administered

Figure 1. Impaired response to erythropoietin in the irradiated polythemic mouse. Number of mice in brackets. (Figure 1 is reproduced by courtesy of the publishers of Nature, MacMillan and Co., London.)

erythropoietin, as measured by Fe^{59} incorporation is presumed to reflect stem cell damage.

Decreased Fe^{59} incorporation by newly-formed red cells is not primarily a reflection of changes in the plasma iron level following irradiation, since we have recently demonstrated a high level of plasma iron in hypertransfused rats, which, unlike the plasma iron in normal animals, is not further elevated following irradiation.¹² In the experiments reported here it must be emphasized that only effective erythropoiesis is being measured.

By varying the time after irradiation at which different groups of plethoric mice are challenged with a constant dose of erythropoietin, we have been able to observe the rate of recovery and the time at which the stem cells have recovered completely after different doses of radiation. Let us consider a dose of 150 r. Figure 2 shows the Fe^{59} incorporation in non-irradiated hypertransfused mice, and the pattern of recovery of the stem cell pool following irradiation. The horizontal scale is the time in days after irradiation at which the challenging dose of erythropoietin was administered. One curious result is seen. The apparent damage of reproductive integrity of the stem cell compartment is not maximal immediately after irradiation, but rather follows it by 24 hours. Such a finding is without precedent in radiobiology, and an alternate hypothesis, first suggested by L. G. Lajtha and considered in greater detail elsewhere,¹³ is there-

Figure 2. Stem cell recovery in transfused mice after 150 roentgens.

fore worthy of discussion. It is that 24 hours after irradiation, some of the stem cells which have escaped radiation injury have entered cell cycle, ultimately to divide and become two stem cells. It is postulated that these stem cells are refractory to stimuli for differentiation while in cell cycle, and hence cannot respond to erythropoietin. Such a possibility warrants further consideration since it may afford further insight into the behavior of the stem cell pool.

SUMMARY

The extent of the damage to stem cells was measured in mice as a function of the dose of whole-body x-irradiation. Transfusion-induced polycythemic mice receiving total-body irradiation immediately prior to administration of erythropoietin showed impairment of response as measured by Fe^{59} incorporation. Since recognizable red cell precursors were absent from the reticulum at the time of irradiation, it is concluded that the reduction of effective erythropoiesis demonstrated in these animals is a measure of the radiation effect on the stem cell. Using this model, it is possible to obtain patterns of recovery of the stem cell compartment after irradiation.

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THE PRODUCTION OF AN ERYTHROPOIETIC FACTOR BY THE IN SITU PERFUSED KIDNEY

By

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The role of the kidney in either production or activation of erythropoietin is still controversial. Conflicting results have been reported after bilateral nephrectomy in rabbits and rats.¹⁻⁵ Under these circumstances it seemed advisable to investigate erythropoietin production in the perfused kidney, isolated in situ. Cobalt was chosen as a stimulant because it not only stimulates erythropoiesis,⁶ but animals injected with cobalt develop a high titer of erythropoietin in their plasma.⁷ Using this technique, it should be possible to establish the significance of the kidney in erythropoietin production. In the studies reported here erythropoietin titers were measured in plasma following perfusion of the dog kidney in situ with blood containing cobalt, and compared with the erythropoietin levels in an isolated lung perfused in the same manner.

MATERIALS AND METHODS

Male and female mongrel dogs, weighing between 15-25 kg, were used throughout these studies, and all surgical procedures were carried out under pentobarbital anesthesia. Approximately 1200 ml blood was removed from an anesthetized donor dog, and defibrinated. The lungs were removed from the donor dogs, mounted over a funnel to allow warm (37°C) saline to bathe the lungs, and connected to a Harvard Respirator Pump. A second dog was anesthetized and cannulae inserted in the renal artery and renal vein. The right kidney was usually perfused and was exposed through a retroperitoneal incision extending from the junction of the last rib and vertebrae laterally approximately 12-15 centimeters.

As shown in Figure 1 a Dale-Schuster heart-lung double perfusion pump, with 2 connecting reservoirs, was used to circulate the blood containing cobalt through the kidney of the recipient dog and the isolated lungs. Cannulae were inserted in the pulmonary artery and A-V valves between the left auricle and ventricle of the donor dog. Pressures were measured in the perfusion system from the pulmonary and the renal arteries with a mercury manometer. Carotid artery pressure was also measured in the recipient dog. When the isolated lungs were perfused alone, a single pump and reservoir were used, thus excluding the kidney from the system. The blood circulated through the system contained 10 micromoles of cobaltous chloride and 1.5 mg papavarine hydrochloride per ml; the papavarine serving to keep the kidney blood vessels dilated for as long a perfusion time as 3 hours.⁸ The plasma was dialyzed at 4°C

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Figure 1. Diagram of kidney perfusion system.

R-1, Blood Reservoir 1
P-1, Perfusion Pump 1
PA, Pulmonary Artery
L, Isolated Lungs
PV, Pulmonary Vein

R-2, Blood Reservoir 2
P-2, Perfusion Pump 2
RA, Renal Artery
K, Dog Kidney
RV, Renal Vein

against Dowex-50 ion exchange resin for 24 hours to remove the cobalt prior to assaying for erythropoietin. Subsequent analysis by a modification of the technique of Almond⁹ showed that each sample contained less than 0.1 micromole cobalt per ml.

Erythropoietin assay was carried out in the starved rat according to the procedure of Fried and co-workers,^{10,11} using 5 male Sprague-Dawley rats in each assay. Young healthy rats, weighing 165 to 195 grams each, were starved for 32 hours prior to the first injection and maintained in the starved state throughout the duration of the bio-assay, water being permitted ad libitum. Two ml of plasma or the test substances were injected subcutaneously daily for 2 days. On the third day, a tracer dose of 1 microcurie of Fe^{59} citrate was given intravenously and standards were prepared for later counting. Sixteen hours later, 1 ml of blood was obtained by cardiac aspiration and the Fe^{59} incorporation into red cells was calculated according to the following formula:

$$\text{Fe}^{59} \text{ uptake} = \frac{\text{net counts per ml blood} \times 0.05 \text{ body weight}}{\text{net counts injected}}$$

In these experiments, the blood volume was assumed to be 5 per cent of the body weight.¹¹

RESULTS

A. Erythropoietin production by the kidney in situ with blood containing cobalt. Seven recipient dog kidneys were perfused for a mean time of 2.14 hours with donor dog blood containing cobalt. Table 1 shows that the plasmas from the perfusion system after 2.14 hours stimulated a significantly ($P < 0.01$) greater Fe^{59} incorporation (11.2 ± 1.28) than the zero time plasma

Table 1

INFLUENCE OF PLASMA FROM KIDNEY-LUNG PERFUSION SYSTEM ON Fe^{59} INCORPORATION IN THE STARVED RAT

Dog No.	Perfusion time (hours)	Per cent Fe^{59} 0^a	Incorporation X^b	Δ Incorporation ^c
1	1-1/2	6.7	11.7	5.0
2	1-1/2	6.2	9.4	3.2
3	1-1/2	5.9	8.0	2.1
4	2	9.0	15.0	6.0
5	2-1/2	5.5	8.6	3.1
6	3	5.1	9.0	3.9
7	3	5.9	16.7	10.8
Mean	2.14	6.3 ± 0.48	11.2 ± 1.28	4.9 ± 2.65

^a0 = Plasma from the same dog blood before kidney perfusion.

^bX = Perfusion time.

^c Δ = The difference between 0 and X time incorporation.

$\pm$ = Standard deviation.

(6.3 ± 0.48). (The high zero time plasma from dog 4 could have been due to some type of anemia in the donor dog.) The mean hematocrit value on all assay rats injected with zero time dog plasma was 53.1 ± 1.89 per cent whereas the mean value from the assay rats receiving plasmas at the end of the perfusion was 51.7 ± 3.74 . The difference between these mean hematocrit values was not statistically significant. Therefore, it seems unlikely that the increase in Fe^{59} incorporation induced by plasma from the kidney-lung perfusion system could have been due to the presence of a hemolysin.

B. Radioactive iron incorporation in starved rats injected with plasma from the isolated lung perfusion system. Eight donor dog lungs were perfused with blood containing cobalt for 2 hours. Table 2 shows that there was no significant difference between the mean Fe^{59} incorporation in red cells of rats receiving the plasma from the 8 lungs before perfusion (5.04 ± 0.97) and the plasma from the blood after it had been perfused through the lungs for 2 hours (5.55 ± 1.39). As for the mean hematocrit values in the assay rats from the lung-kidney perfusion system, the hematocrits from assay rats receiving zero time plasma (52.8 ± 3.2) were not significantly ($P = 0.50$) different from those from the 2-hour perfusion plasmas (53.5 ± 3.9).

C. Radioactive iron incorporation in starved rats injected with sheep erythropoietin and cobaltous chloride. Table 3 presents the pooled results of saline, cobalt and erythropoietin treated rats assayed at the same time as the dog plasma samples from the kidney-lung and iso-

Table 2
INFLUENCE OF PLASMA FROM ISOLATED LUNG
PERFUSION SYSTEM ON Fe⁵⁹ INCORPORATION
IN THE STARVED RAT

Dog No.	Per cent Fe ⁵⁹ 0 ^a	Incorporation 2 hr
1	5.08	5.48
2	6.25	4.32
3	5.95	7.10
4	5.07	7.00
5	4.26	7.04
6	5.93	5.50
7	3.54	3.69
8	4.22	4.26
Mean	5.04 ± 0.97	5.55 ± 1.39

^a0 = Plasma from the same dog blood before lung perfusion.

± = Standard deviation.

Table 3
THE EFFECTS OF COBALTOUS CHLORIDE^a AND SHEEP ERYTHROPOIETIN^b
ON Fe⁵⁹ INCORPORATION IN STARVED RATS

Treatment	Dose (μ m or units)	No. rats	Per cent Fe ⁵⁹ Incorporation	Δ Incorporation ^c
Saline	-	30	5.5 ± 1.6	-
Cobalt ^d	5	20	7.7 ± 2.0	2.2
	10	19	12.6 ± 4.3	8.1
Erythropoietin ^e	1	20	10.7 ± 2.2	5.2
	2	20	13.8 ± 2.8	8.3
	3	15	16.6 ± 3.2	11.1

^aCobaltous chloride hexahydrate

^bArmour (AL-0336) purified erythropoietin from sheep plasma.

^c Δ = Difference between saline controls and treatment groups.

^dTotal dose expressed as micromoles CoCl₂.

^eTotal dose expressed in Armour Units.

lated lung perfusion systems. All dosages of cobalt and erythropoietin tested stimulated a significantly ($P < 0.01$) greater Fe⁵⁹ incorporation than saline treated controls. It is important to note that plasma from the in situ perfused kidney stimulated Fe⁵⁹ incorporation (11.2 per cent) to an extent slightly greater than 1 Armour unit of sheep erythropoietin (10.2 per cent). Goldwasser et al.¹² have shown a dose-response relationship with cobalt and sheep erythropoietin

on Fe⁵⁹ incorporation into red blood cells. They found that 5 micromoles of cobaltous ion stimulated Fe⁵⁹ incorporation in the starved rat to the same degree as 1 unit of sheep erythropoietin. Our data indicate a linear dose-response relationship with sheep erythropoietin although the 5 μ m dose of cobalt (7.7 per cent) was not equivalent to 1 unit of sheep erythropoietin (10.7 per cent). However, the 10 μ m dose of cobalt (13.6 per cent) was equivalent to 2 units of sheep erythropoietin (13.8 per cent).

DISCUSSION

Jacobson and coworkers^{1,2} have found that after bilateral nephrectomy, the administration of cobaltous ion, acute hemorrhage, or the induction of hypoxic anoxia fails to elevate plasma erythropoietin; whereas bilateral ureter ligation reduces but does not eliminate the erythropoietin plasma elevation. These experimental findings have been supported by Osnes,¹³ but have been challenged by the findings that nephrectomized rats³ and rabbits¹⁴ retain a limited but consistent ability to produce erythropoietin in response to hypoxia.

More recently Reissmann *et al.*¹⁵ have shown that bilaterally-nephrectomized or mercury bichloride-poisoned rats did not respond significantly to anemic stimuli. Bilateral nephrectomy is followed by a decrease in erythroblast percentages in bone marrow.¹⁶ Injections of erythropoietin in nephrectomized rats¹⁵ and dogs¹⁶ was followed by a significant increase in erythropoiesis which would certainly indicate that the role of the kidney in erythropoiesis is more than "permissive."

The studies reported here present positive evidence that the dog kidney, when isolated *in situ*, can be stimulated with cobalt to manufacture and/or release an erythropoietic factor. Cobaltous ion did not stimulate the lung or vascular endothelium or activate blood itself to produce erythropoietin. This seems to support the hypothesis of Jacobson and his coworkers that the kidney fulfills the requirements for an endocrine gland controlling the rate of red cell production although the possibility that there may be other sites which produce erythropoietic factors cannot be excluded.^{3,14}

Kuratowska *et al.*¹⁷ demonstrated the presence of an erythropoietic substance in plasma from blood perfused through the isolated hypoxic rabbit kidney. They suggested that the tissue responsible for this activity may not be the renal tissue *per se* but the vascular endothelium or the connective tissue of other organs including the kidney. Our failure to provoke an erythropoietic response with plasma from an isolated lung perfused with blood containing cobalt makes it probable that the vascular endothelium, at least in lungs, is not capable of producing erythropoietin.

Osnes¹⁸ has found that hypophysectomized rats are unable to elevate their titer of kidney erythropoietin after bleeding and concluded that some pituitary substance stimulates the secretion of kidney erythropoietin. The findings presented here, showing that the kidney, when perfused *in situ* with blood containing cobalt, is capable of producing a significant amount of erythropoietin would certainly indicate that the hypophysis is not necessary as a stimulus for the kidney to secrete erythropoietin.

The results of our experiments also have important physiological implications. If the kidney produces or releases an erythropoietic substance, which portion of the kidney is involved? Is the quantity of erythropoietin liberated from the renal cells or produced by them determined by the relationship of renal oxygen supply to renal oxygen demand?¹¹ It has been demonstrated¹⁹

that unilateral partial ligation of the left renal vein in dogs results, in 8-12 weeks, in erythrocytosis associated with increases in circulating RBC volumes and venous pressure. It has also been established that the hypertension following constriction of the renal artery is the result of a substance produced by the ischemic kidney.²⁰ Furthermore, Osnes has postulated that the kidney erythropoietic factor may arise from granules of the juxtaglomerular cells.¹³ It is not yet known whether the renal hypertensive substance and the kidney erythropoietic factor are the same. In this regard it would be of interest to know whether or not ischemia of one or both kidneys would stimulate the kidney to produce erythropoietin. It would also be important to know if the kidney activates a substrate precursor which is manufactured by some other organ. It seems possible that kidney erythropoietin is present in the renal tissue in the form of a precursor, and is activated only after it has been released into the circulation.

The erythropoietic response of the bilaterally nephrectomized animal to hypoxia³ raises the question whether other sites may be involved either directly or indirectly in the production of erythropoietin. The response at a particular site may depend upon the type and intensity of the hypoxic stimulus applied. There is evidence that adrenocortical steroids,^{21,22} thyroid hormone,²³ pituitary growth hormone,²⁴ possibly ACTH²⁵ and a liver factor²⁶ stimulate erythropoiesis. It is quite conceivable that a sufficiently powerful hypoxic stimulus could provoke discharge of multiple erythropoietic substances from some or all of these sites. The limited response of the bilaterally nephrectomized animal could result from the release of several of these hormones, which in summation may produce an erythropoietic effect.

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THE EFFECT OF ADRENALECTOMY ON THE ERYTHROPOIETIC RESPONSE TO SHEEP ERYTHROPOIETIN AND COBALT*

By

J. W. Fisher†

It has been known for over three decades that cobalt stimulates erythropoiesis in experimental animals.¹ Plasma from animals injected with cobalt contains a high titer of erythropoietin.² We have shown previously that, during the 30-day period following adrenalectomy, cobalt injections in rats fail to produce the characteristic polycythemic response. However, during a 30-65 day period following removal of the adrenals, cobalt did produce the typical polycythemia which is attributed to the presence of accessory adrenals.³ Further studies revealed that the adrenalectomized dog treated with cobalt did not display the characteristic bone marrow erythroid hyperplasia or the increase in total circulating red cell volume seen in intact dogs receiving cobalt.⁴ However, when hydrocortisone and cobalt were injected simultaneously into adrenalectomized rats, the increase in circulating red cell volume was greater than that seen in intact rats receiving cobalt.^{3,5}

The present study was undertaken to clarify the response of the adrenalectomized animal to such erythropoietic stimuli as sheep erythropoietin and cobalt. The term "erythropoietin" is here used to designate the factor present in plasma of anemic animals which stimulates erythropoiesis.

MATERIALS AND METHODS

The procedure of Fried and coworkers⁶ was followed for the erythropoietin assay. Male rats of the Sprague-Dawley strain were used, 5 in each assay. Young healthy rats, weighing 165 to 195 g each, were starved for 32 hours prior to the first experimental injection, and maintained in the starved state for the duration of the bioassay, water being permitted ad libitum. Two ml of plasma or test substance were injected subcutaneously daily for 2 days. On the third day, a tracer dose of 1 microcurie of Fe⁵⁹ citrate was given intravenously and standards were prepared for later counting. Sixteen hours later, 1 ml of blood was obtained by cardiac aspiration and the Fe⁵⁹ incorporation into red cells was calculated according to the following formula:

$$\text{Fe}^{59} \text{ uptake} = \frac{\text{net counts per ml blood} \times 0.05 \text{ body weight}}{\text{net counts injected}}$$

In these experiments, the blood volume was assumed to be 5 per cent of the body weight.⁶ The data was evaluated statistically by analysis of variance.⁷

* Summary of a paper which appears in *Endocrinology* 70:243, 1962. The work was supported by USPHS Grant A-2974(C2) from the Institute of Arthritis and Metabolic Diseases and USPHS Senior Fellowship Grant SF-463-C2. Part of it was carried out while a guest investigator at the Argonne Cancer Research Hospital.

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For the adrenalectomy experiments, male Holtzman rats ranging in weight from 150-400 g, were bilaterally adrenalectomized or sham-operated by a standard technique and kept for 12 days after operation to allow clearance of residual adrenal steroids. They were maintained on Rockland Rat diet and 0.9 per cent NaCl solution as drinking fluid during the time preceding their use for the erythropoietin studies. Food but not water was withheld for 32 hours prior to the first experimental injection and throughout the bioassay. The subsequent procedure in the adrenalectomized rats was the same as the starved-rat assay described above.

The total urinary excretion of cobalt during a 5-hour period was measured in male Sprague-Dawley rats weighing 215 ± 5.1 g. Each rat was adrenalectomized or sham-operated and maintained for 12 days before use as described above. Cobaltous chloride* injections were made subcutaneously. Two rats were placed in each metabolism cage. A 1/2-inch mesh wire screen served as a platform, and a finer mesh wire screen was placed under each cage to collect feces. Any dried urine on the sides of the cages was washed into the collecting flasks and transferred with sufficient deionized water to bring the total volume up to the next 5 ml. Each value represents the analysis of the pooled urine of the 2 rats in each cage. The technique of Almond⁸ was used for the cobalt analysis of urine and plasma in the cobalt excretion studies. All plasma samples from rats receiving cobalt were analyzed for cobalt subsequent to erythropoietin assay. All plasma samples analyzed for cobalt before erythropoietin assay contained less than 0.1 micromole Co^{++} per ml, an amount not sufficient to result in a significant increase in radioactive iron incorporation. One unit of sheep erythropoietin[†] exerts an effect on radioactive iron incorporation equivalent to 5 micromoles cobaltous chloride.⁹

RESULTS

A. Radioactive iron incorporation in RBC of adrenalectomized rats injected with sheep erythropoietin. The data summarized in Figure 1 show that 1, 2 and 3 units of sheep erythropoietin are significantly less effective in stimulating radioactive iron incorporation in starved adrenalectomized than in sham-operated rats. The baseline iron incorporation in starved, untreated adrenalectomized rats maintained on saline was also significantly lower than in sham-operated controls. It is of further interest that 1 unit of erythropoietin in the adrenalectomized rat did not completely return the iron incorporation values to the level of untreated, sham-operated controls. Erythropoietin is apparently more toxic to the adrenalectomized than to the sham-operated rat since three units killed 3 out of the 8 adrenalectomized rats tested, whereas none of the sham-operated rats died after equivalent dosages of erythropoietin.

B. Radioactive iron incorporation in RBC of adrenalectomized rats injected with cobalt. Figure 2 shows that 5, 10 and 15 micromoles of cobaltous ion injected into the adrenalectomized rat resulted in a significantly greater Fe^{59} incorporation than equivalent dosages given to sham-operated rats. This increased sensitivity to cobalt was slightly greater at the 10 micromole dose than at either 5 or 15 micromoles. The 5, 10 and 15 micromole dose of cobalt resulted in a 60-70 per cent greater elevation in iron incorporation in the adrenalectomized than the sham-

* Analytical Grade $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ with the maximum limits of impurity for lead of 0.005 per cent.

† Erythropoietin extracted from sheep plasma was obtained through the USPHS Hematology Study Section and the chemical and physical constitution of this material has been described by Jacobson et al.¹⁴

Figure 1. Mean values for per cent Fe^{59} incorporation in red blood cells of starved adrenalectomized and sham-operated rats receiving 0.85 per cent saline, 1, 2 and 3 units of sheep erythropoietin. Asterisk (*) indicates values significantly different from the controls at the 1 per cent level. Vertical lines to the left of the bar represent standard error of the mean. Each value represents the mean value for 15 animals in each of the sham-operated groups and 8 animals in each of the adrenalectomized groups except the 3 unit dose of erythropoietin, which contained 5 animals.

operated rats. The 5, 10 and 15 micromole doses of cobalt in the sham-operated (Figure 2) rats produced Fe^{59} incorporation equivalent to 1, 2 and 3 units of sheep erythropoietin (Figure 1). This is further confirmation that 1 unit of erythropoietin exerts an effect equivalent to 5 micromoles cobaltous ion (Goldwasser et al.⁹).

C. Plasma erythropoietin titers in sham-operated and adrenalectomized rats after cobalt injection. To clarify this increased sensitivity of the adrenalectomized rat to cobalt, a comparison was made between the erythropoietic activity in plasmas after a single Co^{++} injection. Sham-operated and adrenalectomized rats were given single injections of 62.5 and 125 micromoles Co^{++} per kg and the animals were bled 12 hours later. The maximum erythropoietin titer in the intact rat occurs approximately 12 hours after a single subcutaneous injection of cobalt.² Plasma samples from all animals were analyzed for cobalt and were found to contain less than 0.1 micromole per 4 ml. As indicated in Table 1, the 62.5 and 125 micromole dosages of cobalt in adrenalectomized rats resulted in significantly higher plasma erythropoietin titers than equivalent doses in sham-operated rats.

D. Cobalt excretion studies in adrenalectomized and sham-operated rats. It is known that adrenalectomized animals fail to respond to water loading with diuresis.¹⁰ To test whether the higher erythropoietin titers in the adrenalectomized rat was the result of reduced urinary excretion of the Co^{++} ion, the total urinary output of Co^{++} was compared in the sham-operated and adrenalectomized rat 5 hours after a single subcutaneous injection of 75 μm Co^{++} /kg. This time was chosen because the plasma concentration of cobalt reaches a peak in 5 hours after subcu-

Figure 2. Mean values for per cent Fe⁵⁹ incorporation in red blood cells of starved adrenalectomized and sham-operated rats receiving 0.85 per cent saline, 5, 10 and 15 micromoles cobaltous chloride. Asterisk (*) indicates values significantly different from the controls at the 1 per cent level. Vertical lines to the left of the bar represent standard error of the mean. Each value represents the mean value for 15 animals in each of the sham-operated groups and 10 animals in each of the adrenalectomized groups except the 10 micromole dose of Co⁺⁺, which contained 8 animals.

taneous injection (Weissbecker¹¹). Plasma concentrations in adrenalectomized rats were also compared with those of sham-operated controls.

As shown in Table 2 the total volume of urine and cobalt excreted in a 5-hour period was significantly less and the plasma Co⁺⁺ levels were significantly higher in the adrenalectomized than the sham-operated rats. Therefore, it seems reasonable to assume that the higher erythropoietin titers found in the former after an injection of cobalt are due, at least in part, to a delay in renal excretion of cobalt, and because the cobalt is retained longer, iron incorporation in RBC may be stimulated for a longer period.

DISCUSSION

The hematopoietic response of the adrenalectomized rat to sheep erythropoietin is distinctly different from the response to cobalt administration. Sheep erythropoietin injections resulted in significantly less radioactive iron incorporation in adrenalectomized than in sham-operated rats. This would indicate that an adequate supply of adrenocortical steroids is necessary for sheep erythropoietin to stimulate optimal iron incorporation in red cells, and may be another example of a "permissive" action of the adrenocortical steroids.

In contrast to the above, cobalt exerts a significantly greater effect on iron incorporation

Table 1

EFFECT OF COBALT PLASMA FROM ADRENALECTOMIZED AND SHAM-OPERATED
RATS ON Fe^{59} INCORPORATION INTO THE RED BLOOD CELLS
OF STARVED RATS

Surgical procedure ^a	No. assay animals	Dose cobalt to donor rat ($\mu\text{m Co}^{++}/\text{kg}$)	Fe^{59} incorporation in recipient starved rats to donor Co^{++} plasma	Difference from control
None	14	-	4.92 ± 0.36	-
Sham operation	4	62.5	3.89 ± 0.10	-
	4	125.0	8.43 ± 1.63	3.49
Adrenalectomized	5	62.5	$6.64 \pm 1.46^*$	1.71
	5	125.0	$26.69 \pm 0.23^*$	21.65

^a12 days following surgery, the rats (Holtzman, 350-400 g) were injected subcutaneously with cobaltous chloride, and 12 hours later the blood was collected via cardiac puncture. The plasma thus obtained was assayed in the starved rat. Asterisk indicates the difference between this respective dose in the adrenalectomized and sham-operated groups is significant at the 5 per cent level.

$\pm$ = Standard error of the mean.

Table 2

URINARY COBALT^{*} EXCRETION STUDIES IN ADRENALECTOMIZED
AND SHAM-OPERATED RATS

Treatment	No. animals	B.W. (g)	Urine volume (ml/2 rats/5 hrs)	Micromoles Co^{++}	
				Urine (per 2 rats /5 hrs)	Plasma (per ml after 5 hrs)
Sham-operated	18	231 ± 4.09	4.70 ± 1.16	4.40 ± 1.23	0.04 ± 0.009
Adrenalectomized	16	217 ± 7.09	1.48 ± 0.44	1.92 ± 0.67	0.17 ± 0.071

* Total urinary excretion and plasma concentration measured 5 hours after a subcutaneous injection of $75 \mu\text{m CoCl}_2 \cdot 6\text{H}_2\text{O}/\text{kg}$.

$\pm$ = Standard error of the mean.

in adrenalectomized than in sham-operated rats. Previously, we reported that the elevation in peripheral blood values following cobalt injections in adrenalectomized rats is delayed until accessory adrenals regenerate sufficiently to become functional.³ The effect of cobalt on iron incorporation is dissociated from its effect on peripheral blood values. This dissociation may be the result of a dual erythropoietic action of cobalt. Future work may reveal the nature of these two actions of cobalt. The adrenal cortex is apparently necessary for the erythroid hyperplasia resulting from cobalt injections but not for its action on iron incorporation by erythrocytes.

Cobalt not only exerts a greater effect on iron incorporation in adrenalectomized than in

sham-operated rats but it also produces significantly higher erythropoietin titers in the adrenalectomized group. These effects are probably due to a combination of factors. It is generally believed that adrenalectomized animals metabolize endogenous protein with much less facility than intact animals. Thus, gluconeogenesis is inhibited and a metabolic insufficiency ensues. Hepatic gluconeogenesis is dependent upon a supply of adrenal glucocorticoids. Jacobsen and coworkers¹² have indicated that higher erythropoietin titers are found in the plasma of phenylhydrazine treated rabbits which display considerable liver damage, and interpret this to mean that the injury interferes with the inactivation function of the liver or prevents the elaboration of normally occurring inhibitor. Further support for this interpretation of the role of the liver in inactivating erythropoietin is found in Prentice and Mirand,¹³ who have shown that erythropoietin disappearance from plasma of rats is delayed by the administration of the hepatotoxic agent, carbon tetrachloride. It seems possible that the suppressed function of liver after adrenalectomy could result in its failure to metabolize adequately the erythropoietic hormone. This presumably would lead to a higher plasma titer of erythropoietin in much the same way as does the administration of an agent which produces liver damage.

Variation in the constancy of the erythropoietic stimulus may contribute to the higher iron incorporation found in adrenalectomized rats injected with cobalt as compared with those receiving sheep erythropoietin. Our findings (Table 2) of a delayed renal excretion of cobalt and higher plasma concentrations of cobalt in the adrenalectomized rat support the view that cobalt exerts a more constant and prolonged erythropoietic stimulus than does sheep erythropoietin. In contrast, the action of a single dose of exogenous sheep erythropoietin may be relatively transient. It would be of interest to study the time course of erythropoietic activity after a single injection of sheep erythropoietin.

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CONFERENCE ON ERYTHROPOIETIN AND
REGULATION OF ERYTHROPOIESIS*

January 21-22-23, 1962
San Francisco, California

About 100 scientists attended this meeting and participants were drawn from Argentina, Belgium, Canada, Chile, Great Britain, Israel, Japan, Poland and the United States. Thirty-nine universities and hospitals throughout the United States were represented as well as the National Institutes of Health, the United States Atomic Energy Commission, the United States Army and Navy and the Armour Pharmaceutical Company. The general topics discussed included:

- 1) Erythropoietin Assay Methods and Standardization Procedures
- 2) Purification Procedures and Chemistry of Erythropoietin
- 3) Sites of Formation of Erythropoietin
- 4) Metabolic Fate of Erythropoietin
- 5) Kinetics of Erythropoiesis
- 6) Metabolic Effects and Cellular Responses to Erythropoietin
- 7) Special Subjects Related to the Physiology of Erythropoietin
- 8) In vitro Studies
- 9) Relationship of Erythropoietin to Anemia and Various Types of Polycythemia
- 10) Relationship of Erythropoietin to Renal Abnormalities and Cystic Tumors

It was evident from the outset that great strides have been made in the standardization of assay methods of erythropoietin. Production of this purified hormone from the plasma of anemic sheep was undertaken by Armour and Company in collaboration with Argonne Cancer Research Hospital - University of Chicago, and supported by a grant from the United States Public Health Service. Approximately 600,000 units were thus made available to investigators for use in physiologic studies and study of the chemical nature of the hormone. None of this material has been available for clinical studies in human beings.

Using rabbit erythropoietin prepared at the California Institute of Technology, a study of comparative methods of assay was made in 9 different research laboratories across the United States. The results of these assays were in good agreement. The National Institutes of Health, in cooperation with Dr. D. R. Bangham of the National Institute for Medical Research, Millhill, England, have now made available 500 ampoules of partially purified sheep erythropoietin to research laboratories throughout the world. It is intended that this material should serve as Standard A for quantitative assay of erythropoietin. Scientists are invited to standardize their own materials, whether from plasma or urine, and methods against it. In this way a great deal of what may prove to be minor discrepancies will be cleared up, and it will be possible to compare the results obtained using different research methods and assay animals on an empirical basis.

Methods of preparation of a stable purified product from the plasmas and urines of rabbits,

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Summary by L. O. Jacobson and M. Doyle.

sheep and humans were discussed. The huge quantities of sheep plasma necessary for the preparation of this hormone (286 sheep - 428 liters of plasma - 10 mg erythropoietin of a potency of 1000 units/mg) were noted. The chemical structure of the molecule is thought to resemble that of a glycoprotein with a molecular weight by x-ray inactivation of about 28,000. The molecule appears to be asymmetrical.

Experiments with various animals in various physiological states (e.g., starved and hypoxic rats, polycythemic mice, nephrectomized and ureter-ligated rats, mice and dogs, parabiont rats, perfused isolated kidneys, legs and livers), as well as relationships to the presence of erythropoietin in the plasma and urine of patients with tumors were discussed. The general consensus was that the kidney is the main site of elaboration but that other sites probably exist. It was suggested that the renal medulla might be involved, and that other organs, possibly the liver, might function independently to produce an erythropoietic factor.

Wherever the erythropoietin is produced and released it appears in the plasma and the lymph. It is used by the bone marrow, metabolized and probably destroyed in the liver and is also lost via the urine. It is now generally conceded that erythropoietin acts by initiating differentiation of the stem cell population of the blood-forming tissue. Under normal conditions this process is so controlled that the production of new red cells balances the loss of old red cells. How this control is effected has not been clarified. Experiments on the polycythemic mouse suggest that there may be a maximum single dose of exogenous erythropoietin beyond which no increased response can be elicited. It is thought that erythropoietin regulates stem cell differentiation, but it is not known with certainty whether the hormone has other effects in subsequent divisions and maturation of the red cell series.

Methods of studying the behavior of the stem cell compartment and study of the stem cell compartment recovery from irradiation injury were described. These studies included observations on the polycythemic mouse and normal dogs. The recovery pattern was measured by reticulocyte studies, Fe^{59} and tritium thymidine labeling.

In vitro studies on erythropoiesis using purified sheep erythropoietin, anemic, normal, polycythemic plasma alone, and polycythemic plasma plus erythropoietin variously on rabbit, dog, and human bone marrow suspensions and cultures have yielded a wide variety of results.

In the past it has been a matter for speculation that the effects produced in vitro were so very much smaller than those which could be elicited in vivo. Some recent experiments suggest that it may now be possible to demonstrate quantitative results in vitro.

It is evident that the choice of method, handling of tissues, and experimental criteria are all extremely important and that uniform cultures are essential for cell culture work. Some perfusion experiments on the mechanism influencing the release of cells in the marrow, using whole rat legs and isolated femurs, appear to indicate that there is a separate and distinct response of reticulocytes and leucocytes which would indicate that separate humoral mechanisms exist for release of erythrocytes and leucocytes from blood-forming organs.

Data on the relation of erythropoietin to degree of anemia and various types of polycythemia were discussed. The relationship of urine titer of erythropoietin to plasma titer was discussed and a number of clinical cases were presented showing the effect of transfusion on these titers. Data on the anemias of uremia were presented by several investigators; uniformly the plasma titer is very low, indicating that interference with erythropoietin production exists in this clinical condition. These observations are also borne out by the findings in experimental rabbits and

dogs. It has also been shown that erythropoietin-like activity can be isolated from renal carcinomas and cysts and it was suggested by one investigator that the renal vascular tissue is the site of elaboration. In any case, all these studies offer support to the idea that the kidney is responsible, for a large part, of erythropoietin production or elaboration. The difficulty in using clinical patients under experimental conditions was noted, and again the necessity for controlled experiments with detailed information on procedures was stressed.

The indirect relationship between iron absorption and erythropoiesis was described.

In conclusion, the conference was characterized by the sustained interest of the participants and by the lively debates and discussions which followed each session.

THE ENZYMATIC SYNTHESIS OF RNA: NEAREST NEIGHBOR BASE FREQUENCIES*

By

S. B. Weiss and T. Nakamoto†

RNA[‡] polymerase activity, first detected in mammalian cell-free preparations,^{1,2} has now been described in a number of bacterial extracts and plant tissue.³⁻⁶ The reaction catalyzed by this enzyme shows an absolute requirement for all four ribonucleoside triphosphates and DNA and may be represented by the following equation.

The requirement for a deoxypolynucleotide primer raised the question of the role played by this polymer in RNA synthesis. A recent report from this laboratory demonstrated that the base composition of RNA, prepared with a partially purified extract from Micrococcus lysodeikticus, was similar to and dependent upon the composition of the primer.⁷ Similar results have been obtained by Stevens⁸ and Furth *et al.*^{9,10} with extracts prepared from Escherichia coli.

Cellular RNA similar in base composition to DNA has been identified in various organisms by a number of investigators.^{11,12} Recently, Hall and Spiegelman have shown that the RNA which appears after T2 phage infection of E. coli, forms complexes with denatured phage DNA.¹³ Such complexes are believed to occur only between strands of complementary base sequences and point out the complementary relationship between the deoxy- and ribopolynucleotides. The existence of similar DNA and RNA molecules in nature has added impetus to the idea that genetic information is transmitted to other cellular sites by so-called "messenger-RNA."^{14,15} The discovery of a DNA-dependent RNA polymerase has stimulated speculation that this enzyme may be responsible for the synthesis of this specific-RNA. So far, no direct experimental proof has been offered to support these speculations.

Weiss and Nakamoto have presented preliminary evidence indicating that the relative position of ribonucleotides in enzymatically prepared RNA is determined by the primer used for the reaction.⁷ This information, along with the similar base composition found, suggests but does

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‡ The abbreviations used in this report are: RNA and DNA for ribo- and deoxyribonucleic acid; CTP³², ATP³², UTP³² and GTP³² for the triphosphates of cytidine, adenosine, uridine and guanosine labeled with P³² in the ester phosphate; Cp, Ap, Up and Gp for the 2'3'-nucleoside monophosphates of the bases listed above; PP for inorganic pyrophosphate; Tris, tris-(hydroxymethyl)-aminomethane; TCA, trichloroacetic acid; cpm, counts per minute.

not prove, that synthesis occurs with replication of the nucleotide sequence of the primer. Absolute and unequivocal proof of replication can only be supplied by determining the sequential arrangement of bases in both primer and RNA product. In the absence of methods adequate to perform such analysis, a complete nearest neighbor frequency study for the 16 possible nucleotide pairs in RNA has been determined for RNA's prepared with different primers. In view of the elegant studies reported recently by Josse, Kaiser and Kornberg,¹⁶ it is possible to compare our nearest neighbor pairs with the results published for DNA's that have been similarly characterized. We have also carried out a series of CsCl density-gradient experiments designed to determine the complementary relationship of the RNA formed during reaction with respect to the primer employed, and these results are reported in the following paper.

MATERIALS AND METHODS

ATP³², CTP³² and UTP³² were prepared as previously described. GTP³² was prepared by the method of Tener.¹⁷ Each of these labeled substrates contained P³² in the innermost phosphate only. RNA polymerase was obtained from extracts of *M. lysodeikticus* and preparations ranging from 100- to 200-fold purification were used. *E. coli* DNA was a gift from Dr. A. Rich; T2 DNA was a gift from Dr. L. Grossman; calf thymus DNA was purchased from The Sigma Chemical Company; and *M. lysodeikticus* DNA was prepared from lysozyme-treated cells as described by Lehman and co-workers.¹⁸

The reaction mixture for the preparation of P³²-labeled RNA contained 3 μ moles of MnCl₂, 100 μ mole Tris-PO₄ buffer of pH 7.4, 0.10 mg of a DNA primer, 1 μ mole each of ATP, UTP, CTP and GTP, only one of which was radioactive (specific activity of 1 to 10 x 10⁶ cpm per μ mole) and a purified extract from *M. lysodeikticus* in a total volume of 1.0 ml. Sufficient enzyme was used so that a minimum of 100,000 cpm was incorporated into RNA. For each DNA used, four similar reactions were prepared except that each reaction contained a different labeled substrate. After 30 minutes at 25°, the reaction was stopped with cold 5 per cent TCA, iced, and the precipitate collected. The acid-insoluble material was washed 3 times by dissolving in 0.10 M NaOH and reprecipitating with TCA, and once again with ethanol-ether (3:1). One mg of carrier RNA was added and the residue was extracted twice in 1 ml of 10 per cent NaCl, pH 8, for 30 minutes at 100°. Two volumes of cold ethanol were added to the combined extracts and the precipitate collected after standing at 0° overnight. The nucleic acid fraction was dissolved in 1 ml of water, 0.10 ml of a 20 per cent potassium acetate solution (adjusted to pH 5) was added, and reprecipitation was with ethanol. This procedure was repeated until the ethanol supernatant gave a constant amount of radioactivity. The precipitate was dried under vacuum for 30 minutes, hydrolyzed in 0.3 M KOH for 18 hours at 37°, acidified with HClO₄ and the acid soluble fraction subjected to paper electrophoresis as described elsewhere.² The 2',3'-nucleoside monophosphates were identified under ultraviolet light, eluted and assayed for radioactivity.

RESULTS

During alkaline hydrolysis of RNA, the phosphate esterified to the 5' position of one ribonucleotide is transferred to the 2',3' position of the adjacent nucleotide. P³²-RNA, enzymatically synthesized with one ribonucleoside 5' (pyro)-triphosphate labeled in the ester phosphate only, gives rise to four labeled 2', 3'- nucleoside monophosphates when subjected to this type of hydrolysis. Quantitative determination of the amount of isotope contained in each mononucleotide may

be used as a measure of the relative frequency with which the labeled nucleotide occurs next to a given neighbor in the RNA chain. In the synthesis of RNA, if four labeled nucleotides are used separately, in four different reactions, the isotope content of the hydrolyzed mononucleotides can be used to determine the frequencies of the 16 possible nearest neighbor base sequences. For a detailed description of nearest neighbor sequence determination, the reader is referred to Josse and co-workers.¹⁶ The method used here for calculating these values is essentially as reported by these investigators and may be simply described as follows:

$$\text{Fractional base value} \times \text{base-incorporating factor} = \text{relative frequency.}$$

For a given reaction, the isotope content found for each mononucleotide was converted to the decimal fraction of the total to give the fractional base values (Table 1). To obtain nearest neighbor frequencies, the fractional values must be multiplied by a factor which represents the relative amount of each ribonucleotide incorporated into RNA. Since the relative amount of each ribonucleotide incorporated is determined by the base composition of the primer,⁷ the molar proportions of adenine, thymine, guanine and cytosine of the particular DNA employed in each reaction were used as the base-incorporating factors. The DNA base compositions given in Table V of the paper by Josse, Kaiser and Kornberg¹⁶ were used since it was felt that these values might be more accurate than previously reported determinations of the chemical composition for deoxyribonucleic acid.

DNA from M. lysodeikticus contains a relatively high guanine and cytosine content while T2 phage DNA is rich in adenine and thymine. Table 1 and Table 2 show the counts and fractional values determined for the various 2', 3'-mononucleotides formed by alkaline hydrolysis of P³²-RNA when synthesized in the presence of these distinctly different primers. When the fractional values are multiplied by the appropriate base-incorporating factors (0.147, 0.145, 0.354 for M. lysodeikticus DNA and 0.319, 0.318, 0.184 and 0.179 for T2 DNA in the reactions containing ATP³², UTP³², GTP³² and CTP³² respectively) the nearest neighbor frequencies shown in Table 3 and Table 4 are obtained.

A comparison of the figures of similar base-pairs found in RNA with those reported for DNA (values in parenthesis) shows that the frequency of the 16 nucleotide pairs for primer and product is remarkably close. In all instances where the base-pair frequencies are relatively high or low for a given DNA, the same variation is detected in the base-pairs for the synthesized RNA.

E. coli and calf thymus DNA have base compositions which are somewhat alike. The nearest neighbor frequencies for both these DNA's are also fairly similar except in one or two instances, e.g., the base-pairs CpG and GpC. When the nucleotide pairs of the RNA's formed with these two primers are examined, we find that the frequencies are in good agreement with the values reported for DNA (Table 5). In those few instances where there are marked differences in the base-pair frequencies of the primers, there are also similar differences in the RNA synthesized. Thus, when differences in the nearest neighbor sequences of two primers exist, the corresponding differences in the RNA products can be readily detected.

DISCUSSION

A thorough understanding of the enzymatic mechanism for the synthesis of RNA by DNA-dependent enzymes is important for obvious reasons. The implications of this reaction as a possi-

Table 1

P^{32} CONTENT OF MONONUCLEOTIDES ISOLATED FROM ENZYMATICALLY SYNTHESIZED P^{32} -RNA PRIMED BY *M. lysodeikticus* DNA

Isolated 2',3'-ribo- nucleotide	Labeled Triphosphate											
	Reaction No. 1 ATP 32			Reaction No. 2 UTP 32			Reaction No. 3 GTP 32			Reaction No. 4 CTP 32		
	Sequence	C.P.M.	Fraction	Sequence	C.P.M.	Fraction	Sequence	C.P.M.	Fraction	Sequence	C.P.M.	Fraction
Up	UpA	960	0.065	UpU	947	0.108	UpG	3,834	0.151	UpC	8,124	0.185
Ap	ApA	1,612	0.109	ApU	1,411	0.161	ApG	3,237	0.128	ApC	6,667	0.152
Cp	GpA	5,895	0.397	CpU	3,249	0.371	CpG	10,335	0.406	CpC	13,548	0.310
Gp	GpA	6,360	0.429	GpU	3,145	0.360	GpG	8,041	0.315	GpC	15,447	0.353
Sum		14,827	1.000		8,752	1.000		25,447	1.000		43,786	1.000

Table 2

P^{32} CONTENT OF MONONUCLEOTIDES ISOLATED FROM ENZYMATICALLY SYNTHESIZED P^{32} -RNA PRIMED BY T2 DNA

Isolated 2',3'-ribo- nucleotide	Labeled Triphosphate											
	Reaction No. 1 ATP 32			Reaction No. 2 UTP 32			Reaction No. 3 GTP 32			Reaction No. 4 CTP 32		
	Sequence	C.P.M.	Fraction	Sequence	C.P.M.	Fraction	Sequence	C.P.M.	Fraction	Sequence	C.P.M.	Fraction
Up	UpA	3,735	0.258	UpU	3,724	0.340	UpG	6,001	0.350	UpC	8,576	0.343
Ap	ApA	5,400	0.373	ApU	3,544	0.324	ApG	5,098	0.298	ApC	6,818	0.273
Cp	CpA	2,760	0.190	CpU	1,878	0.171	CpG	2,856	0.166	CpC	4,336	0.173
Gp	GpA	2,580	0.179	GpU	1,811	0.165	GpG	3,170	0.186	GpC	5,275	0.211
Sum		14,475	1.000		10,957	1.000		17,125	1.000		25,005	1.000

Table 3

COMPARISON OF NEAREST NEIGHBOR FREQUENCIES OF RNA
WITH DNA FOR PRIMER *M. lysodeikticus* DNA

Reaction No.	Labeled triphosphate	Isolated 2',3'-nucleoside monophosphate			
		Up (Tp)*	Ap	Cp	Gp
1	ATP ³²	UpA (TpA) .010 (.011)	ApA .016 (.019)	CpA .058 (.052)	GpA .063 (.065)
2	UTP ³² (dTTP ³²)	UpU (TpU) .016 (.017)	ApU (ApT) .023 (.022)	CpU (CpT) .054 (.050)	GpU (GpT) .052 (.056)
3	GTP ³²	UpG (TpG) .053 (.054)	ApG .045 (.049)	CpG .143 (.139)	GpG .112 (.112)
4	CTP ³²	UpC (TpC) .065 (.063)	ApC .054 (.054)	CpC .110 (.113)	GpC .125 (.121)

* The values in parenthesis represent the nucleotide pairs and nearest neighbor frequencies reported by Josse et al.¹⁶ for *M. lysodeikticus* DNA.

Table 4

COMPARISON OF NEAREST NEIGHBOR FREQUENCIES OF RNA
WITH DNA FOR PRIMER T2 DNA

Reaction No.	Labeled triphosphate	Isolated 2',3'-nucleoside monophosphates			
		Up (Tp)*	Ap	Cp	Gp
1	ATP ³²	UpA (TpA) .082 (.089)	ApA .119 (.111)	CpA .061 (.061)	GpA .057 (.059)
2	UTP ³² (dTTP ³²)	UpU (TpU) .108 (.106)	ApU (ApT) .103 (.104)	CpU (CpT) .054 (.054)	GpU (GpT) .052 (.051)
3	GTP ³²	UpG (TpG) .064 (.063)	ApG .055 (.057)	CpG .031 (.030)	GpG .034 (.036)
4	CTP ³²	UpC (TpC) .061 (.057)	ApC .049 (.048)	CpC .031 (.034)	GpC .038 (.040)

* The values in parenthesis represent the nucleotide pairs and nearest neighbor frequencies reported by Josse et al.¹⁶ for T2 DNA.

Table 5

COMPARISON OF NEAREST NEIGHBOR FREQUENCIES FOR RNA AND DNA

Nearest neighbor sequence*	Calf thymus		<u>E. coli</u>	
	Primer DNA†	Synthesized RNA	Primer DNA†	Synthesized RNA
ApA, UpU	0.089, 0.087	0.093, 0.082	0.071, 0.076	0.075, 0.079
CpA, UpG	0.080, 0.076	0.082, 0.070	0.071, 0.071	0.071, 0.068
GpA, UpC	0.064, 0.067	0.062, 0.061	0.055, 0.056	0.059, 0.056
CpU, ApG	0.067, 0.072	0.072, 0.067	0.055, 0.055	0.051, 0.051
GpU, ApC	0.056, 0.052	0.058, 0.053	0.055, 0.054	0.054, 0.058
GpG, CpC	0.050, 0.054	0.061, 0.055	0.056, 0.056	0.057, 0.058
UpA	0.053	0.051	0.051	0.044
ApU	0.073	0.068	0.068	0.070
CpG	0.016	0.016	0.067	0.073
GpC	0.044	0.048	0.083	0.077

* The nucleotide pairs listed are for RNA and are the same for DNA except that T (thymine) would be substituted for U (uridine).

† The nearest neighbor frequencies for calf thymus and E. coli DNA have been reported by Josse et al.¹⁶

ble step in transmitting genetic information has already been mentioned. The results tabulated in this paper, together with the similar base compositions of DNA and RNA previously determined, provide further evidence for the sequential assembly of ribonucleotides along a DNA template. The data are consistent with the idea that the arrangement of bases along such a template would occur by hydrogen-bonding of the appropriate base-pairs.

RNA polymerase can be primed by heat-denatured DNA as well as native DNA.¹⁹ No definitive answer can be given to the problem whether one or both strands of DNA determines the base sequence and composition of the enzymatically synthesized RNA. In theory, according to the Watson and Crick base-pairing hypothesis,²⁰ opposing strands of DNA need not have the same base composition, but they may do so on a purely statistical basis. The similar base ratios found for RNA and its primer DNA and the similarity in nearest neighbor frequencies are consistent with two possibilities: (1) the strands are different and both prime, (2) the strands are alike in compositions and base sequence and one or both may prime. In the latter case, the results offer no information on the mechanism of priming.

Although the nearest neighbor frequencies determined in these experiments suggest that the sequential arrangement of nucleotides in RNA is probably the same as that found in the primer, the above results by themselves cannot be considered unequivocal evidence for faithful replication of the base sequences found in DNA. For this reason, we have carried out CsCl density-gradient studies which will be reported in the subsequent paper²¹ and which provide more conclusive evidence that the RNA strands synthesized are complementary in base sequence to the deoxypolynucleotide primer. The term complementary is defined as a specific fit of portions of primer and product by appropriate base interactions.

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THE ENZYMATIC SYNTHESIS OF RNA: COMPLEMENTARY INTERACTION WITH DNA*

By

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The chemical properties of the polynucleotides synthesized by the DNA - primed RNA polymerase have been described in two previous communications.^{1,2} It has been shown that the average composition and the average nearest neighbor frequencies of enzymatically synthesized RNA and DNA primer are identical. In view of this information and of the results communicated here, we have named the product of this reaction "Complementary RNA" (C-RNA).^{**} It is our working hypothesis that C-RNA is structurally and functionally similar to "messenger" RNA,^{8,9} and that the biosynthesis of C-RNA is an initial step in that sequence of reactions in which DNA asserts the organism's potentialities for protein synthesis. Consequently, it is of considerable interest to discover whether higher order DNA nucleotide sequence is translated with any degree of fidelity in RNA and to describe in greater detail the manner in which the DNA template is utilized during RNA synthesis. In this communication we describe the results of experiments along these lines.

MATERIALS AND METHODS

DNA. T2 DNA was isolated from bacteriophage by phenol extraction.¹¹ Pseudomonas fluorescens DNA, used as a density reference marker in analytical CsCl gradient centrifugation, and Escherichia coli DNA were prepared by a method described elsewhere.¹² DNA from testes of the sea urchin Strongylocentrotus dröbachiensis was isolated according to the method of Simmons.¹³ Bacteriophage T2 DNA, labeled with tritium, was prepared as follows: E. coli were grown as described by Kozloff.¹⁴ At late log phase, T2 phage and H³-labeled adenine were introduced into the medium, and aeration was continued for 4 hours. MgSO₄ to 0.005 M, and 1 µg/ml crystalline pancreatic DNAase I were added. After incubation at 37°C for one hour, the entire culture was filtered through celite, and phage was purified from the filtrate.¹⁵ DNA was

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^{**} A number of different enzymes (polynucleotide phosphorylase, RNA polymerase, polyribonucleotide synthetase) from various sources have now been reported for the synthesis of RNA.³⁻⁵ Each of these reactions has different properties and the products formed, although exhibiting characteristic polynucleotide linkages, are not the same. Furthermore, in recent years, a distinction between RNA molecules has been made by various investigators based upon their cellular origin and biological properties, e.g., soluble RNA,⁶ amino acyl RNA,⁷ and their chemical composition, e.g., "messenger" RNA.^{8,9} In view of this, we have tentatively assigned the name of "complementary RNA" to the product of the reaction catalyzed by RNA polymerase, in order to designate a specific type of RNA, that is, one which is formed from a DNA template, contains a base composition and nearest neighbor frequency similar to that of the primer, and exhibits the type of complementary properties with its primer, first described by Hall and Spiegelman.¹⁰

prepared from the phage by phenol extraction.¹¹

The synthesis of C-RNA on a bacteriophage T2 DNA template was carried out as described in the preceding communication.² The total volume of the reaction mixture was adjusted to allow incorporation of 50 to 100 m μ moles labeled substrate into RNA in the presence of 150 to 300 m μ moles primer DNA-P. The specific activity of labeled nucleotides was reduced to conform with the requirements of the centrifugation experiments.

Isolation of DNA and C-RNA for CsCl gradient centrifugation.

Procedure A: The enzymatic synthesis of RNA was terminated by adding an equal volume of 90 per cent redistilled phenol, and shaking for 30 minutes. The phenol extraction was repeated. The aqueous layer containing RNA and DNA was then extracted two to three times with ether to remove dissolved phenol, and residual ether removed with a stream of N₂. NaCl was added to 0.2 M, and CTAB^{*16} to a final concentration of 1 mg/ml. A precipitate containing DNA and RNA formed immediately. The mixture was allowed to stand for 15-20 minutes, and centrifuged. The precipitate was then washed twice with 0.2 M NaCl, and finally dissolved in a small volume of 2 M CsCl. All the above steps were carried out at room temperature. Under these conditions we found that 90 per cent or more of the enzymatically prepared RNA-P³² was recovered, and contained 10 to 20 per cent TCA soluble nucleotides. The recovery of DNA was less complete, some loss from the aqueous phase occurring in the phenol extraction. However, we did not find it necessary to recover any of that material for the experiments reported here.

Procedure B: Phenol extraction was performed as above. The entire aqueous phase was then reduced under vacuum, adjusted to 7.9 molal CsCl, 0.01 M Versene, pH 6.5 in a final volume of 3 ml, and centrifuged at 16°C, 35,000 rpm, for 48 hours. The material which accumulates at the bottom of the tube in the CsCl gradient contains the bulk of the RNA, well separated from DNA. From this fraction RNA was precipitated as follows: to 0.7 ml CsCl solution, 0.4 ml 0.01 M Tris, pH 7 was added, followed by 2.2 ml ice-cold ethanol. The turbid suspension was left standing at room temperature for 20 minutes, centrifuged and washed twice with 70 per cent ethanol (1 molal in CsCl). The RNA was then dissolved in the solvent appropriate to the experiment in question.

CsCl density gradient centrifugation in a bucket rotor. Solutions of DNA and RNA in 7.9 - 8.4 molal CsCl were centrifuged in SW 39 rotors at 35,000 rpm. 3 ml of CsCl solution were placed in 5 ml lusteroid centrifuge tubes and overlaid with 2 ml of light liquid paraffin. For the experiments shown in Figures 4 and 5, temperature was controlled (in a Spinco model E analytical ultracentrifuge) within $\pm 0.1^\circ\text{C}$. For the experiments shown in Figures 2 and 3, the temperature was near 0°C, but not measured. Centrifuge runs were terminated after 65 to 90 hours, and fractions were collected in a manner similar to that described elsewhere.¹⁷ Each fraction was assayed for acid-precipitable counts and absorbance at 260 m μ .

Analytical methods: Acid precipitable RNA-P³² was determined as described previously. Acid precipitable DNA-H³ was determined as follows: nucleic acids, together with exactly 2 mg carrier yeast RNA were precipitated in 5 per cent TCA, centrifuged, and the supernatant fluid

* Abbreviations: C-RNA, complementary (biosynthetic) RNA; T2-C-RNA, complementary RNA synthesized on a bacteriophage T2 DNA template; DNA-C-RNA, a complex between DNA and complementary RNA; CTAB, cetyltrimethylammonium bromide; TCA, trichloroacetic acid; G, guanine; C, cytosine; Tris, tris-(hydroxymethyl)-aminomethane; A₂₆₀, absorbance at 260 m μ ; s, svedberg unit.

removed. The residue was dissolved in one drop of concentrated NH_4OH , quantitatively transferred to a planchet, dried and measured in a windowless gas flow counter.

Properties of RNA synthesized on a phage T2 DNA template. The ultraviolet absorption spectrum of one preparation of T2-C-RNA prepared as above is shown in Figure 1. It has λ_{max} at $258 \text{ m}\mu$ and λ_{min} at $232 \text{ m}\mu$. This material is macromolecular and very heterogeneous, having an average sedimentation constant $S_{20,w}$ of 5.5s, measured at $c = 0.0017$ per cent in 0.1 M NaCl, 0.01 M Tris, pH 7. (By comparison the "messenger" RNA of *E. coli* has a sedimentation

Figure 1. U. V. absorption spectrum of T2-C-RNA in 0.01 M Tris, pH 7. Absorbances measured in 0.7 ml capacity cuvettes on Beckman DU spectrophotometer.

constant of 8s.¹⁸) Accordingly, whatever the molecular configuration of C-RNA, this sample must have a much lower average molecular weight than the T2 phage DNA used as a template. We do not know whether this reflects an intrinsic property of RNA polymerase acting *in vitro*, or whether it is merely due to degradation by enzymes contaminating the partially purified RNA polymerase.

RESULTS AND DISCUSSION

(1) Does C-RNA remain attached to the DNA template? The answer to this question is given by two experiments which show that labeled RNA and DNA are almost completely separated from each other in a CsCl density gradient. In Figure 2, the CsCl gradient centrifugation of the nucleic acids extracted from a T2-C-RNA- P^{32} synthesis is analyzed. It is evident that RNA- P^{32} has accumulated at the bottom of the centrifuge tube and that only a small peak of RNA- P^{32} (less than 3 per cent of the total) can be detected near that portion of the solution which, judged by absorbance measurements, contains the DNA. Figure 3 shows the corresponding experiment in which unlabeled C-RNA has been synthesized on a template of phage T2 DNA- H^3 . As in Figure 2, the peak of optical density at the bottom of the tube is due to C-RNA, but there is no sign of a complex with tritiated DNA. The two experiments show that the two nucleic acids are almost completely separated from each other. At present, it is not possible to decide whether the small fraction of C-RNA associated with the DNA band represents C-RNA

Figure 2. Distribution of P^{32} -labeled T2-C-RNA and template T2-DNA in a CsCl density gradient. The volume is calculated from the bottom of the centrifuge tube. For the absorbance measurements, fractions 1 and 2 were combined, and fraction 3 was not measured.

Preparation of RNA and DNA: Method A (see methods).

Centrifugation: 35,000 rpm, 96 hours, near 0°C , 8.4 molal CsCl.

Analysis: Each fraction is diluted to 0.8 ml and the absorbance measured at 260 $m\mu$. An aliquot is then taken for measurement of TCA-insoluble radioactivity. The concentration of 260 $m\mu$ absorbing material and label in the original fraction is calculated from the known volumes, and shown as A_{260} and cpm/ml respectively.

trapped on the DNA template or whether it is simply due to incomplete disaggregation at some stage of the preparation.

Only two steps have intervened between the RNA synthesis and the gradient centrifugation: phenol extraction at 25°C , and precipitation with ethanol or CTAB. As used here, neither step involves even slightly denaturing conditions. Thus we can say with certainty that if a complex between the bulk of the C-RNA and its DNA template has been disrupted, it is either one which is very much more labile than the DNA double helix, or else it involves a third component (e.g., protein) which is removed during the phenol extraction and nucleic acid precipitation.

Figure 3. Distribution of unlabeled T2-C-RNA and template T2-DNA-H³ in a CsCl density gradient. Preparation of nucleic acids, centrifugation and analysis as described in Figure 2.

(2) Can DNA-C-RNA complexes be formed? We have closely followed the elegant method of Hall and Spiegelman¹⁰ in answering this question. Figure 4a shows that when T2 DNA and T2-C-RNA are heat-denatured and then "annealed" at 40°C, DNA-C-RNA complexes do form. On the other hand, when the heat-denatured DNA and C-RNA are maintained at "quenching" conditions, almost no DNA-C-RNA complex forms. In other words, the concentration of nucleic acids in this experiment is evidently not high enough to give non-specific aggregates that are stable to gradient centrifugation (Figure 4b).

The complex formation shown in Figure 4a is remarkable. At the concentration of RNA (approx. 100 γ /ml) and DNA (approx. 200 γ /ml) employed in the denaturation and annealing, DNA-RNA complex formation occurs to the complete exclusion of DNA renaturation. That is,

Figure 4. Conditions for the formation of DNA-C-RNA complexes.

Figure 4a. Distribution of a heated and annealed mixture of p^{32} -labeled T2-C-RNA and T2-DNA in a CsCl density gradient.

x: A_{260}
o: cpm

Preparation of RNA: Method B

Denaturation and annealing: 0.07 μ moles T2-C-RNA-P and 0.1 μ moles T2-DNA-P in 0.22 ml 2 M CsCl, are heated to 100°C for 10 minutes, quenched and equilibrated 12 hours at 40.1°C. The mixture is then made up to 3 ml of 8.4 molal CsCl, 0.01 M Tris, pH 7.

Centrifugation: 35,600 rpm, 75 hours, 25°C.

Analysis: as in Figure 2.

Figure 4b. Distribution of a heated and quenched mixture of p^{32} -labeled T2-C-RNA and T2 DNA.

x: A_{260}
o: concentration of P^{32} (cpm/ml)

Denaturation and quenching: 0.07 μ moles T2-C-RNA and 0.1 μ moles T2-DNA-P in 0.22 ml 2 M CsCl, heated to 100°C for 10 minutes, quenched and maintained at 0°C for 12 hours. The mixture is then made up to 3 ml of 8.4 molal CsCl, 0.01 M Tris pH 7.

Centrifugation and analysis: as in Figure 4a.

in the competition between the two types of equilibria

and

the former is greatly favored. Upon CsCl gradient centrifugation the entire DNA band is vacated and a new, considerably denser, band due to DNA-C-RNA complexes appears.

(3) Is the DNA-C-RNA complex specific? T2-C-RNA has been isolated by density gradient centrifugation and separately annealed with denatured T2 DNA (35 mole per cent GC), sea urchin (*Strongylocentrotus*) DNA (38 mole per cent GC) and *E. coli* DNA (51 mole per cent GC). In this experiment, both DNA (approx. 90 γ /ml) and RNA (ca. 10 γ /ml) were more dilute in the annealing mixture so that complexation was less extensive than observed in the previous experiment.

The results are shown in Figure 5. T2 and sea urchin DNA are very close in average base composition. Nevertheless, under conditions in which complex formation between T2 DNA and T2-C-RNA is clearly demonstrable, none occurs between sea urchin or *E. coli* DNA and T2-C-RNA. In fact, no interaction between T2-C-RNA and the heterologous sea urchin DNA occurs at the higher concentration of the experiment discussed in the previous section, and shown in Figure 4 (data are not presented here).

(4) Is the DNA-C-RNA complex unique? It appears from the experiments shown in Figures 4a and 5a that the density of DNA-C-RNA complexes depends on the experimental conditions of the denaturation and annealing, and specifically, in these two experiments, on the concentrations of both components. When complex formation is incomplete, the DNA-C-RNA is only slightly heavier than DNA. When complex formation is very extensive, the density of DNA-C-RNA complex (or complexes) is greatly increased. The evidence of these experiments and others not recorded here is that the composition of DNA-C-RNA may vary over a wide range. Undoubtedly this is, at least in part, due to the disparity between the molecular weights of T2 DNA and the T2-C-RNA synthesized by the partially purified RNA polymerase.

(5) Does RNA polymerase priming cause DNA denaturation? Figure 6 shows a comparison of the buoyant densities of unreacted phage T2 DNA and of T2 DNA which has been used as template in a C-RNA synthesis. There appears to be no change of DNA buoyant density as a result of the RNA synthesis. In the light of these experiments, this result is capable of only two interpretations: (1) phage DNA is not irreversibly denatured by its participation as a C-RNA template, or (2) only a small number of the phage DNA molecules act as C-RNA template, and this experiment has failed to demonstrate the behavior of this small fraction. The second alternative is rejected on the grounds that the maximum quantity of T2-DNA having changed density (i.e., having been denatured), which could escape detection is not more than 10 per cent of the total. If, however, only 10 per cent of the molecules of the DNA preparation were active as primer, this could only have come about by 90 per cent primer inactivation during isolation: the inactivation could not entail denaturation. This seems unreasonable, since all the T2 DNA preparations used by us have had comparable priming activities, and compare favorably with DNA primers derived from other sources and isolated in many different ways. Furthermore, the RNA synthesis for this experiment was carried out at a DNA concentration lower than, and enzyme concentration close to, saturation. All of these considerations militate against the possibility that less than 10 per cent of the DNA molecules prime all of the C-RNA synthesis while the bulk of the DNA remains inactive.

COMMENTS

At this point, the relationship between C-RNA and its DNA template has been determined at several levels of structure. It has been shown previously that the average composition of C-RNA's and their DNA primers, correspond,^{1,19,20} and that the nearest neighbor frequencies of four different C-RNA's and their DNA primers, also correspond.² These two experiments suggest, but by no means prove, that C-RNA is a faithful copy of its DNA template. There is a tendency for many of the nearest neighbor frequencies to follow patterns consistent with the average base composition of the DNA, so that the differences between DNA's of comparable average nucleotide composition, while evident, are by no means distinctive (ref. 21 Tables VI, VII, and VIII). Only when longer nucleotide sequences are investigated, do species specificities become

Figure 5. The specificity of DNA-C-RNA complexes. Distribution of heated and annealed mixtures of P^{32} -labeled T2-C-RNA with

Figure 5a: T2-DNA (35 mole per cent GC)

Figure 5b: sea urchin DNA (38 mole per cent GC)

Figure 5c: *E. coli* DNA (51 mole per cent GC)

in a CsCl density gradient.

x: A_{260}
o: cpm/ml

Preparation of RNA: Method B

Denaturation and annealing: a) 0.02 μ moles T2-C-RNA-P, 0.12 μ moles T2-DNA-P; b) 0.02 μ moles T2-C-RNA-P, 0.10 μ moles sea urchin DNA-P; c) 0.02 μ moles T2-C-RNA-P, 0.10 μ moles *E. coli* DNA-P; each in 0.35 ml of 0.6 M CsCl. Samples are heated in boiling water for 10 minutes, quenched, and equilibrated 10 hours at 37°C. Each mixture is then made up to 3 ml of 8.4 molal CsCl, 0.01 M Tris pH 7. Centrifugation: 35,600 rpm; 77 hours; 25°C; 0.01 M Tris.

Figure 6. The CsCl buoyant density of template T2 DNA and T2 DNA which has not taken part in RNA synthesis. *Ps. fluorescens* DNA is used as a density marker. Data are presented in terms of densitometric traces of absorption camera pictures through the region of the DNA "bands." The vertical arrow shows the anticipated location of denatured T2 DNA. The buoyant densities of T2 and *Pseudomonas* DNA are taken from published data.^{27,28}
Centrifugation: 7.7 molal CsCl, 0.01 M Tris pH 8, 44,700 rpm, 25°C, 24 hours. 0.60 ml solution are contained in a 12-mm Kel-F cell equipped with a 1° negative prismatic window.

more clear-cut.^{10,22} In these experiments, therefore, the ability of template T2 DNA to form specific complexes with T2-C-RNA is a test of the presence of entire nucleotide sequences in DNA and C-RNA chains which are simultaneously capable of binding to each other in the face of the competing tendency of complementary nucleotide sequences on DNA strands to recombine.

We have evidence also, that every strand of a T2 DNA sample is capable of forming such complexes, since in one experiment (Figure 4a) we entirely eliminated material banding at the density of DNA. The simplest interpretation of this finding is that C-RNA molecules exist which are complementary to both strands of T2 DNA, and that both DNA strands act as template, either separately or together.*

We turn next to a consideration of the manner in which the DNA is utilized in the C-RNA synthesis. Others have shown, as have we, that a double helix in which only a small portion of the nucleotide sequence is maintained in register at all times, can rewind in such a manner as

* One other, less plausible possibility exists which, however, cannot be excluded. It is that the DNA-C-RNA complexes of Figure 4a consist of networks, each involving many DNA and RNA chains. In such a network DNA-C-RNA complexes might involve one type of DNA strand only, while the complementary DNA strand is carried along solely because of its ability to form intermittent DNA-DNA complexes.

to obliterate, for the purposes of the density gradient experiment, all traces of its unfolding.²³⁻²⁵ In order to account for the unchanged buoyant density of template DNA (Figure 6) we must therefore assume that DNA double helices do not have to unwind entirely in serving as RNA templates. In addition, we know from the annealing experiments (Figure 4a) that DNA-C-RNA complexes involving entire C-RNA molecules must be very stable. The clear separation of T2-C-RNA from its template argues against any C-RNA synthetic mechanism involving such intermediates. We are, therefore, left with two alternatives: (1) the template for RNA synthesis is a single polynucleotide strand of DNA, of which only the small region immediately surrounding the site of terminal nucleotide addition is unwound, or (2) the double helix may act as a template without unwinding.

The similarity of the RNA and DNA syntheses on a DNA template is striking. However, in this study a difference of great importance can be observed. While the *in vitro* DNA template synthesis involves recombination of template and product polynucleotide,²⁶ the *in vitro* synthesized C-RNA is free of its template. Finally, let us suppose that the mechanism of the *in vivo* formation of messenger RNA is the same as that of C-RNA *in vitro*. In that case, it seems probable that as the assembly of the RNA is completed, the two nucleic acids part, and the DNA remains in, or spontaneously returns to, its double helical form.

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THE INCORPORATION OF 5-RIBOSYLURACIL TRIPHOSPHATE INTO RNA IN NUCLEAR EXTRACTS OF MAMMALIAN CELLS*

By

I. H. Goldberg[†] and M. Rabinowitz

Previous reports have described the enzymatic formation of 5-ribosyluracil triphosphate (pseudouridine triphosphate, Ψ UTP)¹ and its utilization in the synthesis of 5-ribosyluracil diphosphoglycosyl compounds.² Mention has also been made of the incorporation of P^{32} from proximally labeled Ψ UTP^{32†} into the interribonucleotide linkage of RNA¹ by the DNA-dependent and four nucleoside triphosphate requiring system derived from nuclear extracts of HeLa cells.³ This report compares the utilization of UTP and Ψ UTP in this reaction and presents evidence of partial specificity in their incorporation into RNA.

Ψ UTP can substitute completely for UTP in the incorporation of label from CTP³² into RNA (Table 1). In addition, Ψ UTP³² is incorporated into RNA as efficiently as UTP³². Treatment of the labeled RNA with purified snake venom diesterase⁴ resulted in the liberation of 5'- Ψ UMP³² as the only labeled nucleotide, indicating that the P^{32} was carried into the polynucleotide as the 5-ribosyluracil nucleotide without prior conversion or transfer. The presence of equimolar concentrations of both unlabeled UTP and Ψ UTP in the reaction did not lead to any

Table 1

COMPARISON OF UTILIZATION OF UTP AND Ψ UTP IN RNA SYNTHESIS

Labeled substrate	Unlabeled nucleoside triphosphate	Labeled substrate incorporated into RNA (m. μ moles)
CTP ³²	UTP, GTP, ATP	0.885
CTP ³²	Ψ UTP, GTP, ATP	0.823
CTP ³²	GTP, ATP	0.018
UTP ³²	CTP, GTP, ATP	0.880
Ψ UTP ³²	CTP, GTP, ATP	0.829
UTP ³² or Ψ UTP ³²	CTP, ATP	0.012

The reaction mixture contained 3 μ moles of $MnCl_2$, 100 μ moles of Tris buffer (pH 8.1), 20 μ moles of NaF, 0.8 μ moles each of the indicated unlabeled nucleoside triphosphates, 0.1 μ moles of labeled nucleoside triphosphate (12.7×10^6 counts/min/ μ mole), 0.1 ml of saturated $(NH_4)_2SO_4$ (4°), pH 8.0, and HeLa aggregate enzyme containing 2.5 mg of protein. The final volume was 1.0 ml and incubation was for 15' at 37°. The enzyme preparation and assay procedures for incorporation of label into RNA were as described.³

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[‡] All P^{32} labeled nucleoside triphosphates referred to in this paper are proximally labeled.

increase in incorporation of CTP³² over that found when only one of the isomers was included in the reaction mixture. It would appear that in the absence of the other, either one of these two nucleoside triphosphates can satisfy the fourth nucleotide requirement of the system. Furthermore, Lineweaver-Burk plots demonstrate that one is able to act as a competitive inhibitor of the other. The K_M for Ψ UTP is approximately 5×10^{-5} and for UTP, approximately 4×10^{-5} . The K_I for Ψ UTP is about 3×10^{-5} and for UTP is about 1×10^{-4} .

The above data fail to demonstrate any significant qualitative differences in the utilization of Ψ UTP and UTP in RNA synthesis. Similarly, the ability of 5-ribosyluracil triphosphate to form diphosphoglycosyl derivatives which can undergo 5-ribosyluridyl transfer reactions² as well as glucosyl transfer reactions to form glycogen[†] does not distinguish an analogue role for the 5-ribosyluracil compounds.

However, studies on the labeling pattern obtained by alkaline hydrolysis of the isolated P³²-labeled RNA have revealed definite qualitative differences in utilization of the two nucleoside triphosphates. Alkaline hydrolysis of the RNA results in the transfer of the P³² from the incorporating nucleotide to its 3'-linked neighboring nucleoside. When either Ψ UTP³² or UTP³² are used for incorporation into RNA in the absence of its unlabeled isomer, the ratios of the 2'(3') nucleotides produced by alkaline hydrolysis are remarkably similar (Table 2). However, the inclusion of unlabeled isomeric nucleoside triphosphate in the enzymatic reaction leads to signif-

Table 2
RATIO OF LABEL IN ALKALINE HYDROLYZED PRODUCTS OF P³²-RNA

Labeled nucleotide μ moles/ml	Unlabeled nucleotide μ moles/ml	2'(3') Nucleotides			
		UMP Ψ UMP	GMP	AMP	CMP
Ψ UTP ³² (0.1)	None	1.59	0.99	1.00	1.28
	UTP (0.1)	1.14	1.01	1.00	1.03
	UTP (1.0)	0.95	1.18	1.00	0.65
UTP ³² (0.1)	None	1.53	0.93	1.00	1.23
	Ψ UTP (0.1)	1.99	0.68	1.00	1.65
	Ψ UTP (1.0)	2.84	0.64	1.00	2.07

The reaction conditions were the same as used in Table 1 except that the specific activity of the labeled nucleoside triphosphate was 40×10^6 counts/min/ μ mole; 0.8 μ moles each of ATP, GTP and CTP were used, and unlabeled UTP or Ψ UTP was added as indicated. The labeled RNA was isolated in the same way, redissolved in 2 ml of 0.01 N NaOH to which 0.2 ml of 20% potassium acetate, pH 5 was then added. The RNA was reprecipitated with two volumes of ethanol in the cold and redissolved in 1.0 ml of 0.3 N KOH for incubation at 37° for 18 hours. The solution was acidified with 35% HC10₄ to pH 1 and the acid-soluble fraction neutralized and subjected to paper electrophoresis in 0.025 M citrate, pH 3.5. The separated 2'(3') mononucleotides were eluted and counted in a gas-flow counter. The counts associated with each 2'(3') mononucleotide are compared with 2'(3') AMP taken as 1.00.

[†] Unpublished experiments.

icant changes in these ratios. The changes are of rather large magnitude, dependent upon the concentration of the unlabeled isomer, and in opposite directions for the two isomers. If no preference were expressed for special sites in the synthesized RNA for UTP or Ψ UTP, one would not expect to find any alteration in the ratio of 2'(3') nucleotides from what is found with either labeled nucleoside triphosphate in the absence of unlabeled isomer. The fact that these changes are in opposite directions indicate that they are probably not due to non-specific effects. Evidence against the utilization of different templates for these reactions is provided by experiments using CTP^{32} or ATP^{32} with different concentrations of unlabeled Ψ UTP and UTP. In such experiments no ratio changes could be found in the alkali released 2'(3')-labeled nucleotides.

The 2'(3') uridylic and the 2'(3') 5-ribosyluridylic acids are not separated by paper electrophoresis in 0.025 M citrate, pH 3.5 but can be by subsequent paper chromatography in ethanol - 0.5 M ammonium acetate (5:2 v/v), pH 3.8. When ΨUTP^{32} and no unlabeled UTP was included in the enzymatic reaction, 96 per cent of the radioactivity associated with the 2'(3') "uridylic" area on paper electrophoresis was shown to be due to 2'(3') 5-ribosyluridylic acid on paper chromatography. When the concentration of UTP was increased to ten times that of ΨUTP^{32} , 90 per cent of the label associated with the uridylic area after alkaline hydrolysis was present as 2'(3') UMP³². The reverse situation pertained when UTP^{32} , with and without unlabeled Ψ UTP, was used in the enzymatic reaction. The decrease in incorporation of label into RNA is less than 40 per cent at equimolar concentrations of labeled nucleoside triphosphate and its unlabeled isomer and 70-80 per cent at ten times concentration of isomer.

These data imply that some degree of discrimination between UTP and Ψ UTP exists when both are available to the enzyme and the DNA template. When only one is available for RNA synthesis, it can satisfy the requirement for both. The nature of the RNA formed in these reactions, as well as the significance of the described labeling differences, remain to be elucidated.

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INHIBITION OF DNA-DEPENDENT RNA SYNTHESIS BY ACTINOMYCIN D IN MAMMALIAN CELL EXTRACTS*

By

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Actinomycin D, one of the most potent anticancer agents so far discovered, exerts a profound influence on cellular nucleic acid.¹⁻³ Actinomycin C forms complexes with DNA, and to a much lesser extent with RNA, similar to those formed by the acridine dyes with nucleic acids.⁴ It is by such complex formation that the antibiotic is thought to act. Although actinomycin is an inhibitor of bacterial DNA polymerase,⁵ in the bacterial or mammalian cell DNA synthesis appears to be unaffected by the antibiotic.^{6,7} However, Reich *et al.* have found a specific irreversible suppression of RNA formation in L-cells exposed to this compound.⁷ Furthermore, their studies on mammalian cells infected with either RNA or DNA virus indicate that actinomycin D interferes with that RNA synthesis which is dependent on either cellular or viral DNA. The present report on the effect of actinomycin D on the cell-free synthesis of RNA supports such a mechanism.

An RNA-synthesizing system dependent on DNA and requiring the presence of all four ribonucleoside triphosphates has been described in nuclear extracts of HeLa cells.⁸ The enzymatically active component is composed of protein tightly bound with DNA and smaller amounts of RNA. This "aggregate" is capable of incorporating P³² from ribonucleoside triphosphate labeled in its innermost phosphate into RNA. In the experiments reported here proximally labeled UTP³² was used as the carrier of radioactive uridylic acid into RNA. The HeLa "aggregate" preparation, as well as the method of assay for RNA synthesis, are as previously described.⁸

Small amounts of actinomycin D produced a striking inhibition of the incorporation of label from UTP³² into RNA (Figure 1A). A similar curve of inhibition was obtained with P³²-labeled 5-ribosyluracil triphosphate.⁹ Actinomycin D was an effective inhibitor in this system at considerably lower concentrations than reported for the DNA polymerase.⁵ Mitomycin (100 γ /ml) and Vancomycin (200 γ /ml) had no effect on the RNA-synthesizing system. With the same amount of "aggregate" with which 2 γ /ml of actinomycin D produced a 51 per cent inhibition of incorporation of label into RNA, proflavine (200 γ /ml) caused a 35 per cent and acriflavine (200 γ /ml) a 69 per cent decrease in incorporation. In these experiments the RNA was isolated by repeated acid washes, since in the presence of high concentrations of acriflavine, ethanol precipitation of RNA was found to be incomplete.

The study of the kinetics of inhibition by actinomycin D is made difficult by the presence of considerable amounts of DNA bound to the HeLa RNA polymerase. It has been possible, however, to study the effect of different concentrations of the antibiotic with varying "aggregate" concentrations (Figure 1B). The degree of inhibition bears an inverse relationship to the concentration of "aggregate" or probably more properly DNA.

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Figure 1A. Inhibition of incorporation of UTP³² into RNA by varying concentrations of actinomycin D. The reaction mixture contained 3 μmoles of MnCl₂; 100 μmoles of Tris buffer (pH 8.1); 20 μmoles of NaF; 0.8 μmoles each of ATP, GTP, and CTP, 0.1 μmoles of UTP³² (2.8 × 10⁶ cpm/μ mole); 0.1 ml of saturated (NH₄)₂SO₄ (4°), pH 8.0; and HeLa "aggregate" containing 1.44 mg of protein, 390 γ of DNA, 144 γ of RNA. The final volume was 1.0 ml and incubation was for 20' at 37°.

Figure 1B. The effect of varying the HeLa "aggregate" concentration on the degree of inhibition of RNA synthesis produced by different concentrations of actinomycin D. The "aggregate" is expressed in terms of both DNA and protein concentrations. Actinomycin D concentrations—Curve #1: 2 γ/ml; #2: 4 γ/ml; #3: 8 γ/ml; #4: 16 γ/ml.

The actinomycin D effect can be reversed by the addition of calf thymus or HeLa DNA to the enzymatic reaction (Figure 2). Heat-denatured DNA, which complexes less readily with the antibiotic,⁵ may act as a substitute for the inactivated natural DNA template. Yeast RNA at about 100 times the concentration of DNA used above, relieved the inhibition only in part.

The inhibition of the HeLa RNA polymerase system by minute amounts of actinomycin D and its reversal by addition of DNA support the mode of action proposed by Reich *et al.*⁷ for this antibiotic in the intact cell.

Figure 2. Reversal of actinomycin D inhibition by addition of calf thymus DNA. Conditions were the same as in Figure 1 except that 2.4 mg protein of HeLa "aggregate" and 4 γ /ml of actinomycin D were used. Absolute incorporation for this amount of "aggregate" with and without the actinomycin D may be obtained from Figure 1B. Heat-denaturation was accomplished by heating a solution of calf thymus DNA (2 mg/ml) in 0.01 M Tris, pH 7.4 and 0.01 M NaCl at 100° for 3' and rapidly cooling in ice.

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IMMEDIATE DECREASE IN RESPIRATORY $C^{14}O_2$ EXCRETION FOLLOWING
SIMULTANEOUS INTRAVENOUS ADMINISTRATION OF $NaHC^{14}O_3$ AND
ACETAZOLEAMIDE IN THE RAT*

By

J. L. Spratt[†] and G. V. LeRoy

Speculating on the metabolic sequelae of carbonic anhydrase inhibition, Berliner and Orloff¹ concluded that the effects of carbonic anhydrase inhibitors on CO_2 elimination would be an immediate, though transient, decrease in CO_2 output and the appearance of a difference between alveolar CO_2 tension and that found in arterial blood. Since that time different authors have reported variable effects of the potent carbonic anhydrase inhibitor, acetazoleamide, on output of respiratory CO_2 .²⁻⁹ The consensus of these various studies is that although acetazoleamide causes an initial hyperventilation that may produce an increased CO_2 output of brief duration, CO_2 output is usually transiently depressed due to inhibition of erythrocyte carbonic anhydrase. The investigation reported here concerns the influence of acetazoleamide on excretion of $C^{14}O_2$ derived from C^{14} -labeled bicarbonate using a device which continuously measures the radioactivity of expired air. Our method allows direct demonstration of Berliner and Orloff's hypothesis in the intact animal, without necessitating recourse to indirect estimates of CO_2 movement based on application of acid-base equations.

METHODS

Female Sprague-Dawley rats weighing 65-85 g were starved overnight prior to experiment. Four experimental animals were injected intravenously with $0.1 \mu c$ of $NaHC^{14}O_3$ (2.39 mC/mmol in 0.1 cc), followed immediately by acetazoleamide-sodium,[‡] 70 mg/kg (in 0.5 cc/100 g body weight). Nearly-simultaneous injection of $NaHC^{14}O_3$ and acetazoleamide-Na (or saline) was accomplished by means of a double syringe with a 3-way stopcock. This procedure allowed essentially simultaneous administration of the two materials with no variation in $NaHC^{14}O_3$ dosage. Four control animals were injected with $NaHC^{14}O_3$ and saline in the same manner.

Similar animals were pretreated with intraperitoneal acetazoleamide (20 mg/kg) at 30 minutes prior to administration of intraperitoneal $NaHC^{14}O_3$ ($5 \mu c/kg$) and their production of respiratory $C^{14}O_2$ likewise studied.

Immediately after injection of the radiotracer each animal was placed in a sealed container attached to a continuous $C^{14}O_2$ monitor. The apparatus, described in detail elsewhere,¹⁰ supplies the animal chamber with a constant flow of CO_2 -free air. The outflow of the chamber

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[‡] Supplied as the sodium salt (Diamox), by the Lederle Laboratories Division, American Cyanamid Co.

passes through a 4 π G-M counter* connected to a scaler,† a rate meter‡ and a graphic recorder.** The graphic data are correlated with the total counts accumulated on the scaler. After corrections for background and counter efficiency, the per cent of dose recovered per unit of time can be calculated.

RESULTS

The results are presented in Figure 1 and Table 1. When $\text{NaHC}^{14}\text{O}_3$ and acetazoleamide (70 mg/kg) were given nearly simultaneously *i.v.*, there was an obvious disparity between the curves for treated and control animals (Figure 1). The difference between the rate curves at 5

Figure 1. Rate of excretion of C^{14}O_2 in expired air following intravenous injection of $\text{NaHC}^{14}\text{O}_3$. Experimental animals (broken line) were administered acetazoleamide, 70 mg/kg, simultaneously with bicarbonate. Points on smoothed curves represent means $\pm$ standard deviations.

*Model 4P5, Instrument and Development Products Co., Chicago, Illinois.

†Model 162A, Atomic Instrument Co., Cambridge, Massachusetts.

‡Model 1620A, Nuclear Chicago Corp., Chicago, Illinois.

**Recti-riter, Texas Instruments, Inc., Houston, Texas.

Table 1

ACETAZOLEAMIDE AND RESPIRATORY CO₂ EXCRETIONCumulated per cent of injected dose. Data expressed as mean $\pm$ mean deviation.*

Time after NaHC ¹⁴ O ₃ inj., min.	Intraper. NaHC ¹⁴ O ₃		Intrav. NaHC ¹⁴ O ₃	
	Saline control (3)	Acetazoleamide, 20 mg/kg I.P. (4)	Saline control (4)	Acetazoleamide, 70 mg/kg I.V. (4)
5	6.12 $\pm$ 1.32	6.80 $\pm$ 1.48	13.78 $\pm$ 1.80	7.69 $\pm$ 0.50
10	21.61 $\pm$ 3.46	20.50 $\pm$ 0.68	30.92 $\pm$ 3.74	23.49 $\pm$ 1.48
15	32.04 $\pm$ 5.19	31.56 $\pm$ 1.95	39.57 $\pm$ 5.10	34.46 $\pm$ 2.70
30	45.06 $\pm$ 7.39	46.96 $\pm$ 5.79	50.46 $\pm$ 5.95	51.21 $\pm$ 5.05

* No. of animals indicated in parentheses.

minutes was highly significant ($p < 0.01$). Similarly, the difference between the cumulated curves at 10 minutes was significant at $p < 0.05$. By 15 minutes the marked changes had subsided.

DISCUSSION

These experiments indicate that the effect of a potent carbonic anhydrase inhibitor on excretion of carbon dioxide can be demonstrated when such an inhibitor, acetazoleamide, and bicarbonate are injected simultaneously intravenously. When the drug is administered 30 minutes prior to injection of the source of tracer C¹⁴O₂, the inhibitory effect on expired CO₂, although evident, is not significant. The inconclusive result may be due to the large variance of the experimental values (Table 1); or may be the consequence of establishment of a new equilibrium among the different forms of carbon dioxide in blood and tissues. The discrepancies between the 30-minute pretreated and the simultaneously treated acetazoleamide animals are consistent with Mithoefer's observations in the dog,⁸ that the primary effect of acetazoleamide on respiratory CO₂ excretion is manifest very quickly and subsides in 20 minutes.

SUMMARY

The effect of acetazoleamide on rate of expiration of carbon dioxide was studied in rats using carbon-14 labeled bicarbonate. A marked transient reduction in expired C¹⁴O₂ was observed during the first 10 minutes after simultaneous intravenous injection of the drug (70 mg/kg) and NaHC¹⁴O₃. The rate of appearance of C¹⁴O₂ was reduced $35 \pm 7\%$ in the treated animals 5 minutes after injection. When the animals were pretreated (30 minutes) with acetazoleamide (20 mg/kg) and when the labeled NaHC¹⁴O₃ entered the blood slowly as a result of intraperitoneal injection, the reduction in rate of C¹⁴O₂ elimination was not nearly as marked.

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EFFECT OF THYROID HORMONE ON THE PATHWAYS OF GLUCOSE OXIDATION IN THE INTACT RAT*

By

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An increase in metabolic activity of the phosphogluconate pathway occurs when human erythrocytes are incubated with physiological concentrations of triiodothyronine.¹ This raises the possibility that thyroid hormone may stimulate the phosphogluconate pathway *in vivo*. Such a mechanism, if operative *in vivo*, would explain the uncoupling of oxidation from phosphorylation observed in the thyrotoxic animal, since increased metabolism via this pathway is accompanied by the reduction of triphosphopyridine nucleotide (TPN), the reoxidation of which does not appear to be closely linked with phosphorylation.² Thus it seems important to evaluate the activity of this pathway of carbohydrate metabolism in the intact animal, both when an excess and a deficit of the thyroid hormone is present.

A widely accepted, although frequently criticized,^{3,4} method for such an investigation is the measurement of the oxidation of the 1-carbon versus the 6-carbon of glucose by the monitoring of expired $C^{14}O_2$ following injection of specifically labeled glucose.

METHOD

Young (100 g) Wistar littermate female rats were either injected subcutaneously with 250 μ g 3,3',5'-triiodo-L-thyronine (Sigma Chemical Co.) daily for 7 days, or had the thyroid surgically removed 7-10 days prior to the experiment. Oxygen consumption of animals in both experimental groups and in an untreated control group was measured⁵ prior to injection of radioactive glucose. Since the average weight of rats in the three groups remained essentially similar (differences were not statistically significant) thyroidectomy apparently did not alter the food intake and cannot be considered to influence the results.

One and five-tenths microcuries of specifically-labeled radioactive glucose (specific activity: glucose-1- C^{14} , 2.8 μ c/mg; glucose-6- C^{14} , 2.0 μ c/mg) (Volk Radiochemicals, Chicago) per 100 g rat was injected intravenously by the dorsal tail vein and the expired $C^{14}O_2$ radioactivity was continuously measured by a flow counter with associated graphic recording apparatus.^{††} The resulting rate curves were manually integrated and the data are expressed as per cent of injected dose.

* This report is taken from a paper that will appear in Proc. Soc. Exptl. Biol. Med.

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^{††} Spratt, J., and G. V. LeRoy. Manuscript in preparation.

RESULTS AND DISCUSSION

Oxygen consumption, in milliliters O_2 consumed per minute per gram total body weight ($\pm$ SE), is as follows for the 3 groups; hyperthyroid (4 rats), 0.049 ± 0.005 ; euthyroid (6 rats), 0.026 ± 0.001 ; hypothyroid (6 rats), 0.021 ± 0.004 .

Results from the tracer study, calculated as per cent of injected glucose- C^{14} appearing as $C^{14}O_2$, are presented in Figure 1. Counting error was less than 5 per cent at all ranges tabulated. In the hyperthyroid animals, rate of oxidation of both the glucose-1- C^{14} and glucose-6- C^{14} is increased (3.4- and 3.7-fold respectively); there being no significant difference between the two labeled positions. In the thyroidectomized rats, however, rate of oxidation of glucose-1- C^{14} was decreased to a much greater extent than that of glucose-6- C^{14} (0.50- versus 0.75-fold respectively).

Figure 1. Cumulative $C^{14}O_2$ following injection of specifically-labeled glucose- C^{14} . x represents individual determinations.

Similar experiments carried out by Spiro and Ball⁶ dealt only with changes in rate of oxidation in the hyperthyroid animals and they concluded that in this subject both phosphogluconate and glycolytic pathways of glucose metabolism were stimulated. Our observations demonstrate that in addition the pathway for oxidation of glucose-1-C¹⁴ appears to be preferentially depressed.

We therefore suggest that the thyroid hormone(s) in some manner control the rate of metabolism via the phosphogluconate pathway which, in the euthyroid rat, is apparently operating at a maximum rate. It has been demonstrated repeatedly^{7,8} that administration of thyroid hormones increases the apparent activity of glucose-6-phosphate and phosphogluconate dehydrogenases. Such effects might explain the data reported here, yet it appears unlikely that this is the whole explanation since our own experiments* and those of others⁷ demonstrate that the thyroid hormones do not increase the in vitro activity of the purified (isolated) enzymes. However, in the case of erythrocytes, the stimulating effect of the hormones can be demonstrated in vitro. Perhaps the effect of the thyroid hormones on the phosphogluconate pathway is a more indirect one.

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ON THE MECHANISM OF KERATIN FORMATION*

By

J. DeBersaques[†] and S. Rothman

It has been suggested that in keratinizing structures cellular proteins are hydrolyzed to free amino acids with subsequent de novo formation of keratins in the keratogenous zone.¹ This hypothesis was based on the finding that keratinized structures contain large amounts and a great variety of free amino acids but no methionine, cysteine or cystine.^{2,3} The absence of sulfur-containing amino acids in aqueous hair extracts fitted the assumption that all available sulfur-containing amino acids are incorporated into the new protein as cystine, hair having a high cystine content, while the other available amino acids are only partially utilized in synthesis, the rest remaining in the cell or in the cellular debris in form of free amino acids.

In recent years several authors have demonstrated the uptake of sulfur-containing labeled amino acids into the keratogenous zone of hair by autoradiography.⁴⁻⁶ However, the uptake of other amino acids was not tested in a similar fashion. Thus it has remained problematic whether in keratinization there is a simultaneous uptake of different amino acids at the same site suggesting de novo synthesis or whether there is an apposition of sulfur-containing material to preformed fibrous proteins ("tonofibrils") in the final phase of keratin formation. This was assumed mainly on the basis of electronmicroscopic findings.⁷ Nevertheless, even the electronmicroscopic pictures permit the interpretation of "a more or less complete breaking up or dissolving of the tonofibrils"⁸ before keratinization occurs.

Harkness and Bern⁹ on the basis of autoradiographic findings in mice with C¹⁴-labeled protein hydrolysates and C¹⁴-labeled carbonates came to the tentative conclusion that keratin is synthesized de novo in the keratogenous zone of hair. Fleischer, Vidaver and Haurovitz¹⁰ came to a similar view after studying the ratio of uptake of C¹⁴ and S³⁵ into hair of rats from double-labeled proteins. A recent contribution by Downes¹¹ on S³⁵ uptake into the wool of sheep strongly suggests that there is an intermediate metabolic pool between the free cystine in blood plasma and the cystine in wool. He also concludes that cystine is incorporated into hair as a free amino acid.

We hoped to obtain more direct information by administering several labeled amino acids and by studying the time sequence of their uptake into the hair and their incorporation into hair keratins.

Hairs on the backs of 300-500 g female guinea pigs were plucked to induce the anagen or growing phase of the hair cycle. Fourteen days later radioactive labeled amino acids were given intraperitoneally. At different intervals after injection the growing hairs were plucked and the specific radioactivity was estimated with a liquid scintillation counter: (1) in the total hair; (2) in its water-soluble fraction; (3) in its water-insoluble fraction. Experiments with each

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amino acid were repeated on at least 3 different animals. The uptake of leucine was estimated in 7 experiments. The results were uniform throughout.

With tritiated alanine, leucine, and lysine and with C^{14} -labeled serine, the results were similar. The specific activity of the total hair rapidly rose to reach a maximum between 1 and 2 hr after injection followed by a gradual decrease. During this early phase all radioactive material was present in the water-soluble fraction. It was not precipitable with trichloroacetic acid and was dialyzable, indicating small molecular moieties.

The specific radioactivity in the water-insoluble fraction of hair showed a very gradual and slow rise reaching a maximum after 1 to 3 days. At the same time, the radioactive material started to disappear from the water-soluble fraction at about the same rate as it was taken up by keratins (Figure 1).

Figure 1. Time curve of specific activity of the water-soluble part (—) and of the water-insoluble part (-----) of hair after injection of tritiated leucine.

The incorporation of S^{35} -cysteine (6 experiments) showed the same time pattern of incorporation as did that of the non-sulfur-containing amino acids. Uptake into the water-insoluble fraction reached its maximum at the end of the second day. The increment of cysteine-uptake as related to its concentration in the water-soluble fraction, however, was much higher than in the case of the other amino acids. In other words, cysteine was taken up with greater avidity.

When S^{35} -methionine was injected (8 experiments) the uptake of S^{35} into keratins was prolonged up to 5 to 6 days. It could be shown that this prolongation of uptake is due to a slow conversion of methionine into cysteine, prior to its incorporation into hair. That the conversion is

slow was shown by analysis of liver tissue for methionine and cysteine in S^{35} -methionine injected animals.

When tritium-labeled methionine was injected (7 experiments) the tritium appeared in the water-soluble fraction of hair as was the case with the other amino acids. Uptake of radioactivity into hair keratins, however, was minimal or absent, indicating that only the S atom of methionine is incorporated into hair keratins.

The uptake of labeled amino acids into keratins had two suggestive features.

First, the incorporation of all amino acids injected (except methionine) occurred uniformly in time. This suggests that keratins are formed at once *de novo* from free amino acids and that there is no superimposed cystine incorporation into preformed proteins or tonofibrils.

Second, in all experiments disappearance of the radioactive material from the water-soluble fraction and its uptake by keratins were complementary (see Figure 1). This behavior suggests that it is the water-soluble small-molecular material (present probably in the non-keratinized lower portion of hair) from which keratin is built. This water-soluble material in hair may well represent Downes' "intermediate pool," the existence of which he deduced from the shape of his curves of S^{35} -uptake.

We assume that physiologically part of this amino acid pool originates from the breakdown of proteins in matrix cells occurring prior to keratinization in the keratogenous zone.

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ORAL RADIATION DEATH IN MICE*

By

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Irradiation of the head with a dose of about 2500 r is fatal to several species of laboratory animals. This type of death has been called "oral radiation death" by Quastler *et al.*¹ who conclude that the mouth and specifically the lower jaw and tongue, are the radiosensitive targets. Although many of the characteristics of oral radiation death have been described, there are conflicting interpretations of the findings,²⁻⁵ and pathological changes have not been described in detail. The present study on mice was undertaken to investigate the gross and microscopic changes in the irradiated tissues of the head as well as the changes in the other major organs which might be used to indicate the sequence of events and changes leading to death.

MATERIALS AND METHODS

The source of radiation in all experiments was a Maxitron unit operated at 250 kvp and 30 ma with 0.25 mm Cu and 1.0 mm Al added filtration. The dose rate averaged 480 r per minute measured by a Victoreen meter in air at a target distance of 30 cm. Shields of 1/4 inch thick lead tubing were modified so that the jaws of the mouse could be separated by an 1/8 inch thickness of lead plate. A removable neck plate facilitated the placing of the mouse in the shield. With a dose rate of 480 r per minute to the unshielded area, 0.25 r per minute was delivered in the shielded jaw compartment and 5.0 r per minute just inside the opening of the neck shield. Groups of six mice were irradiated on a rotating table, the left side of the mouse being toward the beam in every case.

C57BL/6 male mice were used throughout except in experiments concerned with obese animals where C57BL/6J females were substituted. The mice ranged in age from 8 weeks to 1 year, all animals in any given experiment being of the same age. For all procedures the mice were anesthetized by intraperitoneal injections of Nembutal.

The animals were housed in groups of 6 in plastic cages and water and Rockland Mouse Diet Complete were provided ad libitum for most experiments. Where fluid intake and urine output were to be measured, and in starvation experiments, the animals were housed individually.

Hematocrit was determined in duplicate using the micro-capillary technique. For histological study, tissues were fixed in 10 per cent buffered formalin. All soft tissue specimens were embedded in paraffin and sections were stained with hematoxylin and eosin. The heads were decalcified using a formic acid-sodium citrate mixture and then hemisected in the midsagittal plane. Most head specimens were embedded in celloidin and all sections were stained with hematoxylin and eosin. The presence of ketone bodies was tested by the use of Acetest tablets.

* Summary of a paper that will appear in Radiation Research.

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RESULTS

Irradiation of the entire head. Groups of 6 mice were given radiation to the entire head with doses from 1000 r to 6000 r in increments of 500 r. Survival time averaged 9 days and was not dose dependent between 2000 r and 6000 r.

During the first 5 days postirradiation the weight of the animals fell about 6 per cent (Figure 1). After the fifth day postirradiation the weight decreased rather rapidly until death, at which time there was an average of 39 per cent loss, or 9.2 g per animal.

Figure 1. Weight loss in mice after 2000 r irradiation to the entire head.

Twelve mice were irradiated with 2000 r and water intake and urine output were measured daily. There was a slight increase in water intake until the fifth day postirradiation (Figure 2). On the fifth or sixth day, the intake dropped sharply and after the seventh day it was unmeasurable. The urine output decreased slightly on the third or fourth postirradiation day, after which there was a marked decrease.

Blood and splenic tissue from animals irradiated with 2000 r were cultured according to a method described by Miller *et al.*⁶ Three irradiated animals and a normal control mouse were killed each day from the fourth to the ninth postirradiation days. One irradiated animal had a marked bacteremia on day 6. Cultures from other irradiated animals did not differ from those of normal control mice which grew an occasional bacterial colony.

There was a fairly consistent pattern of change in appearance and activity. After recovery from the anesthetic, the mice resumed activity, and there was no sign of injury or change for the first 5 days postirradiation.

On the sixth and seventh days they moved about the cages more slowly and were slower to

respond when handled. The fur was mildly ruffled and less glossy. The hair on the lower jaw became wet and matted, and this change was sometimes also noted on the inferior surface of the neck and between the forelimbs. When attempting to drink from the water tube, the mice did not use their tongues to lap in the normal manner but instead nudged their lower jaws against the tube opening. The eyes seemed dry and some animals had an opaque, white material in the corner of the lid.

Figure 2. Water intake and urine output in mice after 2000 r irradiation to the entire head.

On the eighth postirradiation day the mice hardly moved when stimulated. They stood in a hunched position and if they did move, they walked in a waddling manner. The fur was very ruffled. Edema of the lower jaw and of the snout was evident and the snout had become red. The eyes were almost closed with a dry, gray-white, material. Much of the fur on the lower jaw and to a lesser extent on the inferior surface of the neck was missing. The mice were very emaciated and often had diarrhea. Some made wheezing or choking sounds but this was not a constant finding. At death they were found hunched over and the mouth appeared to be sealed shut with a clear mucoid material.

Upon opening the mouth, the gingiva around the lower central incisors was often hemorrhagic and shafts of hair were sometimes found driven into the gingival sulcus. The mouth was usually filled with debris. In some animals, muco-purulent exudate formed a membrane at the pharyngeal opening. There were a few ulcerations of the buccal mucosa, particularly opposite the molar teeth. The anterior part of the palate was free from ulceration but the posterior part of the soft palate was ulcerated and covered with a thick exudate. The distal portion of the tongue was covered by an apparently intact mucosa as was the floor of the mouth. On the dorsum

of the tongue in the posterior portion of the intermolar eminence,⁷ there was always an ulcer covered by a thick exudate. The shape of the ulcer followed the outline of the intermolar eminence being roughly triangular with its base at the base of the tongue and the apex pointing towards the tip of the tongue. Six mice irradiated with a sublethal dose of 1500 r and killed 8 and 9 days after irradiation had no evidence of such an ulcer, although there was an occasional bleb in the area of the circumvallate papilla. None of the blebs appeared to be ruptured.

Gross findings at autopsy in animals dying after x-irradiation of the entire head included a small pale spleen, and a small liver. Fat in the abdominal cavity was markedly reduced or absent. The lungs and other tissues appeared normal except for the stomach which was distended by gas but had no fluid or solid contents.

No appreciable histo-pathologic abnormalities were noticed until the third day after irradiation when there was some clumping of the chromatin in the epithelial nuclei of the mucosa of the tongue and soft palate. The marrow in the bones of the skull had a decreased hematopoiesis, and on the fourth day, large open spaces were found. On the fifth day there were fewer cell layers in the epithelium of the tongue and palate, particularly in the posterior regions, and by the seventh day only a layer of keratin remained over the submucosa in the posterior regions. On the eighth day the keratin disappeared leaving an ulcerated area covered by a membranous exudate consisting mainly of leukocytes enmeshed in debris. The epithelium of the rest of the oral mucosa was reduced to one or two layers of cells except for small ulcerated areas opposite the molar teeth.

The oral epithelium in the area of the ostia of the ducts of the submaxillary and sublingual glands was sometimes missing but there was no inflammatory exudate. There were no apparent abnormalities in the serous and mucous glands in the posterior area of the tongue, and no changes in the mucous glands in the submucosa of the soft palate. Behind the ulcerated region of the tongue, the epithelium leading into the pharynx was still intact although the number of cell layers was diminished. The ulcer in the soft palate appeared in an area opposite and a little posterior to the location of the tongue ulcer. The salivary glands, the Harderian glands, and the skeletal muscle had no abnormalities. The skin had been removed from the skull during preparation of the specimens for sectioning and was not examined. The major organs were examined only in selected cases after the seventh postirradiation day. The changes were essentially similar to those found at equivalent days after sub-total irradiation of the head.

Sub-total irradiation of the head. Irradiation of the lower jaw and tongue including the base of the tongue (Figure 3) produced death in the same manner as irradiation of the entire head except that the mean survival time was one to two days longer. A dose of 2000 r to the lower jaw and tongue did not produce 100 per cent mortality as in irradiation of the entire head. Five of 12 animals given this dose died within 14 days after irradiation, while all animals irradiated with 4000 r to 6000 r died. The survival time was not dose dependent. The minimal LD₁₀₀ was not determined. For autopsy and histological studies a dose of 4000 r was used.

During the first 5 days after irradiation the weight of the animals fell by about 4 per cent. After the fifth day there was a rapid fall in weight until death, when the total average loss was 39 per cent, or 10 g per animal. This pattern of weight loss is of the same character as that seen in irradiation to the entire head (Figure 2).

The postirradiation behavior and physical changes shown by the animals were essentially

Figure 3. Ulceration of the tongue 9 days after 4000 r irradiation to the lower jaw and tongue. The arrows indicate the area of ulceration. (Mag. 8X)

those found in mice receiving fatal x-irradiation to the entire head.

Three irradiated animals and one normal control mouse were killed at daily intervals after a dose of 4000 r to the lower jaw and tongue. Nine days after irradiation gross changes in the mouth resembled those in mice dying after lethal irradiation to the entire head (Figure 3). Histological sections revealed the same general progression of changes in the tongue epithelium (Figures 4-6). The skeletal muscle and salivary glands of the lower jaw showed no abnormalities and there were no changes in the shielded tissues of the head.

In animals receiving 4000 r to the lower jaw and tongue there were no changes in the internal organs until the seventh day after irradiation when a decrease in the size of the thymus was noticed. By the ninth day after irradiation the thymus was markedly atrophic, the spleen was small and pale and its weight was half that of the previous day. By 11 and 12 days after irradiation the spleen had lost an average of 84 per cent of its weight.

On the eighth postirradiation day the liver weighed 26 per cent less than an average normal liver. By the twelfth day the average loss was 42 per cent. From the eighth day on the stomach was dilated with gas and had no fluid or solid contents. On the ninth day the adrenals were deep pink in color and appeared enlarged, although the average weights remained normal. By the eighth day after irradiation the perirenal and perigonadal fat had disappeared.

Histological sections revealed vacuolization in the liver appearing on the seventh postirradiation day and becoming severe on the ninth and tenth days. There was a consistent decrease in hepatic cell size evident at 9 days after irradiation. Some adrenals exhibited varying degrees of lipid depletion in the first 5 days. From the sixth to the eighth day after irradiation the

Figure 4. Epithelial change in the tongue and soft palate mucosae 5 days after irradiation of the lower jaw and tongue with 4000 r. The epithelium contains fewer cell layers than the usual but is intact.

adrenals had an increase in lipid content. On the eighth and following days the adrenals again appeared depleted of lipid.

The mesenteric lymph nodes had lymphocytic depletion beginning about the sixth day. The follicles became pale and contained only large lymphoid cells with pale staining nuclei. Moderate amounts of nuclear debris were found in the follicles. By the eighth day after irradiation the sinusoids of the lymph node were nearly depleted of lymphocytes. The thymus remained unchanged until the seventh postirradiation day when there was noticeable paling of the cortex with an increase of nuclear debris. By the ninth day there was almost complete depletion of the cortex.

In most animals the lungs showed no changes. After the eighth day, only 4 animals of a total of 14 irradiated exhibited histologic evidence of minimal bronchopneumonia. Purulent exudate was found in the lumen of the upper portion of the thoracic esophagus beginning 8 days after irradiation. On the ninth day the esophageal lumen was dilated and filled with purulent material. The number of cell layers in the mucosa of the upper part of the esophagus was diminished. On the tenth day the findings were more marked; the mucosa was reduced to 1 or 2 cell layers. These esophageal findings were present in all irradiated animals after the eighth postirradiation day.

Figure 5. Oral mucosa 9 days after irradiation of the tongue and lower jaw with 4000 r. An ulcer, between the arrows, is over the mucous glands in the posterior part of the tongue. There is a small, ruptured bleb in the palatal mucosa. (Mag. 40X)

There were no apparent changes in the kidney parenchyma until the seventh day after irradiation when very fine vacuoles were seen in the cytoplasm of the cells of the proximal convoluted tubules. This was found in 2 of 3 irradiated animals killed on the seventh day and was present in all the irradiated animals on the following days. The vacuoles were found to be larger on each successive day. The glomeruli were mildly congested on the eighth day, markedly congested on the ninth day and ischemic on the tenth and eleventh days after irradiation. The heart showed no apparent histological changes during the course of the experiment.

Spermatogenesis occurred in the testes of all the animals but became sharply reduced after the eighth postirradiation day. The hematocrit (determined on blood from the right ventricle) began to rise on the seventh day after irradiation from a normal average of 45 per cent to an average of 59 per cent on the ninth day after irradiation. Ketone bodies not present normally in the urine of these mice, were found in the urine in small amounts on the sixth day after irradiation. By the seventh and eighth days after irradiation moderate to large amounts were found in the urine.

Irradiation of a group of 6 animals with a dose of 2000 r to the area of the head from the tip of the snout to the orbit including the upper jaw and palate but excluding the lower jaw and tongue, produced no fatalities. There was an average weight loss of 26 per cent on the sixteenth

Figure 6. The anterior border of the ulcer on the posterior dorsum of the tongue. There is a heavy inflammatory membrane extending over the intact epithelium on the right. There is no inflammatory exudate in the musculature of the tongue. (Mag. 230X)

day after irradiation. The postirradiation motor activity of these animals was somewhat reduced. The snout became very edematous and reddened.

Another group of 6 animals was given 2000 r to the posterior portion of the skull between the orbit and the atlas, an area containing the major portion of the brain and the pituitary, but excluding the lower jaw and tongue. There were no fatalities, and none of the animals appeared sick, the maximal weight loss of 10 per cent being reached on the eighteenth day postirradiation.

Irradiation of the lower jaw and the tongue anterior to the intermolar eminence with dosages of from 2000 r to 10,000 r produced a 7 per cent mortality in a total of 84 animals. These deaths occurred 12 to 14 days after irradiation with 4000 r and 6000 r.

Studies in non-irradiated mice. The effect of deprivation of food and/or water on survival time was studied in a number of mice housed individually in wire-floor cages. Those deprived of both food and water had an average survival time of 5 days, and the average weight loss at death was 36 per cent (Figure 7). Deprivation of food alone gave a survival time of 6 days with an average weight loss of 38 per cent.

Some animals were killed for histological study when they were moribund. The spleen was pale and greatly decreased in size, weighing 66 per cent less than normal. The thymus was greatly decreased in size. The amount of abdominal fat was decreased. The livers had vacuolization

Figure 7. Weight loss in mice after 4000 r irradiation to the lower jaw and tongue as compared to the weight loss seen in mice denied both food and water.

and decreased hepatic cell size. Vacuoles were found in the cytoplasm of the cells of the proximal convoluted tubules in the kidney. The sinusoids in the spleen were moderately depleted of red blood cells and the lymphoid follicles were depleted and contained nuclear debris.

Obese mice deprived of food had an average survival time of 18 days. By the third starvation day the presence of small amounts of ketones was noted. Obese mice deprived of both food and water had an average survival time of 6 days. When irradiated so as to produce oral radiation death, these obese mice had an average survival time of 10 days, whether the dosage was 2000 r to the entire head or 4000 r to the lower jaw and tongue.

Glossectomy was performed on a group of twelve animals, the most satisfactory method being by the tightening of a ligature around the tongue. Of these twelve mice, 2 died on the fifth and 8 on the sixth post-operative day. The average weight loss at the time of death was 8.5 g or 35 per cent. The 2 survivors had an average weight loss of 31 per cent. In all the glossectomized animals; the fur on the lower jaw became wet and matted, this condition extending progressively down the neck and sometimes including the area between the forelimbs. In order to drink, the animals nudged their lower jaws against the opening of the water bottle tube so that a considerable amount of water ran down the outer surface of the lower jaw. At death these animals were found to have shafts of hair driven into the gingival sulcus of the lower incisor teeth.

DISCUSSION

Male mice of the C57BL/6 strain die about 9 days after exposure to 2000 r x-irradiation of the entire head or to 4000 r of the lower jaw and tongue. The major radiosensitive area appears to be the posterior portion of the tongue and lower jaw. Other areas of the head could be

irradiated with doses of 2000 r without producing a high death rate. These findings suggest that damage to the brain and pituitary are not major factors leading to death after irradiation of the head with doses in the range of 2000 r to 4000 r. Bronchopneumonia was found only occasionally in animals dying after irradiation of the head. Bacteremia could not be demonstrated in moribund mice 7 to 9 days after irradiation of the entire head. Thus generalized infection does not appear to play an important role in oral radiation death. This observation confirms the findings of Quastler *et al.*¹

In the present investigation ulceration of the tongue and damage to the epithelium of the soft palate was found in all animals dying after irradiation of the entire head or the lower jaw and base of tongue. These findings were not present in animals which had received sublethal doses of irradiation. Lesions of the tongue have been reported by Wensinck and Van Bekkum⁹ in mice which received total-body x-irradiation in dose levels from 575 r to 1200 r. The lesions appeared between 11 and 16 days after irradiation and most often occurred in the region of the circumvallate papilla of the tongue. Microscopically, these were characterized by heavy infection of necrotic tissue. Some of these lesions were deeper in the tongue and not connected with the oral cavity. It seems likely that these lesions are different from the ulcerations that develop after irradiation of only the head or lower jaw and tongue. The lesions described by Wensinck and Van Bekkum are abscesses which develop in animals receiving total-body x-irradiation. The defense mechanisms are greatly depressed in such animals.

Most animals subjected to subtotal glossectomy die 5 to 6 days after the operation. Such animals appear to be unable to drink or eat well. They have a marked weight loss of the degree seen after fatal head irradiation (Figure 7). The posterior portion of the tongue is probably important in bringing food and water into the mouth, transporting food and water to the back of the mouth, and swallowing; ulceration here may seriously interfere with these functions particularly when there is associated damage to the soft palate. Animals dying after x-irradiation to the entire head or to the lower jaw and tongue have many of the characteristics of dehydration and starvation, fluid intake and urine output are decreased, weight loss is marked, hematocrit is increased. Gross and microscopic changes in many organs are similar to those changes found in dehydrated and starved animals. Quastler *et al.*¹ considered that starvation was not a major factor since obese mice which normally survive without food for 21 days, died about 11 days after head irradiation. The possible role of dehydration was apparently not considered. Our studies indicate that obese mice deprived of both food and water do not survive much longer than normal mice under the same conditions.

It appears likely that oral radiation death could be prevented if the mouse could be provided with adequate food and water in a manner which is not dependent upon its ability to ingest. Experiments utilizing gastrostomy tubes, subcutaneous feedings, force feedings, and soft diets, are currently being conducted in an attempt to prevent oral radiation death.

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THE RATE OF DISAPPEARANCE OF LABELED VITAMIN B₁₂ FROM THE PLASMA AFTER INTRAVENOUS INJECTION IN THE RAT*

By

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The rate of disappearance from the plasma of intravenous doses of labeled vitamin B₁₂ has been studied in normal subjects¹⁻⁷ and in patients suffering from a variety of diseases. A markedly reduced rate has been found in chronic myeloid leukemia^{1,2,4} and this has been explained as being due to the partial unsaturation of the greatly increased binding capacity of the plasma for vitamin B₁₂ in this condition. Unsaturation of the plasma-vitamin B₁₂ binding protein also occurs in untreated pernicious anemia and other conditions in which vitamin B₁₂ depletion obtains, and it is not surprising that in these conditions the rate of disappearance of intravenously injected vitamin B₁₂ is lower than in the normal,^{1,3,4,6} though not as low as in chronic myeloid leukemia, in which condition the degree of unsaturation is usually much higher. However, it has also been observed^{4,6} that in patients with pernicious anemia in remission, in whom there was no deficiency of vitamin B₁₂, there is still a somewhat lower rate of disappearance than in the normal, although the binding capacity of the serum is within normal limits. The difference from the normal in the rate of disappearance in adequately treated pernicious anemia cannot therefore be explained by postulating a greater degree of unsaturation of the plasma binding factors and resultingly greater avidity for injected labeled vitamin B₁₂.

There is a possibility that, in pernicious anemia, a factor mediating the exchange of vitamin B₁₂ between tissues and plasma is either absent or present in inadequate amounts. The points which need to be examined are: 1) Is this factor identical with, or closely related to intrinsic factor? 2) If so, is it of gastric origin?

There is no evidence that gastric intrinsic factor is essential for the tissue uptake of vitamin B₁₂ from the plasma, but the possibility of its playing some part in the tissue exchanges of the vitamin in the normal subject has not been excluded.

We decided to examine this possibility in the rat, using normal and gastrectomized animals and tracer doses of Co⁵⁸-labeled vitamin B₁₂ of high specific activity.

MATERIALS AND METHODS

Young female rats of Wistar strain weighing approximately 250 g were used. Gastrectomized animals were prepared by resection of the whole stomach under Nembutal and ether anaesthesia and subsequent oesophago-duodenostomy. One to six weeks after operation such animals and intact control rats were anesthetized with Nembutal by intraperitoneal injection. Thirty minutes

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later the saphenous vein on either side was exposed and an injection of either 0.25 ml isotonic saline containing 2.5 μmg Co^{58} -labeled vitamin B_{12} was given, or a mixture of the same dose of the vitamin, together with sufficient hog or rat intrinsic factor-containing material to bind at least 80 per cent of the vitamin B_{12} , as measured by dialysis for 24 hours at 4°C. The labeled vitamin B_{12} was of high specific activity of the order of 100 μc per μg and its purity had been checked by the makers by assay with Ochromonas malhamensis and Lactobacillus leichmannii and by electrophoresis and isotope dilution.

Earlier work has shown that a marked fall in concentration of vitamin B_{12} in the plasma occurs after gastrectomy in the rat⁸ and animals which had been gastrectomized more than 10 days before being used in these experiments received 200 μg cyanocobalamin intramuscularly five days before the intravenous injection of the tracer dose to ensure that they were not depleted of vitamin B_{12} . Control and gastrectomized animals were fed on a standard Purine rat cake.

Rat gastric juice was obtained from healthy rats as follows. The pylorus was tied off under transient ether anesthesia and the animals then given histamine phosphate by subcutaneous injection in a dose of 40 mg per kg. Four hours later the animals were killed and the contents of the stomachs removed, centrifuged to remove all debris and then dialyzed at 4°C against phosphate buffer of pH 6.8 for 48 hours. An amount of this material equivalent to one ml of gastric juice was capable of restoring vitamin B_{12} absorption to within normal limits when tested in gastrectomized rats. Labeled vitamin B_{12} was added to the dialyzed gastric juice and the mixture dialyzed against an equal volume of isotonic saline at 4°C overnight. By measuring the radioactivity of the mixture and of the fluid outside the sac it was possible to calculate the volume of the mixture containing 2.5 μmg of the vitamin (this never exceeded 1 ml) and the degree of "binding," which was never less than 80 per cent.

The hog intrinsic factor was a purified concentrate kindly supplied by Dr. Leon Ellenbogen of Lederle Research Laboratories. It was known to be active, as judged by tests of vitamin B_{12} absorption in patients with pernicious anemia, in doses of 1 mg. This was made up in phosphate buffer pH 6.8 and the Co^{58} -labeled vitamin was added and the mixture dialyzed against isotonic saline in the same way as the rat material. The dose contained 2.5 μmg of the vitamin, of which more than 80 per cent was "bound" and the amount of intrinsic factor present was of the order of 0.05 mg.

Following intravenous injection of the labeled vitamin, 0.25 ml samples of blood were removed from each rat by cardiac puncture at intervals of from 5 to 300 minutes. The total volume of blood removed from each animal before the end of the experiment was 1.25 ml.

Samples were placed in small plastic tubes of equal size, 0.75 ml water was added, with complete lysis and mixing, and activity counted in a standard well counter. Twenty or 300 minutes after injection the final blood sample (1 ml) was taken and the animals were killed, the whole liver (and the stomach in the case of the controls) being removed. The former was homogenized in water and 1 ml aliquots of the homogenates were counted. The stomachs were packed into flat-bottom tubes and counted whole, since they tend to be too tough to homogenize satisfactorily and were of sufficiently small volume to permit accurate counting in the well counter. Counting time was 10 minutes. Standards prepared from the solutions of Co^{58} -vitamin B_{12} used were counted before and after counting of each batch of samples and background counts were made similarly.

RESULTS

Initial studies showed that the rate of disappearance of activity from the blood in the first 30 minutes after intravenous injection of labeled vitamin B₁₂ is extremely rapid, similar to that occurring in the human subject. Cronkite and his colleagues⁵ have discussed the complexity of the pattern of plasma concentration in this period, in which mixing and exchange with tissues both play a part. Our data do not permit any similar analysis.

All count rates per unit volume have been expressed as percentages of the calculated activity per unit volume at zero time, as has been done in similar studies in the human.⁴ The total number of counts injected was known and the total blood volume was estimated from the data of Belcher and Harriss⁹ for the rat, on the basis of body weight.

Previous studies¹⁰ have shown that, following intravenous injection of labeled vitamin B₁₂, the amount of activity present in the red cells in the first six hours is so small that, when counting whole blood, a considerable error may be introduced by a falling hematocrit due to repeated sampling. For this reason, the volume of each sample was kept to a minimum. Check hematocrits did not show changes greater than 5 per cent.

A comparison of the means of values in control and gastrectomized animals is shown in Figure 1, obtained from the data in Table 1. It is clear that there is no significant difference be-

Figure 1. Rate of disappearance of radioactivity after intravenous injection of 2.5 μ mg Co⁵⁸-vitamin B₁₂.

tween the two groups. For reasons referred to above, the shape of the curves for the period of the first five minutes after injection probably depicts only an approximation to the true concentrations. The comparison, however, of the two groups is not invalidated by this, and it seems that in the gastrectomized rat no factors are operating to delay the transfer of labeled vitamin B₁₂ out of the plasma, though the mode of removal in the gastrectomized rat is not necessarily the same as in the normal animal.

The effects of adding material containing homologous or heterologous intrinsic factor to

Table 1

RATE OF DISAPPEARANCE OF RADIOACTIVITY AFTER INTRAVENOUS
INJECTION OF 2.5 μ mg Co⁵⁸-VITAMIN B₁₂

Counts expressed as mean percentages of calculated count rate at zero time								
Time (minutes)		5	10	15	20	60	150	300
Control animals	Mean %	22.4 (3)	17.0 (3)	13.4 (4)	15.2 (3)	6.1 (7)	4.7 (7)	3.2 (7)
	S.E.	0.57	0.53	1.26	1.5	0.49	0.54	0.26
Gastrectomized animals	Mean %	19.6 (3)	15.9 (3)	10.0 (6)	12.4 (3)	6.2 (8)	4.3 (8)	2.1 (8)
	S.E.	0.58	0.55	0.59	2.24	0.73	0.85	0.19

Numbers of observations in parenthesis.

the intravenous vitamin B₁₂ are shown in Table 2, and Figure 2 has been constructed from these data. Both rat and hog intrinsic factor, in amounts sufficient to bind most of the vitamin given, markedly increase the rate of removal of activity from the plasma. The rate is higher with hog than with rat material. Five minutes after injection of hog intrinsic factor with the vitamin there is less than 5 per cent of the injected dose of the latter present in the plasma. In the case of rat gastric juice the values found at five minutes are within the range found when the vitamin is administered alone, but thereafter the concentration falls to significantly lower levels.

Table 2

RATE OF DISAPPEARANCE OF RADIOACTIVITY FROM THE PLASMA AFTER
INTRAVENOUS INJECTION OF 2.5 μ mg Co⁵⁸-VITAMIN B₁₂ WITH
DIALYZED RAT GASTRIC JUICE (A) AND SEMI-
PURIFIED HOG INTRINSIC FACTOR (B)

Counts expressed as mean percentages of calculated rates at zero time								
Time (minutes)		5	10	15	20	60	150	300
A	Mean %	22.4 (2)	10.6 (3)	6.0 (7)	5.2 (3)	4.0 (8)	2.5 (8)	2.1 (8)
	S.E.	0.65	0.45	1.29	0.55	0.66	0.49	0.43
B	Mean %	3.4 (3)	1.8 (2)	1.9 (3)	0 (2)	1.1 (5)	0.8 (5)	0 (5)
	S.E.	0.21	0.61	0.32	-	0.15	0.20	-

Numbers of observations in parenthesis.

The pattern of distribution of radioactivity throughout the whole animal has not been studied, but uptake by liver and stomach has been measured. In the normal animal about 20 per cent of total radioactivity is found in the liver 20 minutes or 300 minutes after injection of the vitamin alone, and in the gastrectomized animal there is no significant difference.

The addition of rat gastric juice markedly increases the hepatic uptake at 20 minutes, but at 300 minutes the percentage uptake is not significantly different from that observed following

Figure 2. Rate of disappearance of radioactivity after intravenous injection of 2.5 μ mg Co⁵⁸-vitamin B₁₂ with and without intrinsic factor.

injection of the vitamin alone (Table 3). With hog intrinsic factor a very much greater percentage of the total activity is found in the liver at 300 minutes than in animals receiving the vitamin alone or together with rat gastric juice. Uptake by the stomach (Table 3) represents only a small part of the total activity administered. It appears to be fairly rapid, so that at 20 minutes there is as much activity present as at 300 minutes. In four animals the pylorus was tied off before administration of the intravenous vitamin B₁₂ so that the gastric contents might be retained and counted; almost all the activity was present in the stomach walls, both at 20 and 300 minutes.

It appears from the results in Table 3 that there is greater uptake by the stomach when the vitamin is "unbound," but this trend is not statistically significant.

Table 3
MEAN PERCENTAGE UPTAKE OF Co⁵⁸-VITAMIN B₁₂ BY LIVER AND STOMACH

Preparation		20 minutes		300 minutes	
		Liver	Stomach	Liver	Stomach
Vitamin B ₁₂ alone	S.E.	22.7 (2) 4.56	2.4 (2) 0.05	22.5 (14) 2.19	2.4 (14) 0.26
Vitamin B ₁₂ + rat gastric juice	S.E.	69.5 (2) 1.4	1.2 (2) 0.21	13.6 (6) 0.94	1.4 (3) 0.20
Vitamin B ₁₂ + hog intrinsic factor	S.E.	-	-	87.0 (4) 2.60	1.2 (1) -

Numbers of observations in parenthesis.

DISCUSSION

The results presented do not support the theory that any gastric secretion plays an important part in the transfer of vitamin B₁₂ from the plasma into tissues in the rat. The rate of disappearance of activity in the plasma and the percentage uptake by the liver are essentially the same in the intact and the gastrectomized animal. If gastric intrinsic factor is secreted into the blood stream as well as into the gastric lumen, in a way analogous to pepsinogen, it does not play an essential part in vitamin B₁₂ exchange.

The reason for the somewhat decreased rate of removal of labeled vitamin B₁₂ from the plasma in treated pernicious anemia and in one patient following total gastrectomy studied by Brody *et al.*⁶ is not apparent. Apart from the absence of a postulated transferase, one possible explanation might be that where gastric intrinsic factor is absent, as in pernicious anemia, uptake of labeled vitamin B₁₂ by the stomach wall is reduced or absent. In the rat this uptake amounts to only 2.5 per cent of the total dose, and the absence of the stomach does not significantly alter the plasma curve of disappearance, but, in the rat, the total glandular stomach is small in relation to body weight; in the human the gastric mucosal area is relatively greater and hence percentage uptake of vitamin B₁₂ by the stomach might be greater. Direct measurements in the human by surface counting might be of interest.

Our finding of rapid uptake of vitamin B₁₂ by the liver in the rat, when the vitamin is bound to rat or hog intrinsic factor, confirms earlier work¹¹ by Chow and his colleagues. Their findings suggest that, in the case of hog material, it is not intrinsic factor activity which mediates the rapid transfer of vitamin B₁₂ into the liver, but some other binding material, since treatment of the hog material by heating in a way known to destroy intrinsic factor activity without decreasing vitamin B₁₂ binding, did not result in diminished vitamin B₁₂ uptake.

These workers also showed that 22 hours after giving the vitamin with hog intrinsic factor the amount of radioactivity in the liver was less than at 15 minutes by some 30 per cent. Our own results show that, with rat intrinsic factor, the retention of a high percentage of radioactivity in the liver is even more transient. By six hours the mean percentage was within the limits of the results obtained using vitamin B₁₂ alone, whereas with hog intrinsic factor almost 90 per cent of the activity was still present in the liver at this time.

At least two factors may promote this abnormally high hepatic uptake. First, intravenous vitamin B₁₂ injected in a bound form may be prevented from binding with the normal vitamin B₁₂-binding proteins of the plasma and so not be retained in the plasma, and, secondly, both impure homologous and heterologous intrinsic factors may be treated as foreign substances by the reticulo-endothelial system in the liver and removed from the circulation, or conversely, become attached to the liver parenchymal cells in the same way as occurs with liver slices and homogenates *in vitro*. It is possible that subsequent enzymatic reactions occur which release the vitamin B₁₂ into the circulation, the intrinsic factor being destroyed or removed by the reticulo-endothelial system. Such a situation might be analogous to the system described by Cooper and Castle¹² in the intestinal wall. Physical fractionation of components of hepatic cells may reveal the site of uptake of the vitamin and show whether it is truly intracellular or merely bound temporarily on the surface of cells.

One of the difficulties in interpreting data in this field is that all preparations of intrinsic factor used are impure and probably possess vitamin B₁₂-binding activity unrelated to their intrinsic factor content. Further, as Nieweg and his colleagues have emphasized,¹³ the prepara-

tions of hog intrinsic factor used in both in vivo and in vitro studies is not active as intrinsic factor in the gastrectomized rat. This is probably due to the presence of inhibitors, which may be species specific.¹⁴ Clearly, data obtained with such preparations should be interpreted with caution.

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