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950 EAST FIFTY-NINTH STREET · CHICAGO · ILLINOIS 60637

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1971

ALEXANDER GOTTSCHALK, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

OPERATED BY THE UNIVERSITY OF CHICAGO
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FLUORESCENT THYROID SCANNING (SCANNING WITHOUT RADIOISOTOPES): INITIAL CLINICAL RESULTS*

By

P. B. Hoffer,[†] and A. Gottschalk
With the technological assistance of J. Foster

Fluorescent thyroid scanning is a new technique of imaging the thyroid gland without administering radioisotope to the patient. The scan can be made with an extraordinarily low radiation dose to the patient (less than 10 millirads), and gives quantitative information about the iodine content of the gland. This communication presents the first clinical results obtained from a group of 76 patients studied by the fluorescent technique.

The fluorescent thyroid scan offers certain unique advantages over other methods of thyroid scanning currently in use. A highly significant feature of the technique is the exceedingly low radiation dose received by the patient. The thyroid is irradiated by a narrow beam of γ -rays. The radiation dose to the remainder of the body is nil. In this series, irradiation to the thyroid was less than 200 millirads as measured directly in realistic phantoms by lithium fluoride and film dosimetry. The scanner has been subsequently modified to deliver a radiation dose of less than 10 millirads to the thyroid. The radiation dose from this method compared to other thyroid scanning techniques is described in Table 1.

Table 1
PATIENT DOSE FOR VARIOUS THYROID SCANNING AGENTS

Scanning agent	Activity ingested	Whole-body dose	Thyroid dose (critical organ dose)	Reduction factor compared to ^{131}I (%)
Na ^{131}I	50 μCi	23 mr	65 rads (ref. 8)	-
Na ^{125}I	50 μCi	20 mr	41 rads (ref. 8)	65.
Na $^{99}\text{TcO}_4$	1 mCi	10 mr	0.1 rads (ref. 1) (0.3 rads)*	0.15
Fluorescent	none	nil	>0.010 rads	0.015

* Stomach

METHODS

The mechanics of the technique have been previously described¹⁻³ and will be only briefly reviewed. When stable iodine (^{127}I), or any other element, is irradiated by an x-ray or gamma

* This report is taken from a paper that appeared in Radiology, 99:117, 1971. The work was supported in part by the Junior Auxiliary of the University of Chicago Cancer Research Foundation.

[†] Scholar in Radiological Research of the James Picker Foundation.

ray beam of sufficient kilovoltage potential, the element itself will produce x-rays. The energy of the x-rays produced is characteristic for the element irradiated and is therefore referred to as "characteristic radiation" (Figure 1). The characteristic radiation produced by most elements in the body is of very low energy and travels only a short distance before it is absorbed

Figure 1. Production of characteristic radiation. The 60 keV gamma ray ($h\nu$) of ^{241}Am displaces a K shell electron (photoelectron) from the target iodine atom. Replacement of this shell vacancy from an outer ring results in production of a 28.5 keV characteristic x-ray. (Characteristic radiation.)

by surrounding tissue. Elements with relatively high atomic numbers, such as iodine, produce more energetic characteristic x-rays which can travel through greater thicknesses of tissue and therefore be detected by instruments placed outside the body.

It is possible to direct a narrow beam of γ -rays from an isotope source (we use 60 keV gamma rays from americium-241) at the thyroid gland and use a lithium-drifted silicon detector to detect the characteristic x-rays produced by the stable iodine within the gland (Figure 2).^{*} The number of characteristic x-rays produced will be proportional to the amount of iodine present. By moving the beam and the detector over the gland in a rectilinear pattern, a scan is produced which depicts the amount of iodine present in various portions of the thyroid.

Case material. The usefulness of this new scanning method was evaluated on a series of 76 patients most of whom were selected randomly from patients who presented to the Nuclear Medicine Section of the Department of Radiology, University of Chicago for radioactive isotope thyroid scans. A few of these patients, however, were selected either because they were candidates for subsequent thyroid surgery (8 cases), or because they were not candidates for routine

^{*}The lithium-drifted silicon detector used in this apparatus was manufactured by the Nuclear Equipment Corporation, San Carlos, California; the americium-241 source was fabricated by the Monsanto Research Corporation, Dayton, Ohio; the amplifier for the detector was manufactured by the Camberra Instrument Corporation, Middletown, Connecticut. The apparatus was mounted on a conventional rectilinear scanner.

Figure 2. Schematic fluorescent scanner. A beam of 60 keV gamma rays (A) from americium-241 (SOURCE) irradiates a portion of thyroid (B). Some of the characteristic radiation produced (C) is seen by the lithium-drifted silicon detector (DETECTOR) which is housed at the base of a liquid nitrogen cooling reservoir.

isotope studies for one of the following reasons: (a) on thyroid replacement therapy (4 cases); (b) flooded iodine pool (2 cases); (c) on anti-thyroid drugs (2 cases).

In 60 cases, an isotope scan and a 24-hour ^{131}I uptake measurement were made in conjunction with the fluorescent scan. Most of the isotope scans were done with 50 μCi of ^{125}I as the tracer; the remainder were done with 50 μCi of ^{131}I . The protocol for these 60 patients was as follows: On the first visit to the Nuclear Medicine Section, the fluorescent thyroid scan was performed. The patient was then given the uptake and scanning dose of the iodine isotope. The following day, the patient returned to the Nuclear Medicine Section where the 24-hour uptake and isotope scans were performed.

In the remaining 16 patients no isotope scan was performed in immediate conjunction with the fluorescent thyroid scan, although many of them had had isotope scans and radioactive iodine uptake studies within one month to one year previously. These latter scans were not considered coincident for purposes of comparison.

RESULTS

Technical quality. The technical quality of the fluorescent scans was compared with the isotope scans. Factors considered were perceptibility of lesions, perceptibility of gland outline,

and completeness of study, i.e., whether all areas of interest were included. The fluorescent scans were judged as either better than, equal to, or poorer than the conventional isotope study.

The results of this evaluation are summarized in Table 2. The bulky size of our original fluorescent scanner and its slow scanning speed (15 cm/min) were responsible for most of the poorer quality fluorescent scans. A more compact design, and a stronger americium-241 source have corrected both problems in our newest fluorescent scanning system.

Table 2
ANALYSIS OF IMAGE QUALITY, FLUORESCENT SCAN
VERSUS RADIOISOTOPE SCAN

	No. of cases	%
Fluorescent scan superior to isotope scan	11	18
Fluorescent scan equal to isotope scan	29	48
Fluorescent scan inferior to isotope scan	20	33
No isotope scan done	16	-

Iodine content evaluation. The scans were also judged for iodine content within the gland. Since the fluorescent scans were made at a constant speed, the density of the scan reflects the iodine content of the corresponding portion of the gland—(the gamma ray source is filtered so as to produce an isometric response to equal concentrations of iodine between 0 and 2.5 cm from the skin surface). The gland density was evaluated subjectively with selected examples demonstrated in Figure 3. A "light" scan was considered to reflect low iodine content within

Figure 3. These three cases demonstrate the subjective assessment of thyroidal iodine concentration discussed in the text. Case A. Low iodine content in a hypothyroid patient. The gland is difficult to perceive. Note the tracheal air column. Case B. Normal iodine content in euthyroid patient. We routinely use "data blending" to display all thyroid scans, i.e., the "fuzzy" readout is not related to the fluorescent technique. Case C. Increased iodine content in a patient with a binding defect. Kinetic analysis (Dr. L. J. DeGroot) shows an eight-fold iodine increase.

the gland and a "dark" scan to indicate high iodine content. No effort was made to express iodine content of the gland in weight per unit volume.

Low iodine content. Twelve of the fluorescent scans demonstrated low iodine content within the gland. Both clinical and laboratory evidence confirmed hypothyroidism in seven of these patients. Two other patients in this group had normal thyroid function studies, but one of them was suspected of having thyroiditis, and is now clinically hypothyroid and on thyroid replacement one year following the fluorescent scan. Of the remaining three patients, one was on propylthiouracil at the time of fluorescent scanning, one was post ^{131}I therapy for toxic nodular goiter, and one had insufficient follow-up. (See Table 3.)

Table 3
PATIENTS WITH LOW IODINE CONTENT IN THYROID GLAND
DEMONSTRATED ON FLUORESCENT SCAN

Patient	Radioactive iodine uptake (24 hours)	Other pertinent information
635892	Less than 1%	Hodgkin's disease, received 3,000 rads to neck, clinically hypothyroid
371601	6%	PBI 1 $\mu\text{g}\%$; BMR - 10%, clinically hypothyroid
1027833	5%	Clinically hypothyroid
789225	9%	Clinically hypothyroid, ^{131}I treated Graves' disease
465802	3%	Clinically hypothyroid
789191	11%	Clinically hypothyroid, congestive heart failure
1006205	18%	Hashimoto's thyroiditis (no biopsy), clinically hypothyroid 1 year later
861201	20%	Clinically euthyroid
980674	-	Toxic nodular goiter, post ^{131}I treatment
963919	-	Graves' disease, on PTU one year
1012299	-	Clinically hypothyroid, "burnt out" Hashimoto's thyroiditis, T_3 24%
1022208	-	Carcinoma of colon, no thyroid follow-up

High iodine content. Nine patients had a marked increase in the iodine content of their thyroid glands. Four of them had clinical or laboratory evidence of Graves' disease; one had Hashimoto's thyroiditis on biopsy; two had congenital thyroid metabolic defects; one had a non-toxic autonomous nodule, and another had a toxic autonomous nodule. None of these patients was clinically hypothyroid. These cases are summarized in Table 4.

Normal iodine content. The remaining fifty-five patients had near normal or normal concentrations of iodine within the thyroid gland on the basis of the fluorescent scan. This group

Table 4
PATIENTS WITH HIGH IODINE CONTENT IN THYROID GLAND
DEMONSTRATED ON FLUORESCENT SCAN

Patient	Radioactive iodine uptake (24 hours)	Other pertinent information
832383	44%	Clinically Graves' disease, T_4 14 $\mu\text{g}\%$ FTI, 18.7; radioactive iodine $T_{1/2}(\text{biol.}) = 22$ days
1027586	84%	Clinically Graves' disease
415880	87%	Clinically Graves' disease
1023868	67%	Congenital re-utilization defect
863511	19%	Hashimoto's thyroiditis on biopsy
793753	36%	Clinically Graves' disease, PBI - 12 $\mu\text{g}\%$, BMR + 7%, T_4 13.1 $\mu\text{g}\%$, FTI - 15.5
892698	14%	On thyroid replacement Rx, flooded iodine pool, question of organic binding defect
371501	18%	Nontoxic autonomous nodule
722702	27%	Toxic nodular goiter

included the vast majority of patients with nodules. In these patients, only the concentration of iodine in the extra nodular portion of the gland was evaluated; the concentration of iodine within the nodule was disregarded.

Most of these patients were judged to be euthyroid on the basis of clinical and/or laboratory data. Only one of them had a low radioactive iodine uptake (5 per cent at 24 hours), but this patient had been on cortisone therapy up to 9 days prior to the radioactive iodine uptake study. Five cases had active Graves' disease. The group is summarized in Table 5.

Table 5
PATIENTS WITH NORMAL IODINE CONTENT IN THYROID
GLAND DEMONSTRATED ON FLUORESCENT SCAN

	No. of cases	%
Euthyroid	43	78
Hyperthyroid (Graves' untreated)	5	9
Treated hyperthyroid (Graves')	3	5
Thyroiditis	2	4
Nontoxic autonomous nodule	1	2
Cortisone therapy (uptake 5% at 24 hours)	1	2
TOTAL	55	100

The Correlation Between Iodine Content and Thyroid Disease

Hypothyroidism. All eight patients with clinical and laboratory diagnosis of hypothyroidism had low iodine content in the thyroid on fluorescent scan. Four other patients had low 24-hour radioiodine uptake values, but no clinical evidence of hypothyroidism. All of these patients had a normal or increased glandular iodine content. One of them had a known flooded iodine pool; another had clinical thyroiditis; the third patient was post-operative Graves' disease; and the fourth was a patient on long-term cortisone therapy.

Graves' disease. Nine patients had clinical and laboratory evidence of Graves' disease. Five of these patients had large thyroid glands with either normal or slightly increased iodine content. These findings are consistent with the observations of Heedman and Jacobson.⁶ The remaining four had markedly increased iodine content within the thyroid gland. It is generally assumed that patients with Graves' disease have a rapid turnover of iodine, the iodine concentration within the thyroid gland actually being low.⁶ Our findings tend to refute this concept. One of our patients with proven Graves' disease and a documented decreased iodine-131 biologic half-life within the thyroid gland, had a thyroidal iodine concentration which, on the basis of the fluorescent scan, was between two and three times normal. (Gland weight 40 g, radioactive iodine uptake 44 per cent at 24 hours, T-4, 14 µg per cent, Free Thyroxine Index 18, T-1/2 biol. 21 days, Teff = 5.9 days.)

"Cold" nodules. Twenty-one patients demonstrated single or multiple "cold" nodules on either the fluorescent or the radioisotope scan. No "cold" nodule seen on the radioisotope scan was missed by the fluorescent scan, although the fluorescent scan did pick up one nodule missed on the original isotope scan. In two cases, a structural pattern was shown within the nodule on the fluorescent scan (Figures 4 and 5). One patient proved to have a "septate, honeycombed large cyst with follicular lining," and the other patient was diagnosed as having an angioinvasive adenoma or "incapsulated carcinoma." The "cold" nodule group is summarized in Table 6.

Table 6

PALPABLE NODULES WITH NO ISOTOPE UPTAKE AND LOW OR NO IODINE CONTENT ON FLUORESCENT SCAN

Etiology	No pattern on fluorescent scan, i.e., no iodine content	Pattern on fluorescent scan, i.e., low iodine content
Hemorrhagic, degenerative or cystic nodules	6	-
Adenoma	2	-
Angioinvasive adenoma (encapsulated carcinoma)	-	1
Septate cyst with follicular lining	-	1
Nonthyroidal mass (Hodgkin's disease)	1	-
Etiology as yet unknown	10	-

Figure 4. Note the septate appearance on fluorescent scan throughout the large nodule of the right lobe. At surgery, a 15 cc cyst, 3 cm in diameter was excised. The cyst was lined with projections of colloid filled follicles.

Figure 5. Angioinvasive adenoma ("encapsulated carcinoma"). A. Regular ^{125}I scan showing large cold nodule in mid to upper pole of the lateral aspect of the right lobe. B. Fluorescent scan showing a diffuse "mottled" iodine content in the lesion.

Autonomous nodules. Three patients demonstrated autonomous thyroid nodules, toxic in one case, and nontoxic in the other two cases. In the case of the toxic nodule and in one case of nontoxic nodules, the fluorescent scan demonstrated increased iodine content in the nodule as well as in the remainder of the gland. In the other nontoxic nodule, the iodine content of the nodule was decreased, although the iodine content of the remainder of the gland was normal.

Thyroiditis. The patients with thyroiditis showed no consistent pattern, ranging from increased to decreased iodine content on the fluorescent scan regardless of iodine uptake or clinical status (Figure 6, and Table 7).

Figure 6. This case of biopsy proven Hashimoto's thyroiditis shows a high thyroid iodine concentration on fluorescent scan. The 24-hour ^{131}I uptake was 19%. No definite correlation between the fluorescent scan, radioactive iodine uptake, and nuclide scan, could be established in thyroiditis patients.

DISCUSSION

The fluorescent thyroid scan depicts the presence and concentration of nonradioactive iodine within the thyroid gland. Since it is not dependent upon uptake of radioactive tracers, it gives information somewhat different from conventional scanning methods. Factors that inhibit thyroidal uptake of radioactive iodine and prevent isotopic scanning do not necessarily influence the fluorescent scan in the same manner. For example, we obtained good fluorescent scans on patients with "flooded" iodine pools. Good quality fluorescent scans are also possible in patients on thyroid replacement therapy. In our series, this is true for over two years after the onset of therapy. Suppressed thyroid can also be visualized in patients with toxic autonomous thyroid nodules, thereby eliminating the need for a TSH stimulation scan (Figure 7).

On the other hand, good fluorescent scans cannot be obtained on patients treated for any

Table 7

COMPARISON OF RADIOACTIVE IODINE UPTAKE AND TOTAL THYROIDAL IODINE
(ON BASIS OF FLUORESCENT SCAN) IN PATIENTS WITH CLINICAL OR
BIOPSY DIAGNOSIS OF "THYROIDITIS"

Patient	Radioactive iodine uptake (24 hours)	Total thyroidal iodine (fluorescent scan)	Clinical information	Biopsy
1	19%	High	Hashimoto's	Hashimoto's
2	30%	Normal	Thyroiditis	Hashimoto's
3	18%	Low	Hashimoto's Hypothyroid 1 year later	None
4	2%	Normal	Hashimoto's	None
5	24%	High	Hashimoto's	None
6		Low	"Burnt out" Hashimoto's	None

Figure 7. Toxic multinodular goiter. A. Routine ^{125}I scan. Note suppression of the entire left lobe. B. TSH stimulation scan showing uptake on the left. C. Fluorescent scan which also shows the suppressed left lobe, and an increased iodine concentration in the multinodular right lobe. Because of the minimal clinical signs of toxicity, conflicting laboratory values, and frequency of "nodules" in the midwest goiter belt, this patient presented as a mild diagnostic problem that was solved by the TSH scan. The routine tracer scan—fluorescent scan combination—would have achieved the same result, obviating the use of TSH.

length of time with antithyroid drugs such as propylthiouracil. We assume that the reason for these poor scans is the rapid output of iodine from the gland and the inhibition of its replacement due to the impaired binding of iodine to thyroglobulin induced by the medication.

"Cold" nodules seen on an isotope scan are equally well seen on a fluorescent scan and the pattern of iodine concentration in the fluorescent scan may give somewhat more information concerning their etiology.⁷

Although overall scan quality in this series was slightly inferior to that produced by conventional isotope techniques, and total scan time was longer than desirable (about 40 minutes), both of these faults, due to equipment design, have been corrected in our newest fluorescent scanner. Patient irradiation is much lower than from any other available method.

We feel that the following conclusions are justified on the basis of this initial series:

1. Fluorescent scanning is possible in a variety of situations when radionuclide scanning is either impossible or undesirable. These include pregnancy, thyroid replacement therapy, and low nuclide uptake (e.g., flooded iodine pool).

2. In this series, the clinically hypothyroid patients have fluorescent scans that always demonstrate diminished iodine concentration. We have not examined any cases of secondary hypothyroidism, but the fact that thyroids "at rest" from replacement doses of thyroxine show normal iodine concentrations (i.e., normal fluorescent scans) suggests that the technique could also be used to separate primary from secondary hypothyroidism.

3. In active Graves' disease, the fluorescent scan demonstrates a normal to large gland with normal to increased iodine concentration.

4. Some nodules that are "cold" on routine nuclide scans contain enough iodoprotein to show "structure" on fluorescent scans. Our series is still too small to determine if etiologic characterization will be possible in these cases.

5. Autonomous nodules may show either increased or decreased iodine concentration. It is possible to see thyroid tissue suppressed by toxic nodules directly by fluorescent scanning without the necessity of TSH stimulation.

6. No consistent appearance is demonstrated in thyroiditis.

Finally, as fluorescent scanning becomes perfected, it will be possible to perform rapid (about 10 minute) scans with doses so low (< 10 mrad) that they can be done routinely on any patient. It is probable that radionuclide scanning and fluorescent scanning will complement each other in many instances.

ACKNOWLEDGMENTS

We wish to thank Professor R. N. Beck for designing the collimators used in the fluorescent scanner, Professor D. B. Charleston and Mr. N. Yasillo for designing and maintaining much of the electronic instrumentation, Mr. J. Stupka for his aid in the instrument design and construction, and Dr. L. J. DeGroot for constant encouragement and constructive advice throughout the course of this project.

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THE DIAGNOSTIC SIGNIFICANCE OF THE "HOT" OR "COLD" SPLEEN IN COLLOIDAL SCINTIPHOTOGRAPHY*

By

C. Bekerman[†] and A. Gottschalk

Rappaport has recently published a detailed review of pathologic states of the splenic pulp.¹ A prominent aspect of the pathologic spleen in most of the diseases referred to in his paper is a widening of the pulp cords. This pathologic feature suggested to us that the appearance of the spleen on the colloidal scan might be affected in such a way that the relative uptake of liver, spleen, and bone marrow could be a useful diagnostic adjunct.

Several authors² have reported their findings in spleen and liver scanning, and bone marrow delineation in conditions such as cirrhosis,³ lymphomas,^{4,5} and hematological disorders,^{6,7} but no systematic attempt to analyze the relative uptake of colloid in the RE system has appeared.

MATERIAL AND METHOD

We have reviewed all the liver and spleen scans done during 1969 in the Section of Nuclear Medicine of the Department of Radiology of the University of Chicago.

From the total of 931 examinations, only 156 had both liver and spleen imaged and an initial referral diagnosis of cirrhosis, anemia (of unknown etiology), or systemic proliferative diseases and tumors of the hematopoietic tissues.⁸ These 156 cases constitute the present series.

It was suggested as early as 1952⁹ that the distribution of colloidal particles is dependent upon particle size. A description of our method of preparing ^{99m}Tc-sulfur-colloid is therefore relevant.

Following methyl-ethyl ketone extraction of ^{99m}TcO₄⁻, and subsequent evaporation of the solvent, the desired activity is dissolved in 0.5 ml of pharmaceutical grade gelatin in 1 N HCl. Ten milliliters of H₂S gas is introduced into the preparation for 15 min at room temperature. The solution is then adjusted to pH 5-6 with 1 N NaOH, and filtered through a 0.45 μ membrane.¹⁰

Previous reports¹¹⁻¹³ have described the normal distribution of this colloidal nuclide; 80-90 per cent goes to the liver, 5-10 per cent to the spleen, and the remainder to the bone marrow.

It is our practice to image the reticuloendothelial system with about 6 millicuries of ^{99m}Tc-sulfur colloid and the gamma scintillation camera. Multiple views of the liver and spleen are taken with a minimum of anterior, posterior, and lateral projections for each organ. We obtain 10⁶ counts per view in exposure times of 1-2 min.

The 156 scans were interpreted by one of us in a single session with no clinical information to determine the following: spleen size,¹² liver size, uptake of radiocolloid by the spleen versus uptake by the liver, and uptake of radiocolloid by the bone marrow.¹¹

RESULTS

Decreased splenic uptake. In 24 scans, the uptake of radiocolloid by the spleen was decreased

* This report is taken from a paper that appeared in J. Nucl. Med., 12:5, May 1971.

[†] Fellow of the International Atomic Energy Agency.

in comparison with the uptake by the liver; the bone marrow delineation was normal or not visible in 21 of these, and bone marrow uptake was increased in the remaining three. The final diagnosis in this group is shown in Table 1.

Table 1
SPLEEN UPTAKE DECREASED (REGARDLESS OF SIZE); BONE
MARROW UPTAKE NORMAL OR NOT VISIBLE

Final diagnosis	
Malignant lymphoma, histiocytic (reticulum cell sarcoma)	1
Hodgkin's disease	6
Malignant lymphoma, poorly differentiated lymphocytic	6
Chronic lymphocytic leukemia	2
Chronic granulocytic leukemia	1
Mycosis fungoides	3
Undifferentiated lymphoreticular neoplasm	1
Sickle cell anemia in crisis	1
Subacute bacterial endocarditis	<u>1</u>
TOTAL	21

An iliac crest marrow biopsy was performed in 17 patients of this group. Twelve were abnormal (e.g., evidence of Hodgkin's disease, malignant lymphoreticular neoplasm, chronic lymphocytic leukemia). Nine of these 12 patients also had splenectomy; 8 of the spleens were abnormal showing infiltration of hematopoietic neoplasms. Three other cases with decreased uptake of radiocolloid by the spleen had increased uptake in the bone marrow with the following final diagnosis: 1 - adenocarcinoma, metastatic to liver; 1 - valvular pulmonary stenosis with iron deficiency anemia; 1 - sickle cell anemia with lead intoxication. The surgical pathology report on the spleen in the last case of the series showed extensive areas of fibrous changes.

Increased splenic uptake. The uptake of radiocolloid by the spleen versus the liver was increased in 13 cases. In only one of these cases, a patient with acute granulocytic leukemia with thrombocytopenia, did the bone marrow appear normal on scintiphotography. The remaining 12 scans showed increased bone marrow uptake. The final diagnosis in this group is shown in Table 2.

Normal splenic uptake. Comparable uptake of radiocolloid by spleen and liver was found in 119 scans. In 56 of these the delineation of the bone marrow was considered normal and the final diagnosis is shown in Table 3.

An iliac crest bone marrow biopsy was performed in 43 of these patients. Thirty of these biopsies were abnormal with substitutions of neoplastic cells, leukemic infiltrates or fibrotic lesions. Splenectomy was done in 16 patients; the pathological report in 11 was normal, while 5 were infiltrated (4 Hodgkin's disease, 1 mycosis fungoides).

Thirty-three scans showed comparable uptake of radiocolloid by the spleen and the liver, but increased uptake of colloid by the bone marrow. These results are shown in Table 4.

The literature is replete with studies that correlate splenomegaly and increased bone mar-

Table 2

SPLEEN UPTAKE INCREASED (REGARDLESS OF SIZE); BONE
MARROW UPTAKE INCREASED

Final diagnosis	
Cirrhosis	
with anemia (hematocrit 35% or less)	5
without anemia	4
Chronic active hepatitis with anemia	2
Iron deficiency anemia	<u>1</u>
TOTAL	12

Table 3

SPLEEN AND LIVER UPTAKE COMPARABLE (REGARDLESS OF
SIZE); BONE MARROW UPTAKE NORMAL OR
NOT VISIBLE

Final diagnosis	
Malignant lymphoma histiocytic (reticulum cell sarcoma)	6
Hodgkin's disease	20
Malignant lymphoma, mixed cell (histiocytic-lymphocytic)	3
Malignant lymphoma, poorly differentiated lymphocytic	7
Malignant lymphoma, well differentiated lymphocytic	2
Chronic lymphocytic leukemia	1
Granulocytic leukemia	1
Idiopathic thrombocytopenic purpura	3
Polycythemia vera	2
Myelosclerosis with myeloid metaplasia	3
Mycosis fungoides	6
Malignant histiocytosis	<u>2</u>
TOTAL	56

row uptake with cirrhosis or chronic hepatitis. Our results indicate that just as good a correlation with anemia is warranted with this scan appearance (see Table 5).

DISCUSSION

From this analysis it is clear that certain colloidal distributions in the reticuloendothelial system have significant diagnostic implications (see Figure 1).

When the uptake of radiocolloid by the spleen is decreased, and the bone marrow uptake is normal or not visible, the vast majority (19/21 - 90 per cent) of this series showed hematopoietic neoplasms. This correlated well with widespread disease, since over 70 per cent of these

Table 4

**SPLEEN AND LIVER UPTAKE COMPARABLE (REGARDLESS
OF SIZE); BONE MARROW UPTAKE INCREASED**

Final diagnosis	
Cirrhosis	
with anemia (hematocrit less than 35%)	11
without anemia	6
Anemia	
iron deficiency	7
other causes	5
Proliferative diseases and tumors of the hemato- poietic system without anemia	2
Others	<u>2</u>
TOTAL	33

Table 5

LARGE SPLEEN; BONE MARROW UPTAKE INCREASED

Final diagnosis	
Cirrhosis	
with anemia (hematocrit less than 35%)	13
without anemia	7
Anemia	
iron deficiency	5
other causes	5
Proliferative diseases and tumors of the hemato- poietic tissues	1
Others	<u>2</u>
TOTAL	33

cases showed positive bone marrow biopsies. Finally, in cases for which pathologic study of the spleen was available, the scan appearance appears to be due to a widespread infiltration of the spleen by neoplastic tissue.

On the other hand, when the spleen shows an increase in uptake compared to the liver, virtually all of our cases also showed increased bone marrow uptake. In this case, a diagnosis of either cirrhosis or anemia is warranted, the correlation with either disease being about the same. When the bone marrow uptake is increased by itself while the spleen is normal in appearance, the correlation with anemia or cirrhosis is also high.

It is important to point out that our interest was directed primarily to diseases of the reticuloendothelial system. This series is deficient, therefore, in conditions such as widespread metastatic adenocarcinoma, which might also alter the appearance of the colloidal scan.

Figure 1. Left - posterior view of the abdomen (diverging collimator) showing markedly increased splenic uptake, increased bone marrow uptake and relative decrease in hepatic uptake characteristic of either anemia or cirrhosis. Right - posterior view of the left abdomen showing marked decrease in splenic uptake compared to the liver, and no visualization of the bone marrow. The spleen was not enlarged even though it was infiltrated with malignant lymphoma.

Figure 2. Normal liver and spleen (surgical proof). Top row, ABC - posterior view of the liver showing the conventional three lens display ($1/2$ f stop increments) from a pho-gamma III scintillation camera. Bottom row, DEF - three lens display of the posterior view of the spleen exposed for the same time as the liver with the same intensity setting. Note that in the normal case, in our institution, the brightest exposure of the spleen (D) corresponds in intensity to the intermediate brightness display of the liver (B). If splenic activity was increased, exposure D would correspond best to exposure A. If it was decreased, exposure D would correspond to exposure C. This simple technique facilitates the subjective analysis of the relative uptake in the two organs. Since the colloidal preparation used will affect the splenic uptake, it is imperative that each institution establish its own normal appearance when using this technique.

A second limitation of this retrospective study is that no specific provision was made in our routine scanning techniques for the correlation of uptake of colloid in the liver and spleen. This can be done easily from the posterior projection either by including both liver and spleen on the same scan, or by taking a timed view of the posterior liver with a gamma camera, and exposing the posterior spleen for the same period of time at the same camera setting. (See Figure 2.)

The technique of subjectively assessing the relative uptake in liver and spleen from the posterior scan or camera study is comparatively easy, being a simple extension of the routine liver scan. This series suggests that it may be a useful adjunct to diagnosis.

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THE APPEARANCE OF INTRAHEPATIC BILIARY DUCT DILATATION ON THE LIVER SCAN*

By

L. L. Heck, and A. Gottschalk

The identification of dilated bile ducts on the liver scan in jaundiced patients has been suggested by several previously published reports.¹⁻⁵

In recent years at our institution, we have observed the presence of dilated biliary ducts in patients referred to us for liver scanning. In contrast to previous cases that were examined with rectilinear scanners, for this study, we utilized the Anger camera to obtain multiple views and views with breath-holding. This method was used because respiratory motion is responsible for a significant loss of resolution in organ imaging.^{6,7} Our purpose was to determine the reliability of this scan diagnosis in jaundiced patients.

MATERIALS AND METHODS

Thirty-five jaundiced patients were selected for evaluation. The liver scans were all made beginning about 15 minutes after the injection of 6 to 8 mCi of ^{99m}Tc sulfur colloid and collecting one million counts per exposure. Counts with breath-holding were collected at the end of a deep inspiration. These cases were randomly mixed with observations on 25 normal subjects and patients with other diseases. The scans were interpreted by one of the authors (A.G.) without any information on the clinical history and using only the anterior view of the liver. Each case was classified as positive, equivocal, or negative for biliary duct dilatation.

Phantom studies were also performed to evaluate the parameters of respiratory motion and total counts collected on the delineation of a suitable biliary duct phantom. The experimental model of a Y-shaped biliary phantom, and a respiratory motion simulator are shown in Figure 1. The "cold" phantom was immersed in 10 cm of water containing sodium pertechnetate. Respiratory excursions of from 1 to 3 cm were made while imaging the biliary phantom. Fourteen oscillations per minute were used to simulate the breathing rate at rest.

RESULTS

The correlation between pathological diagnoses and scan interpretations in the 35 jaundiced patients is given in Table 1. The overall reliability of diagnosis in the jaundiced patients was 26 out of 32 patients followed up, i.e., 78 per cent. There were no false positives among the 25 non-jaundiced patients. The 20 patients in whom dilated ducts were verified had one of three etiologies: (1) Carcinoma of the head of the pancreas, (2) Common duct stone, or (3) Carcinoma of the ampulla of Vater.

After the data were tabulated and interpreted, we established 4 criteria from the scan material which could be used to determine whether or not biliary duct enlargement was present. These were as follows:

* This report is taken from a paper that appeared in *Radiology*, 99:135, 1971. The investigation was partially supported by a grant from the U. S. Public Health Services.

A

B

Figure 1. Top. Biliary duct phantom, constructed of water-filled Y-shaped plastic tubing with an external diameter of 1.5 cm.

Bottom. The phantom is supported on a wire frame 5 cm above a plastic base resting on a large container placed on the platform of the respiratory motion simulator. When in use, the container is filled with pertechnetate-99m, and the phantom is placed inside. The eccentric arm of the simulator can be adjusted to vary the excursion of the platform. A variable speed motor controls the number of excursions per minute.

Table 1

CORRELATION OF THE PATHOLOGICAL DIAGNOSES WITH THE
SCAN INTERPRETATIONS IN 35 JAUNDICED PATIENTS

Scan interpretation	Patients (no.)	Dilatation present	Dilatation not present	Lost to follow-up
Yes	10	9	0	1
Equivocal	9	7	2	0
No	<u>16</u>	<u>4</u>	<u>10</u>	<u>2</u>
	35	20	12	3

1. Accentuation or mass in junction of the right and left lobes of the liver;
2. "Ducts" or defects not extending to the periphery, especially in the right lobe;
3. A linear defect particularly prominent in the left lobe with an oblique orientation to the axis of the junction of right and left lobes;
4. A stellate area of decreased isotope concentration in mid-right lobe or at junction of right and left lobes.

Representative examples of these criteria from the positive and equivocal categories are shown in Figures 2-5.

Figure 2. Three characteristic cases of biliary duct dilatation. In cases a and b note that the duct system is obliquely oriented to the junction of the right and left lobe; is particularly prominent in the left lobe; does not extend to the periphery (especially on the right); and causes an accentuation of the junction of the right and left hepatic lobes. In case c, a similar but more stellate appearance is seen. In cases a and c two camera fields have been spliced to form the image.

Phantom studies demonstrated that the lesion image is more distinct with the collection of one million counts (which we routinely collect on our scans) than with 200,000 counts (Figure 6). The image of the Y phantom is not noticeably degraded by a simulated respiratory excursion of 1 cm. With 2 cm of excursion discrimination of the lesion becomes difficult, and with 3 cm of excursion it is totally lost (Figure 7). When the rectilinear scanner is used, small excursions destroy fine detail of the limbs of the phantom tending to produce a central mass (Figure 8), as was reported by Gamil et al.⁴

Figure 3. Equivocal cases that were suggestive but certainly not diagnostic of biliary duct dilatation. We were able to place these scans into three general groups. Scans (a) and (b) demonstrate a vaguely characteristic pattern. Scans (c) and (d) show accentuation of the junction between the right and left lobes and a second defect in the right lobe. It is important to recognize this pattern so as not to confuse it with metastases. (e) and (f) typify the third pattern, which is that of a single lesion or a defect in the junction between the right and left lobes.

Figure 4. Two scans are shown with cholangiograms. Note that on the cholangiogram the common duct and distal portions of the right and left hepatic ducts are extrahepatic. In (b) the ducts immediately proximal to this area are coiled at the junction of the right and left lobes creating the defect on scan (a). Note that the caliber of the dilated ducts near the periphery of the liver is smaller than our current instrumentation can resolve. The operative cholangiogram (d) demonstrates the dilated hepatic duct faintly seen on scan (c). The anterior position of the left lobe and its characteristically thinner tissue allows the defect in the left lobe to be resolved.

Figure 5. Left - Scan made on 1/8/68 was interpreted as showing a defect between the right and left lobes suggestive of dilated ducts. The patient was explored and a carcinoma of the head of the pancreas obstructing the common duct was found. A cholecystojejunostomy was performed to decompress the biliary obstruction. Right - Scan made on 7/21/69 demonstrates that the central defect has disappeared and that the patient has developed large lesions in the right and left lobes interpreted as metastases. Autopsy later confirmed the metastases and that the biliary tree was not dilated.

Figure 6. Stationary image of the Y-phantom collecting 200,000 and 1,000,000 counts with similar film intensities.

Figure 7. Increased motion destroys the image of the Y-phantom. Stationary views of the phantom are shown to the right for comparison purposes.

DISCUSSION

The presence or absence of biliary duct dilatation was verified by cholangiography, surgery, autopsy or adequate medical follow-up. In some cases it was not possible to make an accurate assessment of the amount of intrahepatic biliary duct dilatation. At surgery, the intrahepatic ducts are not visualized by the surgeon, who reports only the diameter of the common duct. A common duct with a diameter of 2 cm or more is considered to be evidence for intrahepatic duct dilatation.

Figure 8. Top - Rectilinear scan using Ohio Nuclear 17 M collimators and information density of 1200 cts/cm². The limbs of the Y-phantom are discernible with stationary imaging. (The three linear areas of increased density represent an artifact presumably due to a Tc-Fe complex formed on the wire support.) The phantom was obliques 25° with reference to the scan raster in an attempt to simulate the biliary anatomy, thus accounting for the obliquity of the scan raster in the figure. Bottom - Scan made with same factors and information density with 3 cm of simulated respiratory motion. The limbs of the Y are lost and lesion is imaged as an ill-defined mass.

Two equivocal cases that showed no ductal dilatation were reviewed retrospectively using the four criteria established. The review demonstrated that these two cases (cirrhosis and chronic cholecystitis) did not satisfy the criteria but represented error in original interpretation. There

were four false negative cases: One case of intrahepatic stones with focal duct dilatation was interpreted as multiple masses, probably metastases, and would be misdiagnosed again since it did not fit any of our criteria; a second case in this category seemed to demonstrate faint characteristic criteria, and was considered to be at the resolution limit; the two other false negative cases were considered to be normal.

The first two criteria were met in every case interpreted as positive or equivocal. The third and fourth criteria were met in most but not all of the positive cases. In the equivocal cases, the latter two criteria were not evident or only faintly suggested.

An extrinsic mass in the porta hepatis without duct dilatation presented no problem in diagnosis. The mass caused by the enlarged bile ducts is superior to this region and can be recognized as a distinct defect within the confines of the liver. It is important to ascertain that there is no barium in the bowel in the region of the porta hepatis since this is a potential cause of a false positive scan.⁸

In our series, if the scan diagnosis was positive, there was a great likelihood that obstructive rather than hepato-cellular jaundice was present. Among the nine patients in this category, there were no false positive scans. An equivocal diagnosis was highly suggestive of the presence of dilatation since seven of nine patients in this category had dilated ducts. A negative scan is less reliable since some cases have dilated ducts which cannot be resolved with current instrumentation.

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A TECHNIQUE FOR IMAGING PATIENT TREATMENT AREA DURING A THERAPEUTIC RADIATION EXPOSURE*

By

A. G. Haus, S. M. Pinsky, and J. E. Marks

In the radiotherapeutic treatment of patients it is necessary to know that the anatomic area of interest is being irradiated accurately. The radiotherapist requires a precise method of verifying that internal organs to be treated are included but that adjacent normal tissues are not irradiated unnecessarily.

Several factors in the patient treatment set-up can be a source of error. These include:

(a) Shifting of patient's skin resulting in surface anatomy changes in relation to internal anatomy, which may be caused by changes in weight, the looseness of skin found in elderly people, and obesity; (b) Patient movement during treatment, which can occur in disoriented patients, or may be due to wriggling, coughing, sneezing, or a change of position for comfort; (c) Technician's errors in day to day set-up. It is therefore essential to have a quality control method of verifying that the lesion area is being irradiated accurately.

A patient set-up begins with proper location of the internal organs to be irradiated. For example, Figure 1 shows an abdominal radiograph with lead localization dots on the patient's skin. These dots are used to delineate the margins of an upper inverted y and splenic pedicle in a patient with Hodgkins disease. (This is part of the maltese cross set-up.) Figure 2 shows the treatment area outlined on the patient's skin. The gray areas indicate shielding of the lungs, larynx and upper abdominal organs by absorbing blocks on a lucite platform interposed between the patient and the source of radiation. Check radiographs are made to confirm that the treatment field is satisfactory.

The patient then undergoes a series of radiation treatment exposures. Periodic check films of the treatment portal are made. In many therapeutic radiation centers this technique consists of placing a film cassette containing films (e.g., Kodak Industrial Type AA or Type M) under the patient before treatment. The patient is set up in the normal manner and an exposure of perhaps several rads is made to record an image of the treatment area and adjacent anatomy on the film. The cassette is removed and the patient is again set up for the actual treatment exposure, it being assumed that the original set up is reproduced.

This technique is inefficient for several reasons: (a) an image from the actual treatment is not recorded; (b) valuable time is wasted in handling of the patient; and (c) in most cases films have to be hand processed.

A commercially available professional and graphic arts type of film called Kodak Fine Grain Positive Film (on acetate base) has been evaluated and used for imaging a complete patient treatment. The film is placed under the patient for a complete therapeutic radiation exposure and simultaneous imaging of the treatment field including internal anatomy. Treatment films and dose

*This report is taken from a paper that appeared in *Radiology*, 97:653, 1970. The work was supported in part by a grant from U. S. Public Health Service.

Figure 1. Radiograph of an abdomen with lead localization dots on the patient's skin.

Figure 2. The outlined treatment area on the patient's skin.

data have been recorded with both the Cobalt-60 and Van de Graaff generator supervoltage therapy machines. Maximum patient exit dose for exposure of the film ranged from 80 to 160 rads. The Kodak Fine Grain Positive Film was processed—developed, fixed and dried completely—satisfactorily in several of the rapid process X-Omats. Sensitometric curves of the film exposed to cobalt-60 are shown in Figure 3. Curve A shows films processed in a Kodak M6 X-Omat with Kodak RP chemicals (90 second process). Curve B shows films processed in a Kodak M3 X-Omat

Figure 3. Sensitometric curves of Kodak Fine Grain Positive Film exposed to cobalt-60. Curve A shows films processed in a Kodak M6 X-Omat with Kodak RP chemicals (90 second process). Curve B shows films processed in a Kodak M3 X-Omat with Kodak RP chemicals (converted to variable speed process set for 5 minutes). Curve C shows films exposed with lead foil screens and processed as for Curve A.

with Kodak RP chemicals (converted to variable speed process set for 5 minutes). Notice that the film developed by the 5 minute process has approximately twice the speed and a slightly higher contrast than the film processed at 90 seconds. Other Kodak M3 X-Omats (converted) using Kodak RP chemicals and processing times longer than 90 seconds but less than 5 minutes gave characteristic curves falling between curves A and B, but having similar shapes.

Treatment films made with a maximum exit dose of approximately 160 rads had similar film contrast for all processes investigated. When the maximum exit dose is decreased, the film contrast is lower at shorter processing times. To increase film contrast at short processing times, the film was exposed with commercially available lead foil screens (0.005 inch front screen and 0.01 inch back screen).¹ Curve C shows results of the film exposed with lead foil screens and processed at 90 seconds (as in Curve A). Comparing curves A and C, it can be seen that, by using lead foil screens, the film speed is increased by almost a factor of two without changing curve shape. Therefore by increasing film speed, higher film contrast can be obtained for shorter X-Omat processing times. It should be noted that lead foil screens can reduce scattered radiation and therefore increase subject contrast.¹

Figures 4, 5 and 6 are films of the patient shown in Figure 2. Figure 4 is a check film exposure using Kodak Industrial Type AA film and lead foil screens (exit dose approximately 1/2 rad), hand developed in Kodak Rapid X-ray developer. Figure 5 is a treatment film using Kodak Fine

Figures 4, 5 and 6 are films of the patient in Figure 2.

Figure 4. A check film exposure using Kodak Industrial Type AA Film and lead foil screens (exit dose approximately 1/2 rad). The film was hand developed in Kodak Rapid X-ray developer.

Figure 5. A treatment film using Kodak Fine Grain Positive Film and lead foil screens (entrance dose 312 rads, tumor dose 200 rads, and exit dose 112 rads). The film was processed in the Kodak M6 X-Omat (90 second process).

Figure 6. A treatment film using Kodak Fine Grain Film without lead foil screens. The exit dose and process are the same as the treatment film in Figure 5.

Grain Positive Film and lead foil screens (entrance dose 312 rads, tumor dose 200 rads, and exit dose 112 rads), processed in the Kodak M6 (90 second process) X-Omat. Note the similar contrast and image quality between the check film and the treatment film. Figure 6 is Kodak Fine

Grain Positive Film exposed without lead foil screens. The exit dose and process are the same as the treatment film on Figure 5. This film has lower overall film density resulting in lower film contrast, but anatomy and shielding in the treatment field can still be easily identified.

The developing characteristics of this film in various X-Omat processes are quite adequate, as the examples indicate. The major problem encountered is that this film is not directly transportable through an X-Omat process, partly because it is coated on an acetate base instead of a thicker polyester base. At the present time treatment films are taped to a film leader for transporting through the X-Omat. This merely consists of taping the treatment film with Scotch Magic Transparent Tape No. 810 to a film that has been previously processed by X-Omat, e.g., Kodak RPL Medical x-ray film on Estar. Unfortunately this technique is somewhat of a nuisance; the ideal situation would be to have a film of this type which was directly transportable through X-Omat processes.

For the radiotherapist a treatment recording film of similar quality to Kodak Fine Grain Positive Film, but directly transportable through an RP X-Omat would result in improved quality control for treatment verification and increased operational efficiency. The overall usefulness of recording a complete radiation treatment must be evaluated by the radiotherapist. Perhaps daily treatment films should be required in cases where a precise treatment set-up is necessary.

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EXCESSIVE PURINE SYNTHESIS AND NEUROLOGIC DYSFUNCTION IN CHILDREN*

By

L. B. Sorensen, F. Kawahara, D. Chow, P. J. Benke,[†] and L. Coben[‡]

In 1964 Lesch and Nyhan¹ first reported on a familial disorder of excessive purine synthesis and central nervous system function. Clinically, this sex-linked syndrome is characterized by choreoathetosis, spastic cerebral palsy, obsessive destructive behavior, mental retardation and hyperuricosuria. Children with this disorder have now been observed to present all the clinical manifestations of gout, including uric acid lithiasis, gouty nephropathy, acute arthritis and tophi. A virtually complete absence of the enzyme hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8., inosine monophosphate : pyrophosphate phosphoribosyltransferase) (HG-PRTase) in red blood cells from patients with the Lesch-Nyhan syndrome was reported by Seegmiller et al.² In addition it has been shown that brain, liver and other tissues obtained postmortem from a patient with this syndrome contained no measurable enzyme activity.³ The condition is a disease of the male, transmitted as an X-linked recessive character. Migeon et al⁴ demonstrated two cell populations of skin fibroblasts in a mother of two boys with the Lesch-Nyhan syndrome: one population with normal HG-PRTase, and a mutant population lacking this enzyme activity.

When assay of the same enzyme was extended to the gouty population at large, a partial deficiency of HG-PRTase was soon found in a small proportion of the patients.⁵ Neurologic deficits were not uncommon among those patients who had marked decrease in HG-PRTase activity. The accelerated uric acid production in HG-PRTase deficiency is derived from hypoxanthine that is insufficiently reutilized for inosine monophosphate formation and, as a consequence is catabolized excessively to uric acid.⁶

A case of a 13-year-old boy with neurologic signs and grossly excessive uric acid formation, symptomatically manifest by recurrent uric acid stone disease since the age of three has been reported by two of us.⁷ Subsequent study of his red blood cells showed only a moderately decreased activity of HG-PRTase. Recently, Rosenberg et al⁸ have reported a familial syndrome of hyperuricemia, modest increase in uric acid excretion, ataxia, deafness and renal disease that apparently is distinct from other types of familial nephropathy associated with deafness. Red blood cell HG-PRTase levels were normal in this kindred.

The present communication reports on metabolic and enzymatic studies in three boys with all the clinical features of the Lesch-Nyhan syndrome, the asymptomatic mother of one of these patients, and a fourth boy with mild neurologic deficit and excessive purine biosynthesis. A brief clinical description is included in order that the clinical manifestations may be correlated with the severity of the biochemical defect.

* This report is taken from a paper that appeared in *Arthritis and Rheumatism*, 13:835, 1970.

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MATERIALS AND METHODS

Plasma and urine uric acid and its immediate precursors, hypoxanthine and xanthine, were determined by enzymatic spectrophotometric methods. The miscible pool of uric acid and its rate of turnover were determined after intravenous injection of uric acid-2-¹⁴C. The incorporation of a precursor into purines was assessed by determining the concentration of ¹⁴C in urinary uric acid following intravenous injection of glycine-1-¹⁴C. The effect of azathioprine (4 mg/kg body weight per day) upon purine metabolism was studied in two patients by measuring the incorporation of glycine-1-¹⁴C into uric acid. Isotopic glycine was injected 96 hr after administration of azathioprine had begun. The inhibitory effect of allopurinol upon xanthine oxidase and its effect upon purine synthesis de novo were studied in three patients by summing the daily excretion of hypoxanthine, xanthine and uric acid. In one of the three patients the incorporation of isotopic glycine into the three oxypurines was also measured. The uricogenic compound 2-ethylamino-1,3,4-thiadiazole* was administered to three patients, either alone or in conjunction with nicotinamide. The dose of 2-ethylamino-1,3,4-thiadiazole was either 1 or 2 mg/kg body weight for a maximum of 3 days. The activities of HG-PRTase and adenine phosphoribosyltransferase (E.C. 2.4.2.7., adenosine monophosphate : pyrophosphate phosphoribosyltransferase) of dialyzed erythrocyte hemolysates were determined by a radiochemical method using a copper-loaded chelating resin for the separation of purines and nucleotides.

The reader interested in more detailed information on the methods used in these studies should consult Ref. #6.

RESULTS

The clinical data on the four boys and the mother of one of the patients with the Lesch-Nyhan syndrome are summarized in Table 1. In addition, this table contains values for purine phosphoribosyltransferase activities in erythrocytes so that the spectrum of clinical manifestations may be correlated with the activities of these two enzymes.

Mi.I. and Ma.I. are brothers. Although the younger brother had no evidence of self-mutilation at the time of the study, he began to bite his lips and fingers when he was 8 years old, necessitating the use of restraints. These two boys as well as patient D.T. exhibited all the characteristic features of the Lesch-Nyhan syndrome, i.e., mental retardation, spastic cerebral palsy, choreo-athetosis, and aggressive self-mutilating behavior. All had passed gravel and urinary calculi in the past and two had had surgical removal of stones.

HG-PRTase activity in erythrocyte hemolysates from these patients was about 2 per cent of normal. This value is at least one hundred times higher than the values reported by Kelley.⁹ Whether this discrepancy reflects differences in the assay technique or whether it means that some patients with the Lesch-Nyhan syndrome have small, but detectable activities of HG-PRTase remains to be resolved. These three patients had significant increases in the activity of adenine phosphoribosyltransferase, the enzyme that catalyzes the formation of adenylic acid from adenine and PRPP, corroborating previously reported findings by Seegmiller et al.²

Patient C.M. had only mild neurologic deficit. Since the age of three he had experienced recurrent episodes of crystalluria and hematuria reflecting marked hyperuricosuria. When he was 13 years old he had an episode of uric acid obstructive nephropathy with a BUN of 172 mg per

*IND #3973 pursuant to Section 505(i) of the Federal Food, Drug and Cosmetic Act.

Table 1
CLINICAL DATA AND ACTIVITIES OF PURINE PHOSPHORIBOSYLTRANSFERASES

Subject	Age (yr)	Weight (kg)	Gouty arthritis	Tophi	Nephro- lithia- sis	Neurological complications	Self- mutilation	Purine phosphoribosyltransferase activity (mμmole/mg protein/min)		
								Hypoxanthine mean ± S.D.	Guanine mean ± S.D.	Adenine mean ± S.D.
Normal values (26 subjects)								2.14 ± 0.15	2.49 ± 0.24	0.431 ± 0.05
Mi.I.	13	26	0	0	+	Choreoathetosis; double hemi- paresis; combined spasticity and rigidity; mental retarda- tion	Lips; hands	0.054	0.054	0.780
Ma.I.	6	20	0	0	+	Athetosis; mild double hemi- paresis; combined spasticity and rigidity; mental retarda- tion	Began at age 8 yr; lips and hands	0.043	0.049	0.706
D.T.	8	16	0	0	+	Athetosis; rigidity; spastic paraplegia; severe mental retardation	Lips; hands	0.034	0.047	0.890
C.M.	13	56	+	0	+	Choreiform movements; anti- social behavior	Self- induced alopecia over the vertex	1.35	1.09	0.843
N.T.*	33	80	0	0	0	0	0	1.49	1.83	0.318

* Mother of D.T.

cent and a plasma uric acid of 33 mg per cent. A detailed account of this case has been published elsewhere.⁷ The aberration of the activities of the phosphoribosyltransferase enzymes in this patient's red blood cell hemolysates was less spectacular than in the Lesch-Nyhan syndrome; the activity of the enzyme for hypoxanthine and guanine was about one-half of normal, whereas that for adenine was increased twofold.

The asymptomatic mother, N.T. (obligate heterozygote), had moderately decreased HG-PRTase activity, but no increase in adenine phosphoribosyltransferase activity.

The results of studies of the miscible pool and turnover of uric acid, and of the incorporation of glycine-1-¹⁴C into urinary uric acid are summarized in Table 2. Grossly excessive uric acid synthesis is evident in the four boys. The excretion of uric acid in the urine is greatly enhanced. When the daily production of uric acid is adjusted for differences in body weight, the value for this parameter ranges from 42.8 to 62.2 mg/day/kg body weight as compared to a normal value of 10 mg/day/kg. The percentage of the injected dose of radioactive uric acid that was recovered in the urine agreed well with the estimate based on the mean daily urate excretion and the computed daily turnover. Approximately three-fourths of the uric acid formed was excreted in the urine, indicating normal handling of uric acid at the renal level. On a purine-free diet the carrier mother had a urine uric acid excretion of 641 mg/day, which is significantly above the average value for normal women. On the other hand, uric acid production measured with isotopic uric acid was only marginally increased. The efficiency with which the mother excreted uric acid enabled her to maintain a normal plasma urate concentration.

The rate of de novo purine synthesis was assessed by following the incorporation of glycine-1-¹⁴C into urinary uric acid. Each of the four boys studied in this manner had evidence of excessive incorporation of isotopic glycine into uric acid. The cumulative recovery of isotope in urinary uric acid over a 7-day period after injection of glycine is given in Table 2. These values represent a 10- to 15-fold increase in the utilization of glycine for purine synthesis as compared to normal control subjects. In the carrier female the percentage of glycine incorporated into purine was slightly increased, as judged by the measured incorporation into urinary uric acid. When this value is corrected for extrarenal disposal of uric acid, the amount of glycine incorporated into produced uric acid was 0.27 per cent. In our hands normal values range between 0.15 and 0.20 per cent. The pattern of labeling of uric acid following glycine administration is shown in Figure 1 for three patients with the Lesch-Nyhan syndrome, one carrier mother and one normal subject. Strikingly rapid and excessive incorporation of precursor into urinary uric acid is evident in all patients with the Lesch-Nyhan syndrome, maximum isotope concentration being reached about 12 hr after administration of glycine. In two of the boys with the Lesch-Nyhan syndrome the isotope curve is distinctly biphasic. This finding is consistent with previously reported studies of the incorporation of glycine into hypoxanthine in patients with a deficiency of HG-PRTase in whom a second peak of radioactivity was observed in urinary hypoxanthine between 24 and 48 hr after injection of glycine.⁶ Data obtained in that study indicated that excess uric acid in HG-PRTase deficiency originates from hypoxanthine that is insufficiently reutilized and, instead thereof, catabolized to uric acid. The incorporation of glycine into uric acid in the carrier mother is of particular interest. Although the cumulative recovery of ¹⁴C in uric acid over a 7-day period was only marginally increased, the pattern of glycine incorporation deviated distinctly from the customary pattern observed in normal individuals. The isotopic curve was intermediate between that

Table 2
SUMMARY OF DATA ON URIC ACID METABOLISM

Patient	Uric acid							¹⁴ C-glycine in urine uric acid (7 days) (% of dose) [†]
	Plasma	Miscible pool	Turnover		Recovery from urine			
	(mg %)	(mg)	mg/day	<u>mg/day</u> kg	mg/day	% of turn- over	% ¹⁴ C uric acid	
Ma.I.	8.85	695	1,244	62.2	907	72.9	71.5	1.60
Mi.I.	8.74	869	1,454	55.9	1,054	72.5	77.4	1.47
C.M.	12.45	2,695	2,396	42.8	1,741	72.7	76.9	1.35
D.T.	7.73	-	-	-	840	-	-	2.08
N.T.*	4.58	850	821	10.3	641	78.1	82.1	0.21

* Mother of D.T.

[†] Net recovery of ¹⁴C-uric acid, i.e., values are not corrected for extrarenal disposal of uric acid.

Figure 1. Incorporation of glycine-1-¹⁴C into urinary uric acid in 3 patients with the Lesch-Nyhan syndrome (D.T., Ma.I., and Mi.I.), one heterozygous carrier (N.T.) and one normal subject. Specific activities are corrected to a dose of 1 μ Ci/kg body weight of glycine-¹⁴C.

observed in patients with the Lesch-Nyhan syndrome and that in normal subjects. Peak specific activities occurred within the first 12 hr.

The effect of two purine analogues known to affect purine metabolism was studied in several patients. Azathioprine (Imuran) is known to suppress purine biosynthesis in certain patients with primary gout.^{10,11} The mechanism of this inhibition is thought to be due to pseudofeedback inhibition, at the rate-limiting step, of phosphoribosylpyrophosphate amidotransferase by a nucleotide derivative of azathioprine or one of its metabolites.

Azathioprine in a dosage of 4 mg/kg/day was given orally in divided doses to two of the boys, Ma.I. and C.M., and had no discernible effect on the incorporation of glycine-1-¹⁴C into urinary uric acid, nor on plasma or urinary uric acid excretion of patient Ma.I. with the Lesch-Nyhan syndrome (Figure 2). The pattern of incorporation of isotopic glycine into uric acid did not change with administration of azathioprine. The 7-day recovery of isotope in urinary uric acid was 1.58 per cent during the azathioprine study and 1.60 per cent during the control period. The mean daily urinary uric acid excretion was 881 mg during azathioprine administration and 909 mg during the control period. The resistance of the effect of azathioprine on purine synthesis *de novo* in this patient can be attributed to the deficiency of HG-PRTase, since the antimetabolic effect of azathioprine depends on its conversion to ribonucleotide by HG-PRTase.

Figure 2. Effect of azathioprine on the incorporation of glycine-1-¹⁴C (1 μ Ci/kg body weight) into urinary uric acid (top), and on plasma urate and 24-hr urinary uric acid excretion (bottom) of a patient with the Lesch-Nyhan syndrome (Ma.I.).

Comparable results were obtained in a similar study of C.M.⁷ The 7-day recovery of glycine-¹⁴C in urinary uric acid was 1.35 per cent in the control study and 1.37 per cent in the azathioprine study, with mean daily urinary uric acid excretions of 1,741 and 1,778 mg in the two studies, respectively. Since the activity of HG-PRTase was only modestly reduced, the resistance of this patient to azathioprine may be related to insensitivity of phosphoribosylpyrophosphate amidotransferase to its normal allosteric feedback mechanism. This theory is currently being tested in fibroblast culture studies.

Allopurinol, another purine analogue, is a potent inhibitor of xanthine oxidase, the enzyme that catalyzes the conversion of hypoxanthine to xanthine and of xanthine to uric acid. In addition to the inhibitory effect on xanthine oxidase, allopurinol reduces purine biosynthesis in normal man, as demonstrated either by diminished excretion of total urinary oxypurine or by the reduction of glycine-¹⁴C incorporation into hypoxanthine, xanthine and uric acid.^{6,12} The mechanism of the inhibitory effect of allopurinol on purine synthesis may be related to higher concentration of hypoxanthine which results from the inhibition of xanthine oxidase and which—in the presence of HG-PRTase activity—leads to increased formation of inosinic acid via the reutilization pathway.

The effect of allopurinol upon the excretion of the major purines in patient Mi.I. with the

Lesch-Nyhan syndrome is shown in Figure 3. Substantial reduction in both plasma and urinary uric acid shows that xanthine oxidase is being inhibited effectively by allopurinol. However, the fact that the reduction in urinary uric acid is accompanied by an increase in the precursors, hypoxanthine and xanthine, to the extent that the total excretion of urinary oxypurines remains essentially unchanged, indicates that this boy is resistant to the effect of allopurinol upon purine biosynthesis. In the control period the mean urinary uric acid excretion was 6.27 mmoles (1,054 mg) with the immediate precursors making up an additional 0.2 to 0.3 mmoles. When the subject was on allopurinol, the total oxypurine excretion was 6.61 mmoles/day, of which hypoxanthine contributed 2.40 mmoles; xanthine 2.15 mmoles; and uric acid 2.06 mmoles. The ratio of hypoxanthine to xanthine was 1.12.

Figure 3. Effect of allopurinol on plasma urate and 24-hr urinary excretion of hypoxanthine, xanthine and uric acid in a patient with the Lesch-Nyhan syndrome (Mi.I.).

Essentially similar findings have been reported in patient D.T. with the Lesch-Nyhan syndrome.⁶ In his case the effect of allopurinol on the de novo purine biosynthesis was also measured by comparing the incorporation of glycine-1-¹⁴C into urinary uric acid in a control period with that of glycine into all three oxypurines during a period of treatment with allopurinol. The 7-day incorporation values for ¹⁴C were comparable in the two studies (2.77 per cent vs 2.72 per cent), indicating a failure of allopurinol to suppress purine biosynthesis in this condition.

Patient C.M., whose biochemical defect was different from that in the Lesch-Nyhan syndrome, was likewise refractory to the effect of allopurinol on de novo purine synthesis. During the control period he had a mean daily excretion of 10.36 mmoles of uric acid and between 0.7 and 0.8 mmoles of hypoxanthine plus xanthine. On 400 mg of allopurinol per day his daily uric acid excre-

tion fell to 2.05 mmoles, but at the same time there was a rise in urinary hypoxanthine to 5.73 mmoles/day and of xanthine to 5.04 mmoles. Also in this case, there was predominance of hypoxanthine over xanthine in the urine.

Of special interest were experiments to determine whether or not purine synthesis of these children is maximally stimulated. This question was examined by administering the uricogenic compound 2-ethylamino-1,3,4-thiadiazole (EAT) to three of the patients. In 1956 Krakoff and Magill reported a marked increase in uric acid production, which was not related to tissue destruction.¹³ Later, evidence was provided that the increase in uric acid production is due to a marked stimulation in the de novo synthesis of purines.^{14,15} The uricogenic effects of EAT and its oral toxicity could be blocked by the simultaneous administration of nicotinamide in equal or greater doses.^{14,15} The precise mechanism of action of EAT is poorly understood; it would appear that the compound interferes in some way with the operation of the normal homeostatic mechanisms that control purine biosynthesis.

In all studies with EAT special precautions were taken to ensure a high urine output and a urine pH of between 7.0 and 7.5. Figure 4 shows the results of studies on patient C.M. Administration of EAT resulted in a dramatic increase in plasma uric acid accompanied by a threefold increase in urinary uric acid excretion on the second day. This rise occurred with a dose of EAT which on a weight basis was considerably less than those used in previous studies in humans. When administration of EAT ceased, plasma and urinary uric acid returned promptly to their previous levels.

Figure 5 shows the effect of EAT and nicotinamide on plasma urate level and urinary uric acid in a patient, Mi.I., with the Lesch-Nyhan syndrome. EAT was administered orally in a dose

Figure 4. Effect of 2-ethylamino-1,3,4-thiadiazole on plasma urate and 24-hr urinary uric acid excretion in patient C.M.

Figure 5. Effect of 2-ethylamino-1,3,4-thiadiazole and nicotinamide on plasma urate and 24-hr urinary uric acid excretion in a patient with the Lesch-Nyhan syndrome (Mi.I.).

of 2 mg/kg body weight per day divided into three doses. Concomitantly, nicotinamide was given in a dose that blocks the uricogenic effect of EAT in control subjects. Nevertheless, a significant rise in uric acid production occurred. When administration of the compound was stopped, there was a parallel decrease to baseline values for both plasma and urine uric acid. Side effects in the form of glossitis and periorbital erythema appeared the day after EAT had been discontinued and lasted for 3 days. It follows that these patients are unusually sensitive to this compound.

As shown in Figure 6, prior and simultaneous administration of nicotinamide in a dose 15 times greater than that of EAT prevented the rise in plasma and urinary uric acid in patient Ma.I. with the Lesch-Nyhan syndrome. This boy received the smallest dose of EAT, i.e., 1 mg/kg body weight per day for 3 days.

Nicotinamide administered alone did not reduce the excessive production of uric acid in these children.

DISCUSSION

The purpose of the present report is to give an account of the clinical and biochemical features of four boys with neurologic manifestations associated with remarkable aberration of purine metabolism. Three of the boys presented with all of the clinical features of the Lesch-Nyhan syndrome, while the fourth patient, C.M., had only mild neurologic dysfunction and on clinical grounds did not appear to have the Lesch-Nyhan syndrome.

The patients with the Lesch-Nyhan syndrome had HG-PRTase activities in hemolysates of red blood cells which were only about 2 per cent of normal; these values are, however, significantly higher than those reported from Seegmiller's laboratory.^{5,9} As mentioned above, it is not clear whether this difference reflects different techniques or whether, indeed, some children with

Figure 6. Effect of 2-ethylamino-1,3,4-thiadiazole and nicotinamide on plasma urate and 24-hr urinary uric acid excretion in a patient with the Lesch-Nyhan syndrome (Ma.I.).

the Lesch-Nyhan syndrome may have small, but detectable activities of this enzyme. Red blood cell hemolysates from all of seven patients with the Lesch-Nyhan syndrome assayed in our laboratory have contained similar activities of HG-PRTase (with hypoxanthine as substrate the activity ranged from 1.1 to 2.5 per cent of the mean normal value). It is also of interest that Fujimoto and Seegmiller¹⁶ have reported that skin fibroblasts from patients with the Lesch-Nyhan syndrome contain 3 per cent of normal HG-PRTase activity.

Patient C.M. had a 50 per cent reduction of red blood cell HG-PRTase activity. The modest decrease in enzyme activity was felt to be insufficient to account for the severe derangement of purine metabolism in this patient. An alternative explanation, viz., a structural alteration in those subunits of phosphoribosylpyrophosphate amidotransferase concerned with the binding of feedback inhibitors, is currently being tested.

All of the patients had a four- to sixfold increase in uric acid production compared to control subjects of similar age and weight. The de novo purine synthesis was markedly accelerated as reflected in the incorporation of glycine-1-¹⁴C into urinary uric acid.

Allopurinol and azathioprine failed to suppress the rate of purine biosynthesis de novo in either condition. Allopurinol produced dramatic reduction in both plasma and urinary uric acid, but simultaneously there was an equivalent rise in the excretion of uric acid precursors. All three patients who were treated with allopurinol excreted more hypoxanthine than xanthine in the urine. This finding is in striking contrast to the situation in normal subjects treated with allopurinol^{6,17} and in patients with xanthinuria,^{18,19} a congenital absence of xanthine oxidase. In the latter condition xanthine constitutes about three-fourths of the total oxypurine. The present findings imply that hypoxanthine is the major source of the excessive uric acid production in HG-PRTase deficiency.

Studies with 2-ethylamino-1,3,4-thiadiazole have shown that purine synthesis is not maximally stimulated, pointing to the existence of regulatory mechanisms for the control of de novo purine synthesis in these children.

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A NEW ASSAY METHOD FOR HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE*

By

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A virtually complete deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8 inosine monophosphate : pyrophosphate phosphoribosyltransferase) (HG-PRTase), was first demonstrated by Seegmiller et al¹ in children with an X-linked neurological disorder associated with excessive de novo purine synthesis, known as the Lesch-Nyhan syndrome. Since then, a partial deficiency of this enzyme has been observed in certain gouty subjects who produce excessive uric acid.^{2,3} To facilitate studies of purine phosphoribosyltransferase (PRTase) activity, a rapid, enzymatic assay method is therefore important.

In this communication, we describe a method for the separation of purine and nucleotide and its utilization in the assay of purine phosphoribosyltransferase activity. Ligand chromatography⁴⁻⁶ is used to separate nucleotides from their corresponding nucleosides and purines. These bases and nucleosides are strongly adsorbed onto the ligand exchanger, while their nucleotides are not, thus permitting rapid and complete separation of purines from nucleotides.

MATERIALS AND METHODS

Preparation of ligand exchanger. Chelex 100 resin, sodium salt, 200 to 400 mesh (Calbiochem) was converted to the copper ammonium salt as described by Goldstein.⁶ The Chelex ligand exchanger was stored at pH 7 to 9 at 4° and under these conditions was stable for at least six months.

Preparation of micro-columns. Disposable Pasteur pipettes were plugged at the narrow end with fine glass wool and packed to a height of 10 to 12 mm with the Chelex ligand exchanger.

For the purification of ¹⁴C labeled purines, the column bed consisted of 32 mm of Chelex ligand exchanger on top of 5 mm of uncomplexed Chelex resin.

Purification of radioactive purines. 8-¹⁴C labeled hypoxanthine (54 mCi per mmole), guanine (31.6 mCi per mmole) and adenine (52.6 mCi per mmole) were obtained from Schwarz Bio-Research Inc., Orangeburg, New York, and purified in the following manner at 4°.

100 µCi of radioactive purine at pH 7 to 8 were passed through a Chelex ligand exchange column. The column was washed with 20 ml of water and then with successive 1.0 ml portions of 0.7 M NH₄OH until the radioactivity in the effluent was less than 20,000 cpm per ml (Figure 1). Radioactivity was measured by removing an aliquot, adding it to 2 ml of water and 15 ml of Bray's solution,⁷ and counting the sample in a Model 3003 Packard Tri-Carb scintillation spectrometer. The purine was eluted from the column with 7 ml of 3.0 M NH₄OH and the effluent was dried overnight in vacuo over P₂O₅. The residue was dissolved in sufficient 0.01 M NaOH to give 3.2 x 10⁷ cpm per ml. In order to minimize radioactive decomposition, the hypoxanthine and adenine solutions were stored frozen. Guanine was purified prior to use and stored at room temperature since stor-

*This report is taken from a paper that appeared in J. Lab. Clin. Med., 76:733, 1970.

Figure 1. Typical elution pattern of hypoxanthine from a Chelex ligand exchange column.

age at lowered temperature caused the purine to precipitate. It is estimated that commercially available radioactive adenine, hypoxanthine and guanine contain 2.5 per cent to 15 per cent of radioactive impurities. Rechromatography of the purine by the same procedure gave a single peak, whose R_f value was identical to an authentic sample when measured by ascending paper chromatography (Whatman #40 paper, 18 hrs, in 1-butanol:1-propanol:acetone:80 per cent formic acid:30 per cent trichloroacetic acid, in a v/v ratio of 8:4:5:5:3).

PRPP. The dimagnesium salt of 5-phosphoribosyl-1-pyrophosphate (PRPP), purchased from P-L Biochemical Co., Inc. (Milwaukee, Wis.), was purified and converted to the sodium salt by the method of Flaks.⁸ Analyses of a PRPP acid hydrolysate for ribose⁹ and phosphate¹⁰ showed that PRPP was better than 95 per cent pure. The concentration was determined by incubating an assay mixture (see below) containing 0.16 mM hypoxanthine (sp. act., 1.1 μ Ci per μ mole) with an excess of purified HG-PRTase and an aliquot of dilute PRPP. The PRPP solution was then diluted to a concentration of 10 μ moles per ml and stored frozen.

Tris, hypoxanthine, guanine, adenine and other reagents were of the best grades available.

Enzyme preparation. Human blood was drawn by venipuncture and centrifuged at 1,000 x g for 10 minutes; the plasma and the buffy coat were removed with a Pasteur pipette. The erythrocytes were washed three times with two volumes of 0.9 per cent NaCl. The washed cells were lysed by adding two volumes of water and the stroma was removed by centrifugation at 20,000 x g for 20 minutes. The supernatant was dialyzed overnight in the cold room against 100 volumes of 0.01 M sodium/potassium phosphate buffer at pH 6.5.

Protein measurement. The protein concentrations were measured by the biuret method of Gornall et al¹¹ in which a control for the color generated by alkaline hemoglobin was included. Crystalline serum albumin (Pentex Biochemicals, Kankakee, Ill.) was used as the standard for protein measurement.

Assay. The assay mixture consisted of 40 mM Tris-Cl buffer, pH 7.4; 5.0 mM MgCl₂; 0.48 mM of radioactive purine (sp. act. 0.4 μ Ci per μ mole); 1.0 mM PRPP and 0.4 to 1.3 mg of hemolysate protein in a final volume of 250 μ l. After incubating the mixture for 5 minutes at 37°, the reaction was initiated by adding PRPP and terminated 10 minutes later by adding 4.0 ml of an ice-cold, 0.25 mM solution of the corresponding nucleotide. The resulting mixture was applied to a 5 x 12 mm Chelex ligand exchange column and the column was washed with water until 10 ml of effluent had been collected in a volumetric flask. Two ml of the effluent were added to 15 ml of Bray's solution and the sample was counted in a liquid scintillation counter. With each assay a sample without PRPP was included as a control. The total amount of convertible substrate in the HG-PRTase reaction was determined by incubation with an excess of purified HG-PRTase. In general more than 90 per cent of the hypoxanthine and guanine was converted to their corresponding nucleotides. Enzymatic activity was calculated on the basis of total convertible substrate present in the assay mixture, and the specific activity was expressed as m μ moles of nucleotide formed per minute per mg of protein at 37°. In the case of adenine, where a purified A-PRTase (E.C. 2.4.2.7 adenosine monophosphate:pyrophosphate phosphoribosyltransferase) was not available, calculations were based upon the amount of adenine present in the reaction mixture.

For the assay of HG-PRTase in hemolysates from patients with the Lesch-Nyhan syndrome, the reaction mixture differed from that described above in that 1 μ Ci of radioactive purine (sp. act. 30 to 50 μ Ci per μ mole) was used. In addition, hemolysate protein was limited to 0.1 to 0.5 mg and the incubation time was extended to 1 hour.

RESULTS AND DISCUSSION

In this study, the reaction rate for the purine substrates was linear for the first ten minutes (Figure 2). When the guanine concentration was lowered to 0.14 mM,^{1,2,12-14} the reaction rate followed zero order kinetics for only the first three minutes. The reaction rate was found to be directly proportional to the amount of protein used within a range of 0.15 to 1.2 mg (Figure 3).

Table 1 shows the PRTase activities measured in the erythrocytes of normal individuals. The normal values for HG-PRTase activity reported here are higher than those in the literature.^{1,2} We suggest that this is due to the use of highly purified purines, the zero order kinetics in our assay system and the better than 99 per cent recovery of the nucleotides from the ligand exchange column.

All the gouty patients studied are characterized by overproduction of uric acid. In this group, four patients have essentially normal HG-PRTase and A-PRTase activities, while one has a partial deficiency of the first enzyme, equivalent to approximately 12 per cent of the normal values. This patient, A.D., has had recurrent acute gouty arthritis since age 22, and on clinical grounds, he cannot be distinguished from other patients with primary gout. Detailed studies on his purine metabolism have been reported elsewhere.^{15,16} In agreement with the data published by Kelley et al,² patient A.D. also had a 50 per cent increase in the A-PRTase activity of his erythrocytes.

All three patients with the Lesch-Nyhan syndrome presented the classic symptoms of choreo-athetosis, spastic cerebral palsy, mental retardation and compulsive self-mutilation. They all had

Figure 2. Formation of nucleotide as a function of time.

Figure 3. Formation of IMP as a function of enzyme concentration.

Table 1
PURINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN THE ERYTHROCYTES
OF VARIOUS INDIVIDUALS

Subjects	Purine phosphoribosyltransferase activity (m μ mole/mg protein/min)		
	Hypoxanthine mean $\pm$ S.D.	Guanine mean $\pm$ S.D.	Adenine mean $\pm$ S.D.
Normals (26)	2.14 $\pm$ 0.15	2.49 $\pm$ 0.24	0.431 $\pm$ 0.049
Gout: Overproduction of uric acid			
Normal PRTase activity			
M.D.	1.52	1.93	0.400
E.K.	2.22	2.40	0.462
D.K.	1.75	2.24	0.497
E.L.	1.60	1.73	0.326
Decreased PRTase activity			
A.D.	0.260	0.320	0.661
L-N syndrome: D.T.	0.034	0.047	0.890
M.I.	0.054	0.054	0.780
Ma. I.	0.043	0.049	0.706
Heterozygote carrier of L-N; N.T.	1.49	1.83	0.318

hyperuricemia and hyperuricosuria. HG-PRTase in their erythrocytes was about 2 per cent of normal. In previous studies HG-PRTase activity was less than 0.01 per cent of normal for hypoxanthine and less than 0.004 per cent of normal for guanine.¹⁵ HG-PRTase activity associated with the Lesch-Nyhan syndrome will be the subject of a future publication. Significantly increased A-PRTase activities were also found in the red cells of these patients.

The usual methods for the separation of nucleotides from purines include ion-exchange chromatography, paper chromatography, thin-layer chromatography and high voltage electrophoresis. These techniques can be used individually, in combination, or in sequence to achieve the desired separation of purine from nucleotide. These methods are however cumbersome and often time-consuming. Thus, in the assay of HG-PRTase activity, ligand exchange chromatography as presented here has distinct advantages. The method is rapid, sensitive and offers a high degree of resolution, reproducibility and recovery.

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MIGRATORY INHIBITION OF SENSITIZED PERITONEAL MACROPHAGES
BY TUMOR-SPECIFIC TRANSPLANTATION ANTIGEN
IN MORRIS-HEPATOMA 5123*

By

A. P. Kyriazis, R. W. Wissler, and K. Dzoga†

The inhibition of migration of macrophages as an indicator of the presence of delayed hypersensitivity to an homologous antigen has been extensively studied.¹⁻³ Both David⁴ and Bloom et al^{5,6} have advanced the hypothesis that sensitive peritoneal exudates or lymph node lymphocytes, upon interacting with antigen in vitro, elaborate a low molecular weight protein, the migration inhibitory factor (MIF), which reacts with the macrophages to suppress their migration. This phenomenon has been shown to be highly specific in detecting the presence of the sensitizing antigen, and its use in identification of a specific antigenic component in a mixture of various macromolecular substances is feasible. With this in mind, we have used inhibition of migration of sensitized peritoneal macrophages as a method of studying the presence of a specific tumor transplantation antigen in Morris hepatoma 5123, and its absence in normal liver tissue from which the tumor originated.

MATERIALS AND METHODS

Animals. Adult male rats of both Buffalo and Sprague-Dawley strains, their weight ranging from 300-350 g, were used throughout.

Glassware. All glassware was siliconized (Silicad No. J-600, Clay Adams) to prevent cells from adhering to it.

Antiserum production. Antiserum to the tumor was produced in a special hybrid strain of rabbits as described by Wissler et al.⁷

Absorption of antiserum. Absorption of antiserum was performed as follows. Normal serum and homogenates of livers, spleens, lungs, and kidneys from 10 Buffalo rats were pooled separately. To 2 ml of antiserum 0.5 ml of each pool was added at room temperature. The mixture was kept overnight at 4°. It was then centrifuged at 5,000 rpm for 1 hr, after which the supernatant was removed and tested in gel immunodiffusion plates against normal tissue and tumor extracts. The plates were kept at room temperature for 72 to 96 hr under continuous inspection. Antisera that reacted with normal tissue extracts were absorbed for a second or even a third time. In most cases, however, the second absorption was adequate to eliminate any nonspecific reaction with normal tissues.

Antigen preparation. Morris hepatoma tumors of the line 5123 (45th transplant generation) growing in Buffalo rats were removed, minced with scissors and passed through a stainless steel hand-operated press homogenizer to remove most of the connective tissue. The homogenate was suspended in ice-cold saline-0.3 M sucrose, and cell disruption was carried out by freezing the

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homogenate in a bath of dry ice-acetone. Cell membranes were isolated according to the method described by Mann et al.⁸ The isolated cell membranes were incubated with papain (crude papain type II, Sigma) for 1 hr at 37°. Digestion was interrupted by adding iodoacetamide to the incubation mixture to a final concentration of 0.05 M. The digested membranes were centrifuged in a Spinco preparatory ultracentrifuge at 52,038 g for 1 hr. The supernatant was dialyzed overnight in a phosphate buffer (0.01 M, pH 6.2) and fractionated in a CM-Sephadex C-25 column (0.9 x 30 cm). Stepwise elution gave the chromatogram shown in Figure 1. Antigenic activities of the eluted

Figure 1. Fractionation of papain-digested tumor cell membranes on CM-Sephadex C-25 column. Stepwise elution: phosphate buffer, pH 6.2; A: 0.01 M; B: 0.05 M; C: 0.1 M; column bed: 0.9 x 30 cm.

peaks were studied in Ouchterlony micro-diffusion slides and with immunoelectrophoresis. Specific antigenic activity, as demonstrated by using repeatedly absorbed antisera to the tumor, was present in the first peak. This was the partially purified tumor antigen (PPTA) used in the present study. Normal liver tissue from the strain of rats in which the tumor was growing was also processed as described above. The final product was the normal liver antigen (NLA).

Sensitization. One volume of PPTA (protein concentration in PPTA, 8 mg/ml) was mixed with an equal volume of Freund's incomplete adjuvant (Difco Lab.) and half that volume of Borde-

tella pertussis vaccine* (Lilly, strain 37790, Lot T-59 187, Eli Lilly and Co., Indianapolis, Indiana). Each Sprague-Dawley rat was sensitized with 0.5 ml of this antigen emulsion injected intradermally in 6-9 places.

Peritoneal macrophage preparation and migration. Ten days after sensitization, peritoneal macrophage production was stimulated by injecting 15 ml of a light mineral oil (Marcol 52, Enco) into the peritoneal cavity of each rat. Control rats received the same amount of oil. Both sensitized and control animals were bled to death 72 hr after injection of the oil, and the peritoneal content was washed twice with about 100 ml of Eagle's minimal essential medium† (MEM) containing 100 units of crystalline penicillin per ml. The washings were filtered through multilayered cheesecloth, and the aqueous phase was separated from the oil in a separatory funnel. The cell-containing aqueous phase was centrifuged in an international centrifuge at 1200 rpm for 10 min to sediment the cells. The packed cells were washed twice with MacCoy's 5a medium by centrifuging them at 900 rpm for 5 min each time, and finally incubated for 30 min at 37° in a 1:10 dilution (cells to medium). At this stage a differential cell count showed 80-85 per cent macrophages with about 95 per cent viable cells as indicated by exclusion of trypan blue.⁹ Small microcapillary tubes were filled with the cell suspension, sealed at one end with flame and centrifuged for 1 min in an International microcapillary centrifuge model MB to pack the cells. The filled tubes were cut at the cell fluid interface, and the cell-containing portion was mounted in MacKanness-type chambers of 1 ml capacity.¹⁰ Two capillary tubes were fixed in position in each chamber with silicon grease. The chambers were sealed with silicon grease and filled with MacCoy's 5a medium containing 30 per cent fetal calf serum. All chambers were incubated for 48 hr. The areas of macrophage migration were read 24 hr after the incubation had started using an inverted unitron phase contrast microscope.

Peritoneal cells from each animal of the control and sensitized group were placed in separate incubation chambers to establish the following cell-culture-groups: nonsensitized control without antigen; nonsensitized control with NLA; nonsensitized control with PPTA; sensitized (control) without antigen; sensitized with homologous (PPTA) antigen; sensitized with heterologous (NLA) antigen.

Tissue cultures (subgroups) from five control and five sensitized animals were tested simultaneously at any time under identical experimental conditions.

After the experiment all subgroups from each group were statistically compared with each other. Since there was no significant difference among subgroups of any group, all subgroups of a group were combined for further statistical evaluation.

Whenever PPTA or NLA was used its concentration was adjusted to 100 µg protein per chamber.

RESULTS

The results of the migration test are given in Figure 2 and Table 1. Each point on the diagram (Figure 2) represents the migration of cells from a single capillary tube, the brackets indicating one standard deviation from the mean. Table 1 shows the difference between means, the

* Obtained from Dr. D. Rowley, Department of Pathology, University of Chicago.

† Medium 199 was compared with MEM in this step. There was no appreciable difference between results obtained with MEM and 199.

Figure 2. Migration patterns of peritoneal macrophages from normal and sensitized animals before and after the addition into the culture medium of liver (NLA), or tumor (PPTA) antigen. Each point represents the migration of cells from a single capillary tube. Brackets encompass one standard deviation around the mean. (●), normal nonsensitized cells; (○), normal nonsensitized cells + PPTA; (◐), normal nonsensitized cells + NLA; (◑), sensitized cells without antigen; (◒), sensitized cells + NLA; (◓), sensitized cells + PPTA.

percentage change, and the level of significance for each set of compared groups.

The migration of nonsensitized-control cells is practically unaffected by the addition into the incubation medium of either NLA or PPTA (Table 1). The observed decrease of migration by 1.3 mm^2 and 1.1 mm^2 , percentage changes of 4.2 and 3.5 respectively, is statistically not significant at the 95 per cent level of significance.

Comparing the migration patterns of nonsensitized and sensitized-control groups, however, we find that the sensitized cells respond differently. There is a definite decrease in migration of sensitized cells even in the absence of sensitizing antigen from the incubation medium. The 11.5 per cent change is found to be significant.

The migration of the same sensitized cells is further decreased when challenged with NLA. The addition of heterologous antigen to the incubation medium reduces migration by 11.1 mm^2 , a percentage change of 41.1. This same cell preparation responds maximally to the presence of homologous antigen (PPTA). The mean migration value is only 2.5 mm^2 as compared to the 27.0

Table 1

STATISTICAL ANALYSIS OF THE MEAN MIGRATION AREAS OF PERITONEAL
MACROPHAGES FROM NORMAL AND SENSITIZED ANIMALS BEFORE
AND AFTER THE ADDITION TO THE CULTURE MEDIUM
OF NLA* OR PPTA†

Groups compared		Difference between means	Change (%)	P(t)
Normal nonsensitized	Normal nonsensitized + NLA	1.30	4.20	>0.05
Normal nonsensitized	Normal nonsensitized + PPTA	1.07	3.50	>0.10
Normal nonsensitized	Sensitized	3.52	11.54	≤0.001
Sensitized	Sensitized + NLA	11.08	41.06	<0.001
Sensitized	Sensitized + PPTA	24.45	90.62	<0.001

* Normal liver antigen.

† Partially purified tumor antigen.

mm² of the sensitized-control cells, and 16.0 mm² of the same group challenged with the heterologous antigen (NLA). This value represents a 90.6 per cent decrease in migration. The effects of different concentrations of challenging antigens upon the migration of sensitized cells were then evaluated. It was found that the response of sensitized peritoneal macrophages decreased with decreasing amounts of NLA. At a final concentration of 40 µg of NLA no inhibitory effect could be detected.

Variations in concentration of PPTA were found to be less critical in inhibiting migration of these cells. Sensitized cells responded well even at a concentration of 30 µg of PPTA. Below this level, however, PPTA was less effective, and concentrations as low as 20 µg were inadequate in inhibiting the migration of sensitized cells.

DISCUSSION

Although the mean migration values of control nonsensitized cells show a slight variation in the presence of either NLA or PPTA, they remain within the 95 per cent confidence limits and the observed differences cannot be considered as significant.

On the other hand, the difference between the mean values of control nonsensitized and control sensitized cells is much more pronounced, the migratory ability of the sensitized cells being less than that of the nonsensitized cells. It is evident that sensitization has a suppressive effect upon migration. This observation has also been reported by Taubler and Mudd who observed that staphylococcal-sensitive cells from splenic explants failed to migrate as well as nonsensitive cells.¹¹

The decreased migration of sensitized cells when challenged with NLA may be the expression of the presence of antigenic cross-reactivity between hepatoma and normal liver tissue. However, by further diluting both NLA and PPTA the migration values of the sensitized cells challenged with NLA were restored to that of unchallenged cells, although the same dilution did not increase the migration of sensitized cells to which the PPTA was added. A 1:3 dilution of the challenging antigen seemed adequate in greatly reducing the nonspecific suppression of migra-

tion. By this method it was possible to clearly dissociate the tumor-specific reaction and to reach a point where the test reflected only the tumor-specific activity. These effects of dilution may suggest that there are quantitative rather than qualitative differences between normal liver tissue and hepatoma. Although this hypothesis cannot be rejected completely, the results of carefully controlled immunoelectrophoretic studies and a further step in the purification of PPTA do not support it. Furthermore, the results of the present study confirm previous observations which indicate by more indirect means the presence of a specific tumor transplantation antigen in the Morris hepatoma 5123.¹²

While this work was in progress, Kronman et al used the inhibition of migration of sensitized macrophages to demonstrate the presence of tumor specific antigens in a guinea pig hepatoma.¹³

All these data clearly demonstrate that the inhibition of migration of macrophages by sensitized lymphocytes may be helpful in studying the presence of tumor-specific transplantation antigens. Its usefulness, however, cannot be critically appraised until more information is available from application in different systems.

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ON THE MECHANISM OF ERYTHROPOIETIN-INDUCED DIFFERENTIATION
VIII. THE EFFECT OF IRON ON STIMULATED MARROW
CELL FUNCTIONS*

By

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The initiation of red blood cell differentiation in vivo and in vitro by erythropoietin is accompanied by a number of biochemical changes in cell function. The most striking of these is the new synthesis of hemoglobin, but prior to the onset of this, erythropoietin causes an increase in iron-uptake by marrow cells in vitro.¹ This increase is due, in part, to the induced synthesis of ferritin and at least one other non-heme, iron-containing, protein. Since the predominant function of cells that have been induced toward becoming erythrocytes is the eventual synthesis of large amounts of hemoglobin, we thought it advisable to find out whether erythropoietin action in vitro was limited by the availability of exogenous iron. It seemed probable that the amount of iron available would be limiting, since the culture medium used in experiments with bone marrow cells was not designed for that purpose and contains only the amount of iron present in the serum. This small amount of iron is not replenished in culture as it is in vivo. The experiments reported here show clearly that the erythropoietin effect on marrow cells in vitro is potentiated by addition of iron to the medium.

METHODS AND MATERIALS

Rat marrow cell cultures were carried out as described in earlier papers of this series,^{2,3} any special conditions being noted in the legends to the figures and tables. Since earlier work from this laboratory showed that essentially all the labeled heme isolated from marrow cells was derived from hemoglobin,⁴ we used the incorporation of ⁵⁹Fe into heme as an indication of hemoglobin synthesis. Two different methods of extracting labeled heme from the cells were used, (1) direct extraction from the acidified cell lysate with cyclohexanone, and (2) the procedure of Fox and Thomson.⁵ The former method is a slight modification of the method of Teale⁶ in which cyclohexanone replaces butanone because of its lower solubility in water. The direct extraction method is very simple and rapid, but after using it for some time, we found that a small amount of non-heme iron was extracted. In cells incubated for more than 48 hours this component, negligible at shorter intervals, accounted for an appreciable fraction of the extracted radioactivity. For this reason, the Fox and Thomson method, which does not yield any non-heme radioactivity, was used in some of the experiments.

Iron uptake was determined by counting the cell pellet which had been washed three times with 5 ml of a slightly hypertonic salt solution (0.145M NaCl, 0.013M potassium phosphate, pH 7.3). Measurement of total RNA synthesis and sucrose gradient analysis of marrow cell RNA were described earlier.³ Total protein synthesis was measured by the incorporation of ¹⁴C-

* This report is taken from a paper that appeared in *Biochim. Biophys. Acta*, 217:461, 1970.

[†] Life Insurance Medical Research Fund Fellow.

valine into the trichloroacetic acid insoluble fraction; counting was done as described for total RNA synthesis. Labeled iron (^{59}Fe) was counted in a Nuclear-Chicago model 4227 counter while ^3H and ^{14}C were counted in a Packard model 3375 liquid scintillation spectrometer.

The source of carrier iron was reagent grade ferric nitrate added to rat serum to give a final concentration of $73\ \mu\text{M}$. Erythropoietin, a gift from the National Blood Resources Branch of the National Heart Institute, was a step IV preparation with a potency of 15 units per mg of protein. Purified human transferrin was prepared by Dr. A. Dahlberg⁷ in this laboratory.

RESULTS

The data in Figure 1 show the relationships between the amount of iron added and the stimulation of both iron uptake and hemoglobin synthesis by erythropoietin. In both processes, the control cells (no erythropoietin) showed a linear increase that reached a plateau at about 3.6 n moles of added iron per ml. When erythropoietin was present, the curves were sigmoidal, reaching very

Figure 1. Effects of increasing amounts of iron on iron-uptake and hemoglobin synthesis. Cultures contained 15×10^6 nucleated cells per ml. Erythropoietin, at 0.085 units/ml, and the indicated concentrations of iron, in rat serum 100% saturated with iron, were added at the start of incubation. At 25 hours, each group received sufficient iron in rat serum, so that all groups had 7.2 n moles/ml. Then $1.0\ \mu\text{Ci}$ of ^{59}Fe (1.4 n moles) in 0.05 ml of rat serum was added to all groups. Incubation was stopped after a 4 hour pulse. Hemoglobin synthesis was determined by the method of Fox and Thomson. Each point is the mean of quadruplicate, 1.0 ml cultures. Control (○—○); erythropoietin (●—●); Δ cpm due to erythropoietin (▲—▲). Iron uptake is in (A), and hemoglobin synthesis in (B).

much higher plateau levels at about the same iron concentration. The ability to take up iron and synthesize hemoglobin in both control and erythropoietin-treated cells clearly increased with increased exogenous iron.

Iron, at a concentration in the plateau region, produced a striking effect on the rates of hemoglobin synthesis (Figure 2). This effect increased with time of incubation, being sixfold or greater on erythropoietin stimulation at 28 hours, and diminishing, though still large, after that.

Figure 2. Time course of the iron effect on hemoglobin synthesis. Triplicate cultures contained 18×10^6 nucleated cells in 1.0 ml of medium. Carrier iron (3.6 n moles of iron in 0.05 ml of rat serum), and 0.05 units/ml of erythropoietin were added at the start of incubation. At the indicated times, cultures were pulsed for 4 hours with 1.0 μ Ci of ^{59}Fe in 0.05 ml of rat serum, and the rate of hemoglobin synthesis was determined by cyclohexanone extraction. The ordinate indicates the % increase in hemoglobin synthesis due to iron (●—●), erythropoietin (○—○), and erythropoietin plus iron (▲—▲).

We tested the effects of rat serum and iron, separately and in combination, on iron-uptake and hemoglobin synthesis with the results shown in Table 1. Under the conditions used, both of these functions responded only slightly, or not at all, to erythropoietin in the absence of added

Table 1

EFFECTS OF IRON AND RAT SERUM ON ERYTHROPOIETIN ACTION

Addition	Fe uptake			% stimulation	HB synthesis			% stimulation
	Control cpm	Stimulated cpm	Δ cpm		Control cpm	Stimulated cpm	Δ cpm	
A None	3650	4250	600	16	21	24	3	-
B Iron	6150	7910	1760	28	53	123	70	132
C Rat serum	9070	11710	2640	29	125	684	559	446
D Iron + rat serum	4770	7990	3220	67	30	890	840	1700

Cultures contained 12×10^6 nucleated cells per ml: A, no additions; B, 3.6 n moles of Fe^{3+} ; C, 0.05 ml of rat serum and D, 0.05 ml of rat serum containing 3.6 n moles of iron, at zero time. Erythropoietin, at 0.10 units/ml, was added to the stimulated cultures at the start of incubation. After 40 hrs they received, iron plus rat serum, serum alone, iron alone or none of these, and all received 1 μCi of ^{59}Fe in an additional 0.05 ml of rat serum. The cultures were stopped at 44 hrs, and hemoglobin was determined by the Fox and Thomson method. The results are expressed as the means of triplicate 1.0 ml cultures.

Table 2

EFFECT OF IRON BOUND TO HUMAN TRANSFERRIN ON ERYTHROPOIETIN ACTION

	No added iron			% stimulation	Iron-transferrin added			% stimulation
	Control cpm	Stimulated cpm	Δ cpm		Control cpm	Stimulated cpm	Δ cpm	
Total protein synthesis	250	270	20	8	280	400	120	43
Iron uptake	1080	1190	110	10	2100	2840	740	35
Hemoglobin synthesis	130	160	30	23	310	870	560	180

Cultures contained 18×10^6 nucleated cells per ml. Purified human transferrin (saturated with iron) at a final concentration of 0.35 mg per ml was added to half of the dishes at zero time (this transferrin concentration is equivalent to 7.8 n moles of Fe^{3+} per ml). Erythropoietin, at 0.05 units/ml, was added to the stimulated groups. One set of cultures was pulsed for 30 min with 0.2 μCi /ml of ^{14}C -valine at 26 hrs. Another set was pulsed for 4 hrs with 1 μCi /ml of ^{59}Fe bound to 0.35 mg of human transferrin beginning at 35 hrs. Hemoglobin synthesis was evaluated from the incorporation of ^{59}Fe into heme isolated by the cyclohexanone method. The results are expressed as the means of triplicate 1.0 ml cultures.

iron. Iron alone, as ferric nitrate, had a considerable effect on both control and stimulated cells, as did rat serum without additional iron. This latter result was probably due to the endogenous iron content of the rat serum (approximately 18 μ M). It is also possible that rat apotransferrin could make iron bound to the new-born calf transferrin available to the rat marrow cells by exchange. The combination of iron and rat serum permitted the largest erythropoietin effect; in the case of iron-uptake the absolute effect of the two in combination was less than the sum of the separate effects, while for hemoglobin synthesis it was more than additive. Experiment D in Table 1 shows a lower control iron-uptake and hemoglobin synthesis when the first phase of incubation was done in the presence of both iron and rat serum than with either one alone. This apparent anomaly can be explained if one considers that the cells incubated for 40 hours under conditions of maximal iron incorporation contained a larger pool of unlabeled intracellular iron capable of diluting the labeled iron during the later four hour incubation period.

The possibility that the effect of the rat serum used in the previous experiment was due to some factor peculiar to rat serum, rather than to transferrin, was tested in a similar experiment in which purified human transferrin replaced rat serum. Dahlberg⁷ has shown that human transferrin is about 60 per cent as effective as rat transferrin in delivery of iron to rat marrow cells. When we used it as the source of exogenous iron, the erythropoietin effect showed an increase, due to iron-transferrin, of about 5-fold, 3-fold, and 8-fold for total protein synthesis, iron-uptake and hemoglobin synthesis, respectively (Table 2).

Shortly after erythropoietin is added to marrow cell cultures it causes complex changes in RNA synthesis.³ The addition of iron to similar cultures had only a small effect on RNA formation; there was an increase of about 40 per cent in both control and stimulated cells at three hours of incubation, and of about 80 per cent at 28 hours, although at the later time there was, as expected, no erythropoietin effect. It was important to determine whether iron addition caused qualitative changes in the pattern of RNA synthesis. The data in Figure 3 show that iron increased the RNA radioactivity throughout the sucrose gradient profile without appreciably altering the qualitative patterns. Furthermore, the presence of iron did not affect the magnitude of the erythropoietin response anywhere in the gradient.

When iron was first withheld from the cells and then added at later times during incubation we found an apparent first order decay of erythropoietin responsiveness (Figure 4). The loss of responsiveness is more rapid with respect to hemoglobin synthesis than with respect to iron uptake; there was a half life of 15-16 hours for the former and of 20 hours for the latter. This experiment shows, in addition, that the loss of erythropoietin responsiveness due to the absence of iron can be partially restored by adding it at a later time.

DISCUSSION

The effects of iron described in this paper may not have a common underlying mechanism. Iron, obviously, is required for hemoglobin synthesis and in its absence, the switch from general cellular protein synthesis to the specific formation of hemoglobin would be impaired. The effect of iron on the cellular uptake of iron may be analogous to the situation in liver⁸ and HeLa cells,⁹ where iron induces the biosynthesis of ferritin, which may act to sequester iron intracellularly, causing a shift from extracellular to intracellular iron. We know that erythropoietin induces the synthesis of ferritin and at least one other iron-containing, non-heme, marrow cell protein;¹ this action of erythropoietin may be mediated by the prior synthesis of a mechanism

Figure 3. Effect of iron on the pattern of RNA synthesis. Triplicate cultures, containing 20×10^6 nucleated cells per ml in 2.5 ml of medium, were incubated in 60 x 15 mm tissue culture dishes. At zero time, 8.6 n moles of iron, in 0.12 ml of rat serum (Δ — Δ), 0.10 units/ml of erythropoietin ($\circ$ — $\circ$), or iron plus erythropoietin (Δ — Δ) were added to the indicated cultures. Control cultures ($\bullet$ — $\bullet$) received no additions. At 5 hours of incubation, all cultures were pulsed for 15 minutes with 2.5 μ Ci/ml of 3 H-uridine and 2.5 μ Ci/ml of 3 H-cytidine. RNA was isolated from pooled cultures by method II and fractionated on sucrose density gradients as previously described.³ Centrifugation was for 80 minutes at 49,000 RPM in the SW 50 rotor.

for the transfer of iron from extracellular transferrin to either a membrane transport system or to an intracellular "sink" such as ferritin. If this were the case, the erythropoietin effect would be only on iron transport and the resulting intracellular iron would act to induce ferritin formation independent of erythropoietin. The end result would be enhanced cellular iron uptake.

Although there is an important permissive effect of iron on erythropoietin-stimulated hemoglobin synthesis and iron-uptake, iron does not appreciably affect the erythropoietin action on RNA synthesis. The small effect it does have on uridine incorporation (both control and erythropoietin treated) does not seem to be qualitatively important. We have not been able to determine whether iron acts to increase the synthesis of RNA, to retard its breakdown or to increase the cellular uptake of precursor uridine. All these possibilities are consistent with our findings. It

Figure 4. Effect of late additions of iron on stimulated iron-uptake and hemoglobin synthesis. Triplicate cultures contained 10×10^6 nucleated cells in 1.0 ml of medium. Erythropoietin, at 0.25 units/ml, was added to one-half of the cultures at the start of incubation. At the indicated times, 3.6 n moles of iron in 0.05 ml of rat serum was added to one set of control and stimulated cultures. After 32 hours, $1 \mu\text{Ci}$ of ^{59}Fe in 0.05 ml of rat serum was added to all cultures, and incubation was terminated after 48 hours. Hemoglobin synthesis was determined by cyclohexanone extraction. The ordinate represents the % of the erythropoietin response, obtained with iron added at zero time, at each time of delayed iron addition. Iron uptake (●—●); hemoglobin synthesis (○—○). With iron carrier addition at zero time: iron uptake was control, 10590, erythropoietin, 19320; hemoglobin synthesis was control, 3800, and erythropoietin, 8150. The lines were fit by the method of least squares.

appears likely that all the effects of iron are on the "specialization" phase of erythroid cytodifferentiation¹⁰ rather than on the primary inductive phase.

Cells incubated in the absence of added iron lose at least two responses to erythropoietin, iron-uptake and hemoglobin synthesis. This loss is reversible but not completely so, indicating that some essential components, affected by the addition of iron at the beginning of the incubation, decay in its absence. The components that are decaying are not known but we can suggest that they are neither the messenger RNA for globin nor that involved in the formation of the iron-

uptake system. The globin messenger has a half life in excess of 24 hours in contrast to the 16-17 hours seen in Figure 4, while the half life of the iron-uptake system messenger is less than 5 hours¹¹ rather than the 20 hours observed.

Regardless of the detailed biochemical mode of action of iron, its potentiating action on erythropoietin stimulation has allowed us to study cultures of rat bone marrow cells over periods of time greatly in excess of those used in earlier experiments. For instance, although our early work indicated that the erythropoietin effect on hemoglobin synthesis was negligible by the third day of culture,^{2,12} the addition of iron has enabled us to show that erythropoietin exerts its effect after as long as 27 days in culture.¹³

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DETECTION OF T4 AND T5 BACTERIOPHAGE-CODED TRANSFER RNAs*

By

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Although the general process of viral replication is now quite clear, the exact details of the developmental mechanism still await elucidation. Reports from several groups have indicated the induction of phage-specific tRNAs when *Escherichia coli* is infected by T4 bacteriophage.¹⁻³ This laboratory first described the synthesis of leucine and proline tRNA coded by the T4 genome.¹ A subsequent publication from another group⁴ confirmed these findings (in part) by reporting that T4 infection induces the synthesis of leucine, arginine, isoleucine and possibly tyrosine T4 tRNAs. It appears therefore, that several different tRNA species are specified by the T4 genome, although the exact number and identity of these T4 tRNA molecules is uncertain. Whether phage-directed tRNA synthesis is a unique property for T-even phages only or whether genetically different viruses are encoded with similar information, has not yet been satisfactorily answered. Because of the tedious procedure involved in detecting new viral-tRNA species, we sought a relatively simple method of answering the questions posed above.

MATERIALS AND METHODS

E. coli B and *E. coli* F, grown in 3 x D medium,⁵ served as hosts for T4 and T5 phages, respectively. After cell lysis, phage was isolated by repeated differential centrifugation, banded in CsCl, and dialyzed against a medium containing 0.9 per cent NaCl, 5 mM MgSO₄ and 10 mM phosphate (pH 6.5). DNA was prepared from these phage stocks by phenol extraction.⁶ *E. coli* B and *E. coli* F (7 x 10⁸ cell/ml, each) were infected separately at a multiplicity of 6 with the appropriate phage. After 13 min and 20 min of infection at 37°, with T4 and T5 phage respectively, replication was rapidly terminated and cells collected as reported elsewhere.⁷ Transfer RNA was isolated from uninfected and infected cells⁷ and further purified by chromatography on Sephadex G-100.¹ Membrane filters (type B-6, Schleicher and Schwell, Inc., Keene, New Hampshire), were impregnated with denatured phage DNA by the procedure of Gillespie and Spiegelman.⁸ Annealing of ³H-aminoacyl-tRNAs with DNA-filters in 50 per cent formamide, and determination of radioactivity "fixed" to the filters were as previously described.¹

Uninfected and infected tRNA preparations were charged with synthetases prepared from their respective soluble cell extracts by the method of Kelmers et al.⁹ In addition, the enzyme preparations were batch-treated with DEAE-cellulose in 0.3 M KCl (pH 7.0) to reduce endogenous tRNA, filtered, and concentrated by precipitation with (NH₄)₂SO₄ at 70 per cent of saturation (0°). Charging of tRNAs with ³H-L-amino acids (Schwarz BioResearch, Orangeburg, New York) was carried out as reported by Kelmers et al.⁹ After reaction, the ³H-aminoacyl-tRNAs were isolated by 3 extractions with phenol (pH 6), alcohol precipitation, and overnight dialysis against 0.01 M NaCl in the cold. Separation of amino acids was accomplished by column chroma-

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tography using the method of Spackman, Stein and Moore¹⁰ without automatic ninhydrin development. The fractions (1 ml) were spot tested on paper for ninhydrin reactive material and then directly assayed for isotope content by scintillation counting.

RESULTS

Hybridization with acylated tRNAs is possible under conditions of relatively low pH and temperature, which may be met by using formamide in the annealing mixture. Figure 1A shows that when tRNA isolated from T4-infected cells is charged with ³H-L-leucine, optimal radioactivity is fixed to T4 DNA within 3 to 4 hours of annealing. The level of hybridized ³H-leu-tRNA remains fairly constant during 10 hours of annealing, although a small but significant drop in "fixed" radioactivity occurs at the higher temperature. Deacylation of ³H-leu-tRNA is slightly higher at 37° than at 33°, but 75 to 80 per cent of the radioactivity is still precipitable with acid after 10 hours. A 4 hour annealing at 35° was therefore used as our standard protocol.

Transfer RNA from uninfected and T4-infected cells was charged with a mixture of 16 radioactive amino acids, and the isolated ³H-aminoacyl-tRNA mixture was annealed with T4 DNA-filters. The filters were heated in H₂O at 80° (after washing and T₁ RNase treatment) to release radioactivity into the aqueous medium. Table 1 shows the chromatographic distribution of the radioactivity recovered from the filters in the 16 amino acids initially used for tRNA charging. Significant radioactivity was associated with 5 amino acids released from the T4 tRNA-T4 DNA hybrid; viz, arginine, proline, glycine, isoleucine, and leucine; the isotope content of the other amino acids was not much different from those of the *E. coli* tRNA control. The slightly elevated counts for arginine and glycine in the control hybridization probably indicate the presence of trace endogenous T4 tRNAs in the synthetase preparation used for charging. To facilitate the detection of radioactive isoleucine, which chromatographs immediately adjacent to leucine, the specific activity of ³H-leucine was kept relatively low in the tRNA charging reaction; this accounts for the low leucine counts recovered from the T4 tRNA hybrid. The recovery of the total radioactivity subjected to chromatography ranged between 75 to 80 per cent.

Individually acylated tRNAs from T4-infected cells were also used for hybridization in the absence and presence of excess *E. coli* tRNA, which served as a competitor. Table 2 shows that hybridization to T4 DNA, but not to T5 DNA, was observed for the same 5 amino acids and little, if any, dilution of "fixed" radioactivity was seen in those annealing reactions to which *E. coli* tRNA had been added. The low levels of "fixed" counts for isoleucine and proline reflect the low level of radioactivity present in the annealing mixtures containing T4 tRNAs acylated with these amino acids. For the 4 other radioactive T4 amino-acyl-tRNAs tested (tyr, val, phe, ala), no positive hybridizations to T4 DNA were detected under the conditions employed. This agrees with the data obtained from the "mixed" charging experiment.

Another method of distinguishing phage from host tRNA species employs the selective removal of phage-transcribed RNAs from an infected cell RNA preparation by hybridization with phage DNA, dissociation of the DNA-RNA complex, and determination of the amino acid-acceptor activity of the isolated RNA. Table 3 indicates that when T4-induced RNA was isolated by hybridization to T4 DNA, significant acid-precipitable radioactivity was formed in a charging system with labeled arginine, glycine, isoleucine, proline and leucine, but not with any of the other amino acids tested. These results are consistent with those obtained by ³H-aminoacyl-tRNA hybridiza-

Figure 1. The stability of T4 ³H-leucyl-tRNA, and the effect of time and temperature on hybridization to T4 DNA. (A) The annealing mixture (0.6 ml) contained 30 μ g of T4 ³H-leucyl-tRNA, 60 μ g of T4 DNA-filter, 2x SSC, and was 50% with respect to formamide. The final annealing pH was 5.7, and the temperature of incubation was 33° and 37° for the times shown. (B) T4 ³H-leucyl-tRNA (10 μ g, 70,000 c.p.m.) was incubated under identical conditions described for (A) except that the DNA-containing filters were omitted. At the times shown, 0.1 ml aliquots were removed, and the acid-precipitable radioactivity was determined after adding carrier nucleic acid and 10% trichloroacetic acid. 1 x SSC = 0.15 M NaCl - 0.015 M sodium citrate.

tion. Isoaccepting tRNA species for a single amino acid cannot be detected by the procedures described here.

The coding of tRNAs by viruses other than T-even bacteriophages has been suggested¹¹ but not confirmed.¹² We have examined whether T5 phage, which is genetically unrelated to T4, codes for tRNA, by using the same procedure as for phage T4. Uninfected and T5-infected tRNAs from *E. coli* F were charged with a mixture of 15 ³H-amino acids and annealed with T5 DNA. After

Table 1

IDENTIFICATION OF RADIOACTIVE AMINO ACIDS ISOLATED FROM,
T4 ³H-AMINOACYL-tRNA HYBRIDIZED TO T4 DNA

Amino acid	Specific activity C/mM	³ H-amino acids fixed to T4 DNA-filter	
		<u>E. coli</u> ³ H-AA-tRNA c.p.m.	T4 ³ H-AA-tRNA c.p.m.
Arg	18.0	138	6,640
Pro	5.1	29	772
Gly	20.0	285	13,100
Phe	33.0	36	493
Leu	1.6	21	201
Ser	2.8	20	42
Val	18.5	14	57
Met	6.2	10	28
Tyr	50.0	29	57
Phe	40.0	19	28
Lys	28.0	28	56
His	3.0	46	16
Glu	20.0	14	57
Ala	50.0	46	85
Cys	0.5	6	13
Asp	0.4	17	6
		Σ = 758	Σ = 21,651

Uninfected and T4-infected tRNAs were acylated with a mixture of 16 ³H-L-amino acids (indicated above) and used for annealing with T4 DNA. Each annealing reaction contained two 57 mm diameter filters impregnated with a total of 900 μg of T4 DNA; the ³H-aminoacyl-tRNA mixture (either E. coli, 180 μg containing 5.5 x 10⁶ c.p.m., or T4-infected, 170 μg containing 5.2 x 10⁶ c.p.m.); 1.3 ml of 12x SSC, pH 4.3; 3.9 ml of formamide; and water to a final volume of 7.8 ml. After annealing for 4 h at 35°, the filters were treated to remove nonhybridizable material as reported elsewhere.¹ The filters were finally heated for 1 h at 80° in 10 ml of water and this extraction process was repeated once more. To remove nucleic acid from the combined water extracts, which contained 97% of the radioactivity "fixed" to the filters, 150 mg of DEAE-cellulose (dry weight) was added and the suspension was shaken for 2.5 h at 25°. The DEAE was removed by filtration, washed with water, and the filtrate was taken to dryness. The residue was dissolved in 2 ml of 0.2 M sodium citrate, pH 7.2, containing 0.2 μ moles each of the 16 amino acids shown above (nonradioactive) and subjected to chromatographic separation. The number of counts subjected to chromatography for the control E. coli tRNA was 1,040 c.p.m. and for the T4 tRNA, 25,400 c.p.m.

washing, and RNase treatment of the DNA-filters some 21,300 counts were fixed with T5-infected tRNA while only 450 counts were fixed with E. coli tRNA. Chromatographic separation of the T5-released membrane counts indicated that significant radioactivity was associated with 10 different amino acids (Table 4).

Table 5 shows that when the annealing reaction is repeated with individually acylated tRNAs from T5-infected cells, the same 10 radioactive amino acids are complexed to the T5 DNA-filters; in addition, the tRNAs charged with radioactive lysine, alanine, leucine and aspartic acid also show membrane-fixed radioactivity. Each of the T5 aminoacyl-tRNAs that hybridized to T5 DNA

Table 2

ANNEALING OF INDIVIDUALLY CHARGED T4 ³H-AMINOACYL-tRNA WITH T4 DNA

Amino acid	Specific activity C/mM	Charged T4 tRNA μ g (c.p.m.)	DNA fixed on filter		Competitor <i>E. coli</i> tRNA	Radioactivity fixed on filter c.p.m.
			T4	T5		
Arg	45	24 321,000	+	-	-	812
		24 321,000	+	-	+	780
		24 321,000	-	+	-	74
Gly	20	15 337,500	+	-	-	1026
		15 337,500	+	-	+	951
		15 337,500	-	+	-	5
Ileu	33	18 28,000	+	-	-	105
		18 28,000	+	-	+	105
		18 28,000	-	+	-	4
Pro	6	45 21,000	+	-	-	50
		45 21,000	+	-	+	47
		45 21,000	-	+	-	9
Leu	58	16 170,000	+	-	-	630
		16 170,000	+	-	+	610
		16 170,000	-	+	-	65
Tyr	42	20 88,000	+	-	-	14
		20 88,000	+	-	+	21
		20 88,000	-	+	-	22
Val	18.5	20 246,000	+	-	-	8
		20 246,000	+	-	+	9
		20 246,000	-	+	-	9
Phe	40	20 259,000	+	-	-	94
		20 259,000	+	-	+	90
		20 259,000	-	+	-	116
Ala	7.1	42 130,000	+	-	-	12
		42 130,000	+	-	+	13
		42 130,000	-	+	-	6

T4-infected tRNA, acylated individually with different ³H-L-amino acids, was used in these experiments. Each annealing reaction contained one ³H-aminoacyl-tRNA in the amount shown above; 40 μ g of either T4 or T5 DNA; 0.1 ml of 12x SSC, pH 4.3; 0.3 ml of formamide; and water to a final volume of 0.6 ml. Where indicated, 150 μ g of uncharged *E. coli* tRNA was included in the reaction. After annealing at 35° for 4 h, the filters were removed, washed and treated with T1 RNase as previously reported,¹ dissolved in a scintillation mixture containing Triton X-100 and counted directly.

showed no significant dilution in the presence of competitor *E. coli* tRNA and no significant hybridization to T4 DNA. Infection of *E. coli* F with T4 phage results in the appearance of T4-specific tRNAs; hybridization to T5 DNA was not observed (not shown here).

DISCUSSION

Hybridization with tRNAs acylated with a mixture of radioactive amino acids offers a relatively rapid scanning procedure for detecting the presence of multiple phage-induced tRNA spe-

Table 3

CHARGING OF T4-INFECTED tRNA AFTER ISOLATION FROM T4 DNA HYBRID

Amino acid	Specific activity C/mM	Acid-insoluble radioactivity		
		(A) Omit tRNA c.p.m.	(B) Add hybrid tRNA c.p.m.	(B) - (A) Difference c.p.m.
Arg	45.0	250	2,235	1,985
Gly	20.0	56	1,460	1,404
Ileu	33.0	67	517	450
Pro	5.1	49	250	201
Leu	58.0	100	1,660	1,560
Ser	2.8	69	71	2
Val	18.5	60	63	3
Phe	40.0	300	300	0
Lys	28.0	265	230	0
His	3.0	97	83	0
Glu	20.0	50	40	0
Ala	50.0	82	101	19
Thr	0.1	43	39	0
Asp	0.4	11	14	3
Try	6.5	589	599	10

T4-infected tRNA (390 μ g) was annealed to 2.82 mg of T4 DNA adsorbed onto 6 membranes (57 mm diameter, each), in a 15 ml annealing reaction containing 2x SSC and 50% formamide as described in Table 1. After annealing, filters were washed on both sides with 50 ml of 6x SSC and on one side with 3 ml of 50% ethanol, extracted twice at 57° for 45 min in 15 ml of 50% formamide, and the combined extracts were dialyzed against water. DNA was removed by passage through 3 membrane filters (57 mm diameter) after adjusting the dialyzed solution to contain 2x SSC. The filtrate was dialyzed against 0.1 mM magnesium acetate and concentrated for assay of amino acid acceptor activity. The acylating system (0.05 ml) contained 0.086 to 0.36 μ moles of one 3 H-L-amino acid indicated above; 3 μ g of synthetase enzyme, DEAE-treated; 10 mM Mg(OAc)₂; 5 mM KCl; 2 mM ATP; 100 mM Tris, pH 7.5; and the T4 tRNA preparation equivalent to an amount recovered from hybridization to 50 μ g of T4 DNA. Incubation was for 15 min at 37° after which the reaction was stopped by the addition of carrier ribosomal RNA and 10% trichloroacetic acid. The precipitate was collected, washed and assayed for radioactivity. Acetylation with 3 H-methionine gave very high wash-outs (> 5,000 c.p.m.) and therefore, is not reported. Reactions containing 3 H-tryptophane were precipitated with ethanol, dialyzed and then acid-precipitated as described above.

cies. Using tRNA from T4-infected *E. coli*, this procedure indicated the presence of phage-induced tRNAs for arg, gly, leu, pro, and ileu, in good agreement with hybridization assays conducted with individually esterified T4 tRNAs and by aminoacyl-charging of T4 RNA isolated from T4 DNA-RNA hybrids. With T5-infected cells, annealing with mixtures of acylated tRNAs was partially incomplete compared with the number of aminoacyl-tRNAs which hybridized with T5 DNA when annealed singly. A partial explanation for this may be that the analysis of the amino acids "fixed" to the DNA-filters by the mixed charging procedure involves several additional steps (deacylation, elution, chromatography) compared with the direct counting of reaction filters when single aminoacyl-tRNAs are used for annealing; thus, the chances of incurring losses

Table 4

IDENTIFICATION OF RADIOACTIVE AMINO ACIDS ISOLATED FROM
T5 ³H-AMINOACYL-tRNA HYBRIDIZED TO T5 DNA

Amino acid	Specific activity C/mM	³ H-amino acids fixed to T5 DNA-filter	
		E. coli ³ H-AA-tRNA c.p.m.	T5 ³ H-AA-tRNA c.p.m.
Arg	18.0	100	3,890
Pro	5.1	4	243
Gly	20.0	18	940
Ileu	33.0	35	4,360
Ser	2.8	5	944
Val	18.5	30	1,850
Met	6.2	20	732
Tyr	50.0	60	3,230
Phe	40.0	24	386
His	3.0	32	216
Lys	28.0	12	80
Glu	20.0	13	0
Ala	50.0	20	71
Cys	0.5	0	0
Leu	58.0	3	14
		$\Sigma = 376$	$\Sigma = 16,956$

Uninfected and T5-infected tRNAs were acylated with a mixture of 15 ³H-L-amino acids as indicated above, and used for annealing to T5 DNA. The conditions for annealing and the procedure used for the analysis of "fixed" radioactivity to membranes were the same as described for Table 1 except for the following: The annealing mixtures contained 1 mg of T5 DNA and either 150 μ g of T5-infected ³H-aminoacyl-tRNA (2.63×10^6 c.p.m., total) or 210 μ g of *E. coli* F ³H-aminoacyl-tRNA (2.55×10^6 c.p.m., total). The radioactivity "fixed" to the T5 DNA-membranes was removed as described under Table 1 and subjected to chromatography for amino acid separation as described under Methods.

are increased in the former method. Moreover, it is not possible to obtain optimal charging conditions for each amino acid in the charged mixture procedure, i.e., optimal amino acid concentrations varied from 0.2 to 2 mM for the different amino acids.

In most instances, hybridization reactions containing singly-charged tRNAs yielded more "fixed" ³H-aminoacid per μ g of DNA than when mixtures of aminoacyl-tRNA molecules were employed (correcting for differences in amino acid specific activities). This apparent discrepancy probably reflects the higher weight ratio of acylated-tRNA:DNA generally employed in the experiments of Tables 2 and 5 which achieved a higher degree of DNA saturation by the viral tRNA. Since saturation of the phage DNA with aminoacyl-tRNA was not achieved, calculation of the number of tRNA cistrons in the T4 and T5 genomes are not possible from these data. Furthermore, if an amino acid tRNA species was not 100 per cent acylated, both charged and uncharged tRNAs would compete for the same DNA site. Another factor contributing to variations in "fixed" radioactivity might be attributed to the different sites of enzymatic cleavage by T1

Table 5

ANNEALING OF INDIVIDUALLY CHARGED T5 ³H-AMINOACYL-tRNA WITH T5 DNA

Amino acid	Specific activity C/mM	Charged T5 tRNA μ g (c.p.m.)		DNA fixed on filter		Competitor <u>E. coli</u> tRNA	Radioactivity fixed on filter c.p.m.
				T5	T4		
Arg	45	20.8	310,000	+	-	-	2,270
		20.8	310,000	+	-	+	1,980
		20.8	310,000	-	+	-	220
Gly	20	20.8	288,000	+	-	-	2,201
		20.8	288,000	+	-	+	2,120
		20.8	288,000	-	+	-	6
Ileu	33	20.8	54,300	+	-	-	600
		20.8	54,300	+	-	+	552
		20.8	54,300	-	+	-	4
Pro	5.1	24.0	8,960	+	-	-	44
		24.0	8,960	+	-	+	38
		24.0	8,960	-	+	-	1
Tyr	50	17.3	109,000	+	-	-	1,095
		17.3	109,000	+	-	+	1,050
		17.3	109,000	-	+	-	20
Lys	28	19.0	80,000	+	-	-	174
		19.0	80,000	+	-	+	205
		19.0	80,000	-	+	-	10
Met	3.3	20.8	9,600	+	-	-	210
		20.8	9,600	+	-	+	210
		20.8	9,600	-	+	-	0
Ser	2.8	20.8	18,050	+	-	-	190
		20.8	18,050	+	-	+	180
		20.8	18,050	-	+	-	8
Val	18.5	24.0	183,200	+	-	-	180
		24.0	183,200	+	-	+	205
		24.0	183,200	-	+	-	8
Phe	40	11.0	170,000	+	-	-	1,590
		11.0	170,000	+	-	+	1,650
		11.0	170,000	-	+	-	70
Leu	58	20	360,000	+	-	-	445
		20	360,000	+	-	+	400
		20	360,000	-	+	-	95
Ala	8.1	21	68,000	+	-	-	368
		21	68,000	+	-	+	382
		21	68,000	-	+	-	1
Asp	12	20	157,000	+	-	-	832
		20	157,000	+	-	+	735
		20	157,000	-	+	-	2

Transfer RNA for T5-infected cells was acylated with the individual ³H-amino acids indicated above and used in annealing reactions with T5 DNA under conditions described in Table 2. The amounts of T5 tRNA and radioactivity used in each reaction are listed above; where indicated, 150 μ g of E. coli tRNA was also included. The membrane filters contained 40 μ g of either T5 or T4 DNA and were used as shown above. The procedure for analysis of "fixed" radioactivity was as described in Table 2.

RNase for different tRNA species.¹³ If hydrolysis of tRNAs by T1 RNase was too close to the aminoacyl-ACC end, the chances of losing "fixed" counts would be increased. For those experiments where T1 RNase was not used (Table 3), the quantitative amounts of the different T4 tRNA species appear to be more consistent with each other if the differences in specific activities of the amino acids are taken into consideration.

Littauer and Daniel,⁴ using T4 N-acetyl-³H-aminoacyl-tRNA, found hybridization with T4 DNA for the N-acetyl derivatives of leucyl-, arginyl-, and isoleucyl-tRNA. We agree with these findings except that our results also include proline and glycine tRNA species. Of the remaining 15 tRNA species, 14 showed no sequence homology to T4 DNA by the assay procedures used here, and one, tryptophane, gave unreliable results because of high washout values.

The experiments with tRNA from T5-infected cells suggest the presence of phage specific tRNAs for most of the amino acids so far tested, except glutamic acid and cysteine. Under our conditions for acylation, glutamyl-tRNA formation was extremely poor and the specific activity of cysteine was among the lowest of the amino acids used. It is quite possible, therefore, that the T5 genome may code for a complete set of 20 tRNA species. On the other hand, the experiments with T4 tRNA suggest that only a limited number of tRNA species⁵ are newly induced. With the present hybridization technique it is difficult to exclude the presence of a particular tRNA species based on negative findings. Inability to detect a new tRNA could be attributed to (a) inadequate tRNA charging conditions for the new tRNA species, (b) the presence of extremely small quantities of the new tRNA, or (c) the use of radioactive amino acids low in specific activity. But even if some of these difficulties were responsible for the detection of only a few T4 tRNA species, they apparently did not hinder the detection of T5 tRNA species for most amino acids.

We conclude, therefore, that both T4 and T5 bacteriophages carry information for the synthesis of phage-specific tRNAs although the number and kinds of tRNA species differ for these two viruses. Whether or not these viral tRNAs play a functional role in T4 or T5 phage development remains to be established. If we assume that they do, then the known variations in the mechanisms of replication already established for different viruses would also be reflected in the tRNAs coded by the different viral genomes.

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THE SELECTIVE INHIBITION OF PROTEIN INITIATION BY T4 PHAGE INDUCED FACTORS*

By

E. B. Klem, W.-T. Hsu, and S. B. Weiss

Various reports have now described specific changes in the translational machinery of *Escherichia coli* when infected by T-even bacteriophage. These changes include alterations in host tRNAs¹⁻³ and the enzymes that modify them,^{4,5} changes in aminoacyl-tRNA synthetases,⁶ and synthesis of phage-coded tRNAs.⁷⁻⁹ Another induced alteration was found by Hsu and Weiss¹⁰ who observed that ribosomes from T4 infected cells efficiently translate T4 template RNA but are restricted in their translation of host and MS2 RNAs. This phenomenon is an early phage function and is not manifested when infection is carried out in the presence of chloramphenicol or with T4 ghosts (unpublished results of the authors).

In the same report, Hsu and Weiss found that a high salt extract of T4 infected ribosomes (T4 factors) imparts a similar property of "selective" T4 RNA translation when added to uninfected ribosomes. These findings were interpreted as a form of translational control exerted by the invading phage on its host. Subsequent reports by Schedl, Singer and Conway,¹¹ Dube and Rudland¹² and Steitz et al¹³ confirm the above observations. The latter investigators^{12,13} have also shown that T4 factors restrict the binding of host, f2 and R17 RNA to ribosomes but permit the binding of T4 RNA. In this communication, we provide independent evidence that T4 factors exert their restrictive effect at the level of initiation complex formation and that the apparent selective translation of T4 template RNA, in vitro, is not exclusive. In addition, evidence is presented which indicates that T4 factors exert no effect on polypeptide chain elongation.

MATERIALS AND METHODS

Ribosomes, factors and template RNAs. The preparation of ribosomes and ribosomal factors from uninfected and T4 infected *E. coli* and template RNAs from *E. coli*, T4 infected cells and MS2 phage, were as previously reported.¹⁰ Washed ribosomes from normal *E. coli* were prepared by extraction of unwashed ribosomes with 1M NH₄Cl and then treated with DEAE-cellulose by the method of Iwasaki et al.¹⁴ T5 template RNA was isolated from *E. coli* F infected with T5 phage at 37° (MOI = 20) for 20 minutes.

Radioactive template RNAs and fMet-tRNA. *E. coli* was grown in a salts-glucose medium supplemented with 0.04 volumes of 3xD medium¹⁵ as reported elsewhere.⁵ MS2 ³H-RNA (1 x 10⁴ cpm/μg) was isolated from MS2 phage grown on *E. coli* K12W1485 in the presence of ³H-uracil (1 mc/40 ml of culture). *E. coli* ³H-RNA (1 x 10⁴ cpm/μg) was isolated from log phase *E. coli* B which had been pulsed for 2 min at 37° with ³H-uracil (2 mc/40 ml of culture). T4 and T5 ³H-RNAs (0.97 x 10⁴ and 61 x 10⁴ cpm/μg, respectively) were isolated from *E. coli* B which was pulsed from 5 - 15 min after infection (MOI = 10) with ³H-uracil (1 mc/40 ml of culture). *E. coli*

*This report is taken from a paper that appeared in Proc. Nat. Acad. Sci. (U.S.), 67:696, 1970.

^3H -fMet-tRNA_f was prepared by charging E. coli tRNA_f^{Met} (97 per cent pure, a gift of Dr. D. Novelli, Oak Ridge National Laboratory) with ^3H -methionine in the presence of leucovorin using a dialyzed E. coli S165 supernatant fraction as enzyme. The ^3H -fMet-tRNA_f was isolated by phenol extraction, alcohol precipitation and dialysis and had a specific activity of 57,000 cpm/ μg .

Ribosome binding assay for template ^3H -RNA and ^3H -fMet-tRNA_f. The components of the reaction mixtures are indicated in the footnotes to Table 3. Experiments using ^3H -RNAs were assayed by centrifugation on a 5 ml 5-20 per cent linear sucrose gradient (containing 5 mM Mg(oAc)₂, 50 mM Tris-HCl(pH 7.8) and 80 mM NH₄Cl) for 65 min at 50,000 RPM in a Spinco SW No. 50 rotor. Fractions were collected and their radioactive content was determined by scintillation counting. Binding of ^3H -fMet-tRNA_f to ribosomes was determined by trapping on nitrocellulose filters.¹⁶ The filters were pretreated with 0.5 N NaOH for 20 min and washed prior to their use as described by Smolarsky and Tal.¹⁷

Incorporation of ^3H -fMet into polypeptides. The reaction mixtures are indicated in the footnotes to Table 3. Incorporation of ^3H -fMet into a hot acid-precipitable fraction was as described elsewhere.¹⁰

Isotopic compounds. The ^3H -uracil and ^3H -methionine used in this report had a specific activity of 25.4 c/mM and 3.3 c/mM, respectively, and were purchased from Schwarz BioResearch, Orangeburg, New York.

RESULTS

Since the T4 "selective" translational factor is associated with ribosomes and behaves similarly to initiation factors with respect to certain properties (i.e., extraction with high salt and chromatographic distribution on DEAE-cellulose) it seemed likely that the T4 factors might exert their effect on the initiation steps of protein synthesis. Studies on ribosomal complex formation showed that in the presence of fMet-tRNA, GTP, and magnesium acetate, binding of T4 ^3H -RNA to washed E. coli ribosomes requires the addition of factors extracted with high salt from either uninfected (EC) or infected (T4) E. coli ribosomes (Figure 1). With MS2 and E. coli (EC) ^3H -RNAs, however, effective complex formation occurs only with EC factors while T4 factors exhibit a marked inhibition. On the other hand, T4 factors do not restrict the binding of T5 ^3H -RNA to washed ribosomes and are equally effective as EC factors for promoting complex formation.

Using the nitrocellulose trapping technique of Nirenberg and Leder,¹⁶ results obtained from experiments on the binding of ^3H -fMet-tRNA_f to ribosomes were found to be in agreement with the above findings. Table 1 shows that in the presence of washed ribosomes, GTP, and magnesium ions, the trapping of labeled fMet-tRNA_f on filters requires the participation of both template RNA and ribosomal factors. With RNAs from T4 and T5 infected cells, the trapping of ^3H -fMet-tRNA occurs equally well with either E. coli or T4 factors, but with MS2 and E. coli template RNAs, T4 factors significantly inhibit label accumulation on the filters. When similar experiments with ^3H -fMet-tRNA_f were carried out under conditions of active polypeptide synthesis, incorporation of radioactivity into hot acid-precipitable material was also dependent upon the presence of ribosomal factors (Table 2). Both E. coli and T4 factors support good incorporation of ^3H -fMet into protein with RNA templates from T4 and T5 infected cells, but with MS2 and E. coli RNAs, T4 factors are inhibitory.

Although the experimental data shown above indicate that T4 ribosomal factors restrict pro-

Figure 1. Sucrose gradient analysis of template (^3H)RNA binding to ribosomes. The reaction system (0.125 ml) contained 5 mM $\text{Mg}(\text{oAc})_2$; 50 mM Tris (pH 7.8); 80 mM NH_4Cl ; 14 mM mercaptoethanol; 40 μg of *E. coli* tRNA charged with fMet; 100 μg of washed *E. coli* ribosomes; one ^3H -labeled RNA (2×10^4 cpm for *E. coli*, T4 and T5 RNAs, and 1.5×10^4 cpm for MS2 RNA); nonradioactive RNA from the same source as carrier (80 μg of *E. coli* and T4 RNAs, 20 μg of T5 RNA and 26 μg of MS2 RNA); and 60 μg of either *E. coli* or T4 factors where indicated above. After 15 min at 37° , the mixtures were subjected to sucrose density centrifugation as indicated under Methods. Only the bottom half of the gradients (70S region) are shown.

tein synthesis at the level of initiation with certain templates, they do not exclude the possibility that these same factors might exert an additional effect on the process of protein chain elongation. Experiment 1 of Table 3 shows that with MS2 RNA as template, the binding of ^3H -fMet-tRNA_f to unwashed ribosomes occurs in the absence of added ribosomal factors and is completely blocked by aurintricarboxylic acid (ATA), a chemical agent previously shown to inhibit protein chain initiation.^{18,19} The addition of *E. coli* factors has no effect on labeled fMet-tRNA_f binding, although T4 factors reduce the amount of ^3H -fMet-tRNA_f complexed by nearly 40 per cent as compared to *E. coli* factors, suggesting a partial reversal of the binding process. On the other hand, once chain elongation has begun, T4 factors have no significant effect on ^3H -fMet incorporation into a poly-

Table 1
EFFECT OF T4 FACTORS ON ^3H -fMet-tRNA_f BINDING TO RIBOSOMES

Source of ribosomal factors	CPM of ^3H -fMet-tRNA _f bound with different templates				
	No RNA	T4 RNA	T5 RNA	MS2 RNA	<u>E. coli</u> RNA
None	108	212	76	100	125
<u>E. coli</u>	118	3450	3513	2150	2260
T4	136	3521	3400	448	1200

The reaction mixtures (0.125 ml each) were the same as in Figure 1 except as follows: No labeled template RNAs were used but non-radioactive template RNAs were present (as indicated) in the following amounts: T4 (160 μg), MS2 (40 μg), T5 (66 μg) and E. coli (250 μg). Purified ^3H -fMet-tRNA_f (0.35 μg , 20,000 cpm) was added to each reaction. After incubation at 37° for 15 min, the mixtures were assayed for ribosomal bound radioactivity by trapping on nitrocellulose filters.¹⁶ The E. coli ribosomes used were washed with 1M NH₄Cl.

peptide form (Table 3, Experiment 2). In this last experiment, ATA was included to prevent initiation of new chains during elongation and at the time of factor addition (30 seconds after adding the elongation components) the amount of ^3H -fMet incorporation into protein was negligible; maximum incorporation was achieved by 2.5 minutes under the conditions used. Similar results were obtained using other labeled amino acids.

DISCUSSION

The present study shows that factors derived from T4 infected-cell ribosomes permit the formation of initiation complexes with T4 and T5 template RNAs but inhibit similar complex formations with E. coli and MS2 RNAs. These results support and explain our previous findings that T4 infected ribosomes, or factors derived therefrom, allow efficient translation of T4 RNA but not of host and MS2 RNA. An explanation is also provided for the earlier observations that RNA-phage replication is blocked by infection with T-even phage.²⁰⁻²² Similar defects in the transla-

Table 2
EFFECT OF T4 FACTORS ON ^3H -fMet INCORPORATION INTO PROTEIN

Source of ribosomal factors	CPM of ^3H -fMet incorporated into an acid-insoluble form			
	T4 RNA	T5 RNA	MS2 RNA	<u>E. coli</u> RNA
None	86	100	112	47
<u>E. coli</u>	5411	4445	2182	3929
T4	6145	5163	765	2810

The reaction mixture (0.125 ml) contained 40 mM Tris-HCl (pH 7.8), 10 mM Mg(oAc)₂, 80 mM NH₄Cl, 4 mM mercaptoethanol, 2 mM ATP, 0.2 mM GTP, 2 mM PEP, 3 μg of PEP kinase, 8×10^{-3} mM each of 20 amino acids, 50 μg of E. coli tRNA, 0.35 μg of ^3H -fMet-tRNA (20,000 cpm), 100 μg of E. coli washed ribosomes, 75 μg of soluble S165 protein, and where indicated, 160 μg of T4 RNA, 40 μg of MS2 RNA, 66 μg of T5 RNA and 250 μg of E. coli RNA as template, and 60 μg of either E. coli or T4 factors. Radioactivity incorporation into a hot, acid-insoluble fraction was determined as described elsewhere.¹⁰

Table 3

EFFECT OF T4 FACTORS ON POLYPEPTIDE INITIATION AND ELONGATION

Expt. no.	Binding reaction	Additions	^3H -fMet-tRNA _f binding cpm	Incorporation of ^3H -fMet into protein cpm
1	Complete	None	2306	
	Omit MS2 RNA	None	184	
	Complete	ATA	193	
	Complete	EC factors	2442	
	Complete	T4 factors	1559	
2	Complete	Elongation mix + ATA		3003
	Omit MS2 RNA	Elongation mix + ATA		42
	Complete	Elongation mix + ATA + EC factors		3556
	Complete	Elongation mix + ATA + T4 factors		3136

Experiment 1. The binding mixtures (0.065 ml) contained the same components as indicated in Figure 1, except that 30 μg of MS2 RNA, 230 μg of unwashed *E. coli* ribosomes and 37,000 cpm of ^3H -fMet-tRNA_f (^3H -methionine, SA = 3.3 c/mM) were used. The binding mixtures were incubated for 10 min at 37° before the "additions" shown above were made. After incubation for another 10 min, each reaction was assayed for ^3H -fMet-tRNA_f trapping on filters as described under Methods. The concentration of aurintricarboxylic acid (ATA) was 0.15 mM and 30 μg of either *E. coli* (EC) or T4 factors were used.

Experiment 2. The binding mixture (0.065 ml) was the same as described for experiment 1. After reaction for 15 min at 37°, the additions were made in the following order: elongation mix and ATA simultaneously, 30 sec incubation, and then the ribosomal factors. Incubation was continued for another 10 min, the reaction terminated and assayed for ^3H -fMet incorporation into an acid-insoluble form.¹⁰ The concentration of ATA was 0.15 mM and in addition to the binding mixture, the elongation mix (0.065 ml) contained all of the components required for protein synthesis, as shown in Table 2. ^3H -fMet-tRNA, EC and T4 factors were the same as described above. At the time of factor addition, no significant incorporation of ^3H -fMet was detectable.

tion of host and RNA-phage templates have now been observed with ribosomes from T5 infected cells (unpublished results from this laboratory) and with ribosomes from T7 infected cells (P. Leder, personal communication).

In view of the fact that T4 factors support ribosomal binding and fMet incorporation into polypeptides with T5 RNA, the translational selectivity of T4 infected ribosomes can no longer be considered exclusive for T4 RNA templates. Since T4 ribosomal factors recognize some, but not all template RNAs, this suggests that nucleotide sequences which specify sites for polypeptide chain initiation may be similar for certain templates but, most likely, are different for others.

Besides blocking the formation of MS2 RNA initiation complexes, T4 factors also seem capable of partially reversing these complexes, once formed, suggesting some type of reversible competition between *E. coli* factors and the T4 inhibitory component. Similar observations have been made by Dube and Rudland.¹² However, once the process of polypeptide chain elongation begins, T4 factors demonstrate little or no effect on the incorporation of amino acids into peptide linkage using MS2 RNA as template. This information reinforces the idea that the T4 translational inhibitory factor exerts its action solely at the level of initiation and not on peptide chain extension.

The mechanism by which ribosomal factors from T4 infected cells prevent the binding of certain template RNAs to ribosomes and their function in the phage replicative process is still not known. We have previously suggested that the T4 induced ribosomal phenomenon could play a role in the shut-off of host protein synthesis, reflecting an effective regulatory mechanism for translational control. It is possible that such a mechanism might be operative in phage infected cells as well as in other biological systems, but experimental proof for this remains to be established.

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HEME a, CYTOCHROME c AND TOTAL PROTEIN TURNOVER IN MITOCHONDRIA FROM RAT HEART AND LIVER*

By

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The hypothesis that the mitochondrion turns over as a unit was first advanced by Fletcher and Sanadi¹ based on their observations that several components of rat liver mitochondria showed similar exponential decay rates. The calculated half-lives of total phospholipids, soluble and insoluble mitochondrial proteins, and cytochrome c labeled with ³⁵S-methionine or ¹⁴C-acetate were all about 10 days. The isotopic precursors used in these studies are now known to be efficiently reutilized for resynthesis of macromolecules, and hence, reutilization may have considerably prolonged the observed turnover rates. We have recently reported a modified approach, based on measuring turnover rates of specific mitochondrial cytochromes labeled with ³H-ALA.^{†2} ALA[†] is a specific heme precursor, efficiently incorporated into the prosthetic group of cytochromes. Moreover, heme is degraded to bilirubin, and the radioactive label is not reutilized. We have previously shown that the half-lives of cytochromes b and c, both localized on the inner mitochondrial membrane, were similar, about 5-6 days in rat liver.² In contrast, cytochrome b₅, found on the outer mitochondrial membrane, turned over more rapidly ($t^{1/2} = 4.4$ days). In this paper we extend these findings to heme a in rat liver mitochondria, and heme a and cytochrome c in heart mitochondria. The turnover rates of these cytochromes are compared with the turnover rates of total mitochondrial protein labeled with two different amino acid precursors: ³H-leucine[†] and ¹⁴C-guanidoarginine.[†] Since ¹⁴C-guanidoarginine is a non-reutilizable protein precursor,³ differences in measured turnover rates using these two precursors yield an index of ³H-leucine reutilization after protein degradation in liver and heart.

MATERIALS AND METHODS

Liver mitochondria were isolated from adult, female Sprague-Dawley rats using the method of Parsons and Williams⁴ as previously described. Microsomal contamination, with glucose-6-phosphatase activity as a marker, was less than 5 per cent with this technique.² A 10 per cent (w/v) heart muscle homogenate was prepared in 0.27 M sucrose, 10 mM Tris chloride, 10 mM KCl, 0.1 mM EDTA, pH 7.5. The homogenate was incubated with Nagarse (Enzyme Development Corporation, New York), 0.5 mg per ml, at 0° for 20 minutes.⁵ The pH was readjusted to 7.5 with NaOH, and after rehomogenization cell debris was removed by centrifugation at 800 x g. This step was repeated. Mitochondrial pellets were obtained by centrifugation at 9000 x g; pellets were twice resuspended and recentrifuged. Two more centrifugations were made at 800 x g, and the pellets were discarded. Mitochondria were collected after a final centrifugation at 9000 x g. All

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† The abbreviations used are: ALA, δ -aminolevulinic acid; ³H-ALA, 3,5- δ -aminolevulinic acid-³H; ³H-leucine, *l*-leucine-4,5-³H; ¹⁴C-guanidoarginine, *l*-arginine-guanido-¹⁴C.

centrifugation steps were made for 10 minutes at 4°. Mitochondrial purity was checked by electron microscopy; * contaminating cellular structures were not seen.

Cytochrome c was extracted from mitochondria with 0.15 M KCl, and the protein was purified by chromatography on XR-64 resin. After extracting cytochrome c, the residual pellets were homogenized with acetone-water (4:1, v/v) for 10 minutes and centrifuged for 10 minutes at 6500 x g. The pellets were dried under an N₂ stream and were further delipidated by three extractions with CHCl₃. Heme was extracted twice from delipidated mitochondria with CHCl₃-pyridine (2:1, v/v) for 30 minutes each time.⁷ After this step, light was excluded from vessels that contained heme. Heme was concentrated by evaporation in vacuo at 30°; the dry residue was dissolved in several drops of CHCl₃-pyridine (1:1, v/v). The heme solution was applied to a 0.6 x 6 cm column containing Sephadex LH-20 (Pharmacia), equilibrated with CHCl₃-pyridine. Heme a was eluted with the lower phase of the following mixture: 50 ml pyridine, 25 ml CHCl₃, 25 ml 0.1 per cent NaHCO₃, 3.3 ml isooctane.⁷ Heme a, which appeared as a green band, was eluted prior to and distinct from heme b, a red-brown band. Fractions of heme a were pooled, washed with 0.05 M acetic acid, and were evaporated to dryness in vacuo. The heme a was dissolved in pyridine, and reduced with dithionite. Absorption spectra were recorded with a Cary Model 14, and the concentration of heme a was calculated from the following relationship:

$$A_{583} - \frac{A_{550} + A_{510}}{2} = 21.7 \text{ mM}^{-1} \cdot 7$$

Separate experiments were performed using ³H-leucine and ¹⁴C-guanidoarginine to label mitochondrial proteins. Mitochondrial protein was precipitated with trichloroacetic acid and delipidated by solvent extractions.⁸ An aliquot of protein was dissolved in formic acid and used for counting. The protein concentration of an aliquot of the same solution was measured by the Lowry method⁹ with bovine serum albumin as a reference standard.

Rats were given intraperitoneal injections of isotopic precursors. For labeling heme a and cytochrome c, each rat received 100 µCi of ³H-ALA (Schwarz BioResearch); for measurement of total mitochondrial protein turnover, each rat received 10 µCi of ¹⁴C-guanidoarginine (Amersham Searle) or 50 µCi of ³H-leucine (Schwarz). Rats were sacrificed at 48 hour intervals, after fasting overnight.

³H labeled heme a, cytochrome c and total mitochondrial proteins (from ³H-leucine) were counted with a liquid scintillation system (Packard 544). Results were corrected for quenching, and are expressed as DPM. ¹⁴C-guanidoarginine labeled proteins were counted with a low background gas flow system (Beckman Low Beta II). Less than 2 mg of protein was dried on each planchet; under these conditions, there is no self-absorption.

RESULTS

In preliminary experiments, heme a was purified by solvent extraction¹⁰ or by chromatography on celite⁷ or silicic acid.¹¹ With small scale preparations, celite chromatography after pyridine-CHCl₃ extraction yielded heme a, but more consistent results were obtained when Sephadex LH-20 was substituted for celite. A typical spectrum of dithionite reduced heme a as shown in Figure 1.

* Electron microscopy was kindly performed by Dr. Z. Hruban.

Figure 1. Absorption spectrum of dithionite reduced heme a prepared from heart muscle with Sephadex LH-20. $\alpha_{\text{max}} = 583 \text{ nM}$; $\gamma_{\text{max}} = 430 \text{ nM}$.

Turnover rates for heme a and cytochrome c were measured with mitochondria isolated from both rat heart and liver. For liver, calculated half-lives were 5.8 days for heme a and 5.6 days for cytochrome c, as shown in Figure 2. These values are essentially identical, $p > 0.5$.* With heart mitochondria, turnover rates of heme a and cytochrome c were very similar, as shown in Figure 3. Heme a was renewed with a 5.9 day half-life; the half-life of cytochrome c was 5.8 days. Turnover rates for heart muscle heme a and cytochrome c are not significantly different, $p > 0.5$.

To measure turnover of total mitochondrial proteins, separate experiments were performed, and protein was counted after injecting ^3H -leucine or ^{14}C -guanidoarginine. In experiments with heart mitochondria, similar protein half-lives were measured with ^3H -leucine or ^{14}C -guanidoarginine, as shown in Figure 4. With liver mitochondria, the apparent turnover rate of mitochondrial protein differed, depending upon which isotopic precursor was injected (Figure 5). With ^3H -leucine the half-life was 7.0 days, and with ^{14}C -guanidoarginine the half-life was 5.0 days, significantly less ($p < 0.05$).

DISCUSSION

In this paper we have shown that heme a and cytochrome c of rat liver and heart turn over at essentially the same rates. These data, added to our previous observations that rat liver heme b and cytochrome c also turn over with identical half-lives of about 5-6 days, provide further support for our hypothesis that the inner mitochondrial membrane, or a significant fraction thereof,

* p values were determined as described by Goldstein.²³

Figure 2. Heme a, ●—● and cytochrome c □---□ turnover in liver mitochondria after labeling with ^3H -ALA. Half lives are 5.6 days for heme a and 5.5 days for cytochrome c.

Figure 3. Turnover of ^3H -ALA labeled heme a ●—● and cytochrome c □---□ in heart mitochondria. For heme a, $t_{1/2} = 5.9$ days; for cytochrome c, $t_{1/2} = 5.8$ days.

Figure 4. Total protein turnover in heart mitochondria. After ^3H -leucine incorporation, $\bullet$ — $\bullet$, $t_{1/2} = 6.1$ days; after ^{14}C -guanidoarginine, $\square$ --- $\square$, $t_{1/2} = 6.2$ days.

Figure 5. Total protein turnover in liver mitochondria. After ^3H -leucine incorporation, $\bullet$ — $\bullet$, $t_{1/2} = 7.0$ days; after ^{14}C -guanidoarginine, $\square$ --- $\square$, $t_{1/2} = 5.0$ days.

is synthesized and degraded as a unit.¹² It is also significant that the turnover rates of total mitochondrial proteins in liver and in heart, which in turn reflect predominantly the renewal of the inner mitochondrial membrane, are also of the same magnitude. Furthermore, rat heart mitochondrial DNA is renewed at a similar rate, with a half-life of 6.7 days. Thus, in heart mitochondria, ³H-ALA labeled cytochromes a, b, and c, total mitochondrial protein, and mitochondrial DNA all turn over at similar rates. The same appears true for liver mitochondria, excepting mitochondrial DNA.¹² The longer half-life of liver mitochondrial DNA (9.4 days)¹² may reflect reutilization of precursor ³H-thymidine in this organ.

It is, however, apparent that matrix and outer mitochondrial membrane components turn over independently of the inner mitochondrial membrane. We have shown that cytochrome b₅, localized in the outer membrane, is renewed with a half life of 4.4 days, which is significantly faster than the turnover of heme b and cytochrome c.

These observations agree with measurements of total protein turnover of inner and outer membranes made after labeling with the reutilized precursors ³⁵S-methionine¹³ or ¹⁴C-leucine,¹⁴ and with experiments¹⁵ that used a double isotope technique¹⁶ from which conclusions independent of reutilization may be made.

δ-ALA synthetase, an enzyme found in the matrix fraction¹⁷ turns over with a half-life of only 20 hours.¹⁸ This enzyme, and probably other matrix enzymes, can therefore be degraded independently of the mitochondrial membrane systems.

Our data also indicate that reutilization of amino acid precursors for protein synthesis differs in heart and liver. Measurement of turnover of total mitochondrial proteins in liver and heart using ³H-leucine and ¹⁴C-guanidoarginine clearly shows differences in patterns of reutilization of precursor amino acids in these two organs. Turnover measurements may be factitiously prolonged by significant isotope reutilization following protein degradation. Labeling of hepatic mitochondria with the non-reutilized precursor, ¹⁴C-guanidoarginine yielded more rapid turnover rates than labeling with ³H-leucine. These results resemble data reported by Swick, et al¹⁹ and Arias, et al²⁰ which showed a more rapid protein turnover rate with ¹⁴C-guanidoarginine in liver. In contrast, protein turnover rates in heart mitochondria are identical after ³H-leucine or ¹⁴C-guanidoarginine labeling. Based upon these data, we now suggest that heart mitochondria do not readily reutilize some amino acids, nor perhaps nucleic acid precursors.

During preparation of this manuscript Kadenbach²¹ reported a 43-day half-life for heart cytochrome c after ¹⁴C-lysine incorporation. Protein turnover in rat heart mitochondria has been measured²² and results similar to our data have been reported. The fivefold disparity between half lives of heart cytochrome c is not presently explicable. Possibilities include an extraordinary reutilization of ¹⁴C-lysine, or differing turnover rates for the heme and apoprotein moieties of cytochrome c. In preliminary experiments,* our data suggest that the latter possibility is unlikely. Incorporation of isotopic labels into the heme and protein moieties of cytochrome c occurs at similar rates, and both rates are similarly reduced by inhibition of protein synthesis.

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CONTROL OF MITOCHONDRIAL TURNOVER UNDER THE INFLUENCE OF THYROID HORMONE*

By

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Many previous reports¹⁻¹⁰ deal with the turnover of mitochondrial constituents in mammalian tissues in the normal physiological state. Since thyroid hormone has marked effects on mitochondrial function^{11,12} it is of interest to determine whether the turnover rate differs in the hypothyroid, normal, and hyperthyroid states, and since mitochondrial mass is a function of both the rate of synthesis and the rate of degradation, one may also ask how changes in these two rates contribute to any changes in mitochondrial mass following the administration of thyroid hormone.

An experiment in which the rate of turnover of mitochondrial constituents is changed may also contribute information on whether there is a major subunit of the mitochondrion which turns over as a single entity, since if such a subunit exists, the turnover rates of its constituents would be affected in the same manner.

In the experiments reported here the effect of thyroid hormone administration on mitochondrial DNA and protein turnover was studied to determine the following: the turnover rates in different thyroid states; whether changes in synthesis or degradation or both result in changes in the mass of mitochondrial protein; whether the turnover rates of mitochondrial DNA and protein are affected in the same manner and to the same extent; and whether mitochondria synthesized before thyroid hormone administration turn over after thyroid hormone administration at the same rate as newly synthesized mitochondria.

METHODS

Two experiments of similar design were performed. In Experiment I, the hypothyroid state and its transition to the euthyroid or slightly hyperthyroid state were studied. In Experiment II, the normal or euthyroid state and its transition to the strongly hyperthyroid state were studied.

Experiment I was performed on female Sprague-Dawley rats surgically thyroidectomized (Hormone Assay Laboratories, Chicago) at 10 weeks of age. The rats were maintained on Purina rat diet ad libitum, and water supplemented with a small amount of CaCl_2 , for 10 weeks. Their weights were checked weekly and any animals whose weight increased were discarded. In rats thyroidectomized at this age the total absence of thyroid function can be diagnosed by the cessation of growth. After several months some increase in weight does occur but this is probably due to myxedema. At the start of Experiment I the rats weighed between 173 and 197 g; they were divided into eight matched groups of 3 rats each. A group was killed each week for 7 weeks with an additional group at 2-1/2 weeks (groups 0, 1, 2, 2-1/2, 3, 4, 5, and 6). Groups 0, 1 and 2 re-

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ceived no thyroid hormone. Groups 2-1/2 to 6 received 20 μ g each of *l*-thyroxine (Sigma Chemical Company, St. Louis, Missouri) on alternate days by intraperitoneal injection starting with the day on which group 2 animals were killed and continuing to the end of the experiment. Experiment II was performed in a similar manner on normal adult female Sprague-Dawley rats weighing between 225 and 247 g. Eight groups of 3 rats were killed on the same time schedule as in Experiment I. Thyroid hormone was administered similarly but at a dose of 300 μ g per rat on alternate days.

Preparation of tissue fractions from liver. The rats were killed by decapitation and the livers were removed, cut into small pieces with scissors, and chilled in 0.3 M sucrose, 0.01 M Tris HCl, pH 7.4, 0.1 mM EDTA (S.T.E. buffer). All subsequent steps were performed at 0-4°C. The livers were pooled, blotted dry, weighed and homogenized as described previously.¹⁰ An aliquot of the homogenate was kept for protein, DNA and enzyme determinations, and the rest was centrifuged at 500 g for 10 min in the SS-34 rotor of the RC2-B Sorvall centrifuge. Mitochondria were purified from the supernatant by one further centrifugation at 500 g for 10 min, centrifugation of the supernatant at 12,000 g for 12 min, incubation of the resuspended pellet with pancreatic DNase and $MgCl_2$, three further centrifugations at 9,000 g for 10 min, centrifugation through a sucrose gradient (0.8 - 2.0 M) in the 25.2 rotor of the Spinco L2-65 ultracentrifuge and reprecipitation of the mitochondria in the Sorvall centrifuge. All subcellular fractions were finally resuspended in S.T.E. buffer and frozen.

Preparation of tissue fractions from heart. After removal of the livers, the hearts were removed and the coronary arteries were flushed with 5 ml of cold 0.3 M sucrose, 0.05 M Tris HCl, pH 7.4, 0.0003 M $MgCl_2$ (S.T.M. buffer). The atria were trimmed away and the ventricles were weighed and homogenized as previously described.¹⁰ A small fraction of the ground muscle was removed, weighed and extensively homogenized separately for determinations of total protein, total DNA, and enzyme. Mitochondria were isolated as described for liver, but omitting the DNase step. All fractions were finally suspended in S.T.M. buffer and frozen.

Chemical determinations. Protein determinations were made in duplicate on all homogenate and mitochondrial samples by the method of Lowry et al.¹³ DNA determinations were made in duplicate on all homogenate samples by Burton's modification of the diphenylamine method.¹⁴

Respiratory and enzymatic determinations. Oxygen consumption was determined in a chamber constructed in this laboratory and similar to that described by MacLagan and Sheahan.¹⁵ Because of considerable variations in successive determinations on the same rats, changes in oxygen consumption following thyroid hormone administration were determined in a separate series of experiments. Six thyroidectomized rats and six normal rats were studied approximately 3 times weekly over a 4-week period. Thyroid hormone was administered as described above and the oxygen consumption was determined over the next 8 weeks. Each determination was made on two rats simultaneously over a 15 to 20 minute period after 10 min equilibration.

Succinate cytochrome *c* reductase activity was measured on all homogenate and mitochondrial samples at 37°C in a 3-ml system by the method of Rabinowitz and de Bernard.¹⁶

Succinate cytochrome *c* reductase determinations on any one tissue were all made at the same session on an aliquot that had been extensively dispersed in an all-glass homogenizer and stored separately for this purpose. Duplicate determinations were made on the refrozen rethawed aliquot. This method was employed because freezing and thawing results in an increase in activity of the enzyme as compared with the fresh preparation, and subsequent freeze-thaw cycles re-

sult in only a slight reduction in activity. Since prolonged storage in the frozen state produced no detectable change in activity, greater consistency could be obtained by analyzing all samples at the same time.

Cytochrome *c* oxidase determinations were made on most homogenate and mitochondrial samples from Experiment I by the method of Cooperstein and Lazarow¹⁷ as modified by de Duve et al.¹⁸ These determinations were performed on unfrozen tissue only. The results for heart were fairly consistent, but the liver showed considerable variation even after stimulation of cytochrome *c* oxidase activity by the addition of Lubrol.¹⁹

Isotopic labeling. Animals were labeled similarly in Experiments I and II. Proteins were labeled with the nonreutilizable isotope guanidine-¹⁴C-arginine⁶ (New England Nuclear, 5 mCi/mM). Each animal in groups 0, 1 and 2 received 10 μ Ci ¹⁴C-arginine by intraperitoneal injection two days before group 0 was killed. (In Experiment II, animals in group 2-1/3 were also labeled at this time; in Experiment I, this group was not labeled.) Animals in groups 3, 4, 5, and 6 received an injection of ¹⁴C-arginine one day before group 3 was killed, i.e., 6 days after the first injection of *l*-thyroxine.

DNA was labeled with methyl-³H-thymidine (Schwarz BioResearch, 6 mCi/mM) and with methyl-¹⁴C-thymidine (Schwarz BioResearch, 45 mCi/mM). All animals received 465 μ Ci ³H-thymidine by intraperitoneal injection 2 days before group 0 was killed. Each animal in groups 3, 4, 5, and 6 received 9 μ Ci ¹⁴C-thymidine by intraperitoneal injection one day before group 3 was killed (in addition to the initial injection of ³H-thymidine).

Purification of liver mitochondrial DNA. DNA was purified from liver mitochondria as described previously (method 2);¹⁰ this method entails deproteinization with phenol, incubation with ribonuclease, chromatography on a column of methylated albumen kieselguhr, denaturation and renaturation and preparative CsCl gradient centrifugation. When mitochondrial DNA is purified by this method, fractions of the final CsCl gradients are obtained which contain pure mitochondrial DNA as characterized by analytical ultracentrifugation.¹⁰ Twenty micrograms of highly purified SP-01 (*Bacillus subtilis* phage) DNA was added to each mitochondrial preparation at the start of the purification procedure. The main purpose of this was to attempt a rough approximation of the amount of mitochondrial DNA in each mitochondrial preparation from the recovery of SP-01 DNA in the final CsCl gradient; it also served as a density marker and may have improved yields of mitochondrial DNA by acting as a carrier. Purification of DNA from heart mitochondria was not attempted since the yield of mitochondria from 3 rat hearts is too low to permit reliable determinations of the specific activity of the DNA.

Reactivity determinations. Specific activity of proteins was determined in duplicate on all mitochondrial preparations. Approximately 5-10 mg of protein was precipitated in the cold by addition of 25 per cent TCA (trichloroacetic acid) to a final concentration of 5 per cent. The precipitate was collected by low speed centrifugation, washed once with 5 per cent TCA and collected in the same way. It was then resuspended in 3 ml 5 per cent TCA and incubated at 90°C for 30 min to hydrolyze DNA, cooled and collected by centrifugation again. The pellet was dissolved by addition of 3 ml 0.5 N NaOH, and incubated at 45°C for one hour, after which it was centrifuged at 9,000 g for 10 min and the small pellet was discarded. The supernatant was neutralized with 1.5 ml 1 N HCl and brought to 5 per cent with respect to TCA concentration by addition of 25 per cent TCA in the cold. The re-precipitated proteins were collected once more by low speed centrifugation. Lipids were extracted by washing successively with 5 ml ethanol-ether (3:1) twice, and once

with 5 ml acetone at room temperature. The final pellet was dried overnight in the centrifuge tubes and dissolved the next day in 1.2 ml concentrated formic acid. Of this solution, 1.0 ml was plated onto planchets and the formic acid was evaporated by heat. Radioactivity was determined in a Beckman low-beta 11 counter, a total of 2,000 counts being collected for each sample. Twenty and 50 microliter duplicate aliquots were taken and evaporated for protein determinations.

The specific activity of purified mitochondrial DNA was determined by collection of the appropriate fractions from each gradient on millipore filters, as described previously.¹⁰ Radioactivity was determined in a Packard Tri-Carb Scintillation Spectrometer and corrections were made for quenching and "spillage" of each isotope by internal standardization.

RESULTS

As described under Methods, the number identifying each group of three animals corresponds to its age in weeks after the initiation of the experiment. In each experiment, thyroid hormone was administered in the doses described above to all remaining animals at the time of death of group 2. Experiment I studies the transition from the hypothyroid to the euthyroid, or more properly to the slightly hyperthyroid state, and Experiment II the transition from the normal or euthyroid state to the strongly hyperthyroid state.

Oxygen consumption. In the thyroidectomized rats, oxygen consumption increased from $2.50 \pm 0.21 \times 10^{-2}$ ml/min/g before administration of thyroid hormone to $3.01 \pm 0.24 \times 10^{-2}$ ml/min/g after 3 weeks of treatment. In the normal rats, oxygen consumption increased from $2.96 \pm 0.28 \times 10^{-2}$ ml/min/g to $3.53 \pm 0.20 \times 10^{-2}$ ml/min/g in the corresponding period after treatment with large doses of thyroid hormone. The increase in oxygen consumption occurred in the first 3 weeks of administration of thyroid hormone. These values for oxygen consumption are somewhat higher and the changes are relatively smaller than those reported by others,²⁰⁻²² but they serve to establish that the dose of thyroid hormone was approximately a replacement dose in Experiment I, and was sufficient to produce hyperthyroidism in Experiment II.

Liver mitochondrial DNA turnover.^{*} The results of Experiment I are shown in Figure 1. The initial decline in specific activity in the hypothyroid animals (groups 0, 1, and 2) is exponential and corresponds to a half-life of about 14.0 days. Following the start of treatment with thyroid hormone there is a very rapid decrease in ³H specific activity (group 2-1/2), which appears to level off to some extent (groups 3 and 4) and then to decline rapidly and exponentially (groups 5 and 6). The half-life determined from ³H DNA specific activity over the last two weeks is 4.9 days. ¹⁴C-thymidine was administered 6 days after the first injection of thyroid hormone and one day before group 3 was killed. During the last two weeks the ¹⁴C DNA specific activity declines exponentially with a half-life of 10.0 days. This is more than twice the half-life of the ³H labeled DNA in the same preparation. The ratio of ¹⁴C specific activity to ³H specific activity, also shown in Figure 5, increases threefold between groups 3 and 6. The difference between the slopes of the two DNA labels is significant at the 1 per cent level (using the t test) over the period from group 4 to group 6.

^{*} It should be emphasized that the figures given for half-lives of mitochondrial DNA and protein in the following sections should only be taken literally where the specific activity declines exponentially and there is no evidence of a change in the total quantity of the constituent. Where these conditions are not satisfied a "half-life" is given only to simplify description of the changes seen in the specific activity curves.

Figure 1

Figure 2

Figures 1 and 2. Turnover of liver mitochondrial DNA in thyroidectomized and normal (euthyroid) rats. All animals received 465 μCi methyl- ^3H -thymidine 2 days before group 2 was killed; animals in groups 3, 4, 5, and 6 received 9 μCi methyl- ^{14}C -thymidine one day before group 3 was killed. Administration of l -thyroxine (T_4) was started at the time and in the dose indicated. Specific activities of ^3H -labeled DNA (○—○) and ^{14}C -labeled DNA (●—●) are indicated on logarithmic scales. Ratio of ^{14}C activity to ^3H activity of the DNA of groups 3, 4, 5, and 6 (□—□) is indicated on a linear scale. A free-hand curve has been drawn through each set of points.

In Experiment II (Figure 2) the initial decline in ^3H DNA specific activity in normal animals is exponential and corresponds to a half-life of 9.1 days; this is similar to the half life of 9.4 days we reported previously.¹⁰ There is little change in the ^3H specific activity immediately after the start of thyroid hormone administration (group 2-1/2), but there is a subsequent rapid decline in specific activity. The half-life of ^3H labeled mitochondrial DNA during the last two weeks is 8.4 days. Over the same period the ^{14}C labeled DNA has a half-life of 12.0 days. The ratio of ^{14}C to ^3H specific activity increases by a factor of nearly 2 during the last 3 weeks of the experiment.

In order to rule out the possibility that the difference in turnover of ^3H and ^{14}C labeled DNA observed in both experiments is due to differential reutilization of the two isotopes, in spite of the fact that they are both labeled in the 5-methyl group, a separate series of experiments was performed. Groups of thyroidectomized, normal, and hyperthyroid animals were simultaneously given the two isotopes used in Experiments I and II. Liver mitochondrial DNA was purified from animals killed 2 and 8 days later and the ratio of ^3H to ^{14}C DNA specific activity was determined. This ratio was constant for animals in each thyroid state. This shows that the apparent half-life of mitochondrial DNA is the same whichever isotope is used to determine the results.

In contrast to the results obtained for mitochondrial DNA, the ratio of ^{14}C to ^3H specific activity of nuclear DNA was constant in each experiment. This rules out the possibility that the changing ratio of ^{14}C to ^3H specific activity of mitochondrial DNA is due to random variations in uptake and incorporation of the two isotopes.

Quantitation of liver mitochondrial DNA. The approximate amounts of mitochondrial DNA in each mitochondrial preparation were estimated from the recovery of SP-01 DNA added in a known amount to each sample at the start of the purification procedure. In the final preparative CsCl gradient the SP-01 DNA was easily identified as a band heavier than the mitochondrial DNA band and separate from it, and also separate from a small band of denatured nuclear DNA which consistently contaminated all preparations. [The buoyant densities in CsCl of native SP-01, denatured rat nuclear, and renatured rat mitochondrial DNA are, respectively, 1.742, 1.717, and 1.706 g/ml.¹⁰ It is assumed that denatured SP-01 DNA renatures rapidly and returns to within 0.003 g/ml of its native buoyant density, as does mitochondrial DNA.] The amount of DNA in each band was calculated from the optical density at 260 m μ of the appropriate fractions of the gradient. From the recovery of SP-01 DNA an estimate can be made of the amount of mitochondrial DNA in the original mitochondrial sample. It is assumed, however, that recovery of the two DNA's is equal, or that any difference in recovery is consistent for all preparations. The ratio of mitochondrial DNA to mitochondrial protein in each preparation of mitochondria is given in Table 1. There is some variability in

Table 1

DNA CONTENT OF LIVER MITOCHONDRIA IN DIFFERENT THYROID STATES

Group	0	1	2	2-1/2	3	4	5	6
	μg DNA per mg mitochondrial protein							
Experiment I	0.095	0.110	0.168	0.090	0.169	0.133	0.213	0.140
Experiment II	0.098	0.130	0.064	0.091	0.070	0.110	0.149	0.100

these results as would be expected, but no obvious trend in the ratio of mitochondrial DNA to mitochondrial protein can be seen. A 50 per cent change in mitochondrial DNA/protein ratio would probably not be detected with certainty; but an increase of 300 per cent, such as has been reported to follow administration of thyroid hormone²³ could probably be detected. DNA/protein values reported here are all lower by a factor of 2-4 than those reported in a recent review.²⁴ This may reflect the different methods of determination.

Quantitation of liver and heart mitochondrial protein. The total liver and heart mitochondrial protein of each group of rats was calculated from the recovery of succinate cytochrome *c* reduc-

Table 2

TOTAL MITOCHONDRIAL PROTEIN CALCULATED FROM SUCCINATE CYTOCHROME c REDUCTASE ACTIVITIES
IN WHOLE TISSUE AND MITOCHONDRIA

	Experiment I*				Experiment II†			
	Mean of groups 0,1,2	Mean of groups 4,5,6	% change	p‡	Mean of groups 0,1,2	Mean of groups 4,5,6	% change	p‡
Liver								
Total enzyme activity, μ moles/min/liver	138	191	+ 38	< .01	190	261	+ 37	< .02
Mitochondrial specific activity, μ moles/min/mg protein	0.350	0.538	+ 54	< .01	0.414	0.597	+ 44	< .02
Total mitochondrial protein, g/liver	0.39	0.36	- 10	N.S.	0.46	0.45	- 2	N.S.
Heart								
Total enzyme activity, μ moles/min/heart	5.43	16.4	+ 202	< .01	17.6	26.0	+ 48	< .05
Mitochondrial specific activity, μ moles/min/mg protein	1.00	1.07	+ 7	N.S.	0.95	0.98	+ 3	N.S.
Total mitochondrial protein, mg/heart	5.43	15.3	+ 182	< .01	18.9	26.5	+ 41	< .05

* In Experiment I, groups 0, 1 and 2 were thyroidectomized untreated rats; groups 4, 5 and 6 were thyroidectomized rats each treated with 20 μ g *l*-thyroxine on alternate days.

† In Experiment II, groups 0, 1 and 2 were normal rats; groups 4, 5 and 6 were normal rats each treated with 300 μ g *l*-thyroxine on alternate days.

‡ Probability values refer to the significance of the difference (using the *t* test) between groups 0, 1 and 2 combined and groups 4, 5 and 6 combined.

tase and cytochrome *c* oxidase. As these enzymes are localized in the mitochondrial inner membrane^{19,25} the ratio of the total activity in the organ and the activity per unit of protein in the purified mitochondria gives an indication of the total mitochondrial protein of the organ. The mitochondrial protein mass calculated from the succinate cytochrome *c* reductase results are shown in Table 2. The total activity of this enzyme in both liver and heart increases following the administration of thyroid hormone; only in the heart, however, was an increase in the total mitochondrial protein demonstrated.

Liver mitochondrial protein turnover. Figure 3 shows the results of specific activity determinations on mitochondrial proteins in Experiment I, in which the transition from hypothyroidism to euthyroidism is studied. In untreated thyroidectomized animals the total mitochondrial proteins turn over with a half-life of 9.1 days. After 2 weeks of treatment with thyroid hormone in replacement doses the half-life is 6.3 days. Data for the corresponding periods in Experiment II, Figure 4, indicate a half-life of 4.0 days for mitochondrial proteins in normal rats. Four days after the start of thyroid hormone administration (group 2-1/2) the slope decreases considerably: Three days later the decline in specific activity is exponential and corresponds to a half-life of 5.3 days.

Heart mitochondrial protein turnover. The results of Experiments I and II are shown in Figures 5 and 6 respectively. In untreated thyroidectomized rats (Figure 5) the half-life of mitochondrial proteins is very long. After thyroid hormone administration the apparent turnover remains very slow. If a correction is made for the increase in total mitochondrial protein during this part of the experiment (by multiplying the specific activity by the total mitochondrial protein at each time point), there is very little or no loss of labeled mitochondrial protein. There is evidence that in heart, as in liver, guanidino-¹⁴C-arginine is not reutilized.²⁶ In untreated normal rats, Figure 6, heart mitochondrial protein has a half-life of 5.2 days; this is similar to the half-life of 5.0 days also reported from this laboratory.²⁶ The decline in specific activity ceases in the first 4 days of thyroid hormone administration. The specific activity of the mitochondrial proteins labeled 6 days after the start of thyroid hormone administration appears to decline slowly at first and more rapidly later. Again, correction for the increase in total heart mitochondrial protein suggests that there is very little degradation of mitochondrial protein for at least two weeks following administration of the second isotope.

DISCUSSION

The results may be summarized as follows: mitochondrial DNA and protein turn over at a slower rate in hypothyroid rats than in normal rats; mitochondrial DNA and protein turnovers are altered in the same direction and to the same extent following thyroid hormone administration; in heart, a decreased rate of degradation of mitochondrial protein contributes to the increase in the total mitochondrial protein. The observation that we consider to be most interesting is that liver mitochondrial DNA labeled before administration of thyroid hormone turns over at a different rate from DNA that is newly synthesized after administration of thyroid hormone.

Effect of thyroid state on mitochondrial turnover. Liver mitochondrial DNA of untreated thyroidectomized animals turns over more slowly than in untreated normal animals (groups 0, 1, and 2; Figures 1 and 2). The half-lives are approximately 14 days and 9 days, respectively. Liver mitochondrial protein also turns over more slowly in thyroidectomized animals (Figures 3 and 4). The difference in the half-life of mitochondrial DNA and protein can probably be explained on the basis of reutilization of the thymidine isotope. The guanidine group of arginine has a very small

Effect of Thyroxine on Turnover of Mitochondrial Proteins
Rat Liver (^{14}C Arginine)

Figure 3

Effect of Thyroxine on Turnover of Mitochondrial Proteins
Rat Liver (^{14}C Arginine)

Figure 4

Figures 3 and 4. Turnover of liver mitochondrial proteins in thyroidectomized and normal (euthyroid) rats. Animals in groups 0, 1, 2, and 2-1/2 received $10\mu\text{Ci}$ guanidine- ^{14}C -arginine 2 days before group 0 was killed; animals in groups 3, 4, 5, and 6 received $10\mu\text{Ci}$ guanidine- ^{14}C -arginine one day before group 3 was killed. Administration of ℓ -thyroxine (T_4) was started at the time and in the dose indicated. The specific activity ($\circ\text{---}\circ$) is indicated on a logarithmic scale of the ordinate. Free-hand curves have been drawn through each set of points.

**Effect of Thyroxine on Turnover of Mitochondrial Proteins
Rat Heart (^{14}C Arginine)**

**Effect of Thyroxine on Turnover of Mitochondrial Proteins
Rat Heart (^{14}C Arginine)**

Figures 5 and 6. Turnover of heart mitochondrial proteins in thyroidectomized and normal (euthyroid) rats. Animals in groups 0, 1, 2, and 2-1/2 received 10 μCi guanidine- ^{14}C -arginine 2 days before group 0 was killed; animals in groups 3, 4, 5, and 6 received 10 μCi guanidine- ^{14}C -arginine one day before group 3 was killed. Administration of *l*-thyroxine (T_4) was started at the time and in the dose indicated. The specific activity ($\circ\text{---}\circ$) is indicated on a logarithmic scale of the ordinate. The activity corrected for increase in mass of heart mitochondrial protein ($\bullet\text{---}\bullet$) was calculated by multiplying the specific activity by the total mitochondrial protein for each of groups 3, 4, 5, and 6. Corrected protein specific activities are expressed in arbitrary units. Free-hand curves have been drawn through each set of points.

probability of being reutilized;²⁷ relatively short half-lives are a feature of experiments that employ arginine labeled in the guanidine group,⁶ and problems related to the size and turnover of the precursor pool are avoided.

Heart mitochondrial protein also turns over more slowly in thyroidectomized animals than in normal animals (groups 0, 1 and 2; Figures 5 and 6).

The results do not permit any conclusions about mitochondrial turnover in the hyperthyroid state because a steady state has probably not been reached in the first few weeks of thyroid hormone administration.

Comparison of the effect of thyroid hormone on protein and DNA turnovers in liver mitochondria. It was noted above that both DNA and protein turn over more slowly in the hypothyroid state than in the normal state. There is additional evidence that mitochondrial DNA and protein are similarly affected by a change in thyroid state. In Experiment I (Figure 1), the turnover of newly synthesized DNA is more rapid after administration of thyroid hormone (¹⁴C-labeled DNA, groups 4, 5, and 6) than before it (³H-labeled DNA, groups 0, 1, and 2). Figure 3 shows that newly synthesized protein also turns over more rapidly after thyroid hormone administration (groups 4, 5, and 6) than before it (groups 0, 1, and 2). The similar effect of thyroid hormone on DNA and protein turnover can also be seen in Experiment II (Figures 2 and 4). It is notable that the change in turnover rate of DNA and protein is of the same relative magnitude.

The proteins labeled by a single injection of ¹⁴C arginine necessarily include a very large number of mitochondrial constituents from outer and inner membranes and matrix. It has been shown^{3,7-9} that significant differences exist between the turnover rates of these major components. However, the similarity of the effect of thyroid hormone on the turnover of mitochondrial DNA and uncharacterized protein suggests that mechanisms which control mitochondrial turnover may be predicated upon the whole mitochondrion or upon some large subunit consisting of the DNA and much of the protein, rather than upon individual components. Detailed turnover data for several purified mitochondrial constituents would be needed to confirm this.

Effect of changes in synthesis and degradation rates on mitochondrial mass. The total mitochondrial protein of heart increases following administration of thyroid hormone (Table 2). The increase could occur by an increase in the rate of synthesis or by a decrease in the rate of degradation of mitochondrial proteins. The turnover curves (Figures 5 and 6) suggest that degradation has been reduced. This is most clearly shown in Experiment II (Figure 6) where turnover is relatively rapid before thyroid hormone administration. The turnover rate is greatly reduced in the second part of the experiment, but increases toward the end (groups 5 and 6). Correction of the specific activity changes for the increase in total mitochondrial protein shows that very little of the newly synthesized protein is degraded until week 5 by which time the mitochondrial mass has stabilized at a new level. One must be cautious in concluding that degradation of all mitochondrial proteins has slowed to the extent suggested by the changes in specific activity of groups 3, 4, and 5. It was shown for liver mitochondria that newly synthesized DNA turns over more slowly than previously synthesized DNA. This could apply to protein in the heart mitochondria. It is noteworthy that the specific activity changes in heart mitochondrial protein which suggest decreased degradation are not apparent in liver mitochondrial protein (Figures 3 and 4) where no net increase in mass was detected.

Differential turnover of ³H and ¹⁴C-labeled DNA in liver mitochondria. There is a difference in the turnover rates of ³H and ¹⁴C-labeled DNA following administration of thyroid hor-

mone which cannot be explained on the basis of differential reutilization of the two thymidine isotopes. Mitochondrial DNA synthesized after the administration of thyroid hormone is evidently more stable than pre-existing DNA. A difference in the replication rate of the new and old populations of mitochondrial DNA would not by itself account for the difference in turnover. Two other features suggest that the difference in turnover is due to selective degradation of pre-existing mitochondrial DNA. In Experiment I, a replacement dose of thyroid hormone was administered to thyroidectomized animals. The half-life of newly synthesized (^{14}C labeled) DNA in the treated animals during the 4- to 6-week period is 10.0 days, about the same as in normal rats. The half-life of the pre-existing DNA (^3H labeled) is much shorter, suggesting that the pre-existing DNA is selectively degraded.

There is a steep decline in the specific activity of ^3H -labeled DNA following the start of thyroid hormone administration. This is most obvious in Experiment I (Figure 1, group 2-1/2), and cannot be explained solely on the basis of dilution of labeled DNA by newly synthesized DNA. The total mitochondrial DNA would have to increase by a factor of almost 3 to account for the three-fold decrease in ^3H -labeled DNA specific activity. Our estimate of the total mitochondrial DNA (Table 1) indicates that no net increase in total mitochondrial DNA occurs. We may conclude, therefore, that ^3H -labeled DNA is being replaced by newly synthesized unlabeled DNA in the first week of thyroid hormone administration. It is noteworthy that at the same stage of thyroid hormone administration the liver mitochondria become more active with respect to succinate cytochrome *c* reductase (Table 2). If a new population of mitochondrial DNA is replacing the pre-existing population the phenomenon most likely reflects the behavior of the whole mitochondrion rather than that of the DNA alone. We cannot, however, exclude the possibility that mitochondrial DNA is being replaced within relatively stable mitochondria. Nor can we exclude the possibility that heterogeneity of the liver cell population or nonuniform response to thyroid hormone may result in the apparent replacement of one population of mitochondrial DNA with another.

The increase in the turnover rate of mitochondrial DNA in the first week of treatment with thyroid hormone is similar to the phenomenon reported by Tata²⁸ in thyroid induced amphibian metamorphosis. An increase in the turnover of previously labeled cytoplasmic RNA occurs in the liver 2 to 4 days after the administration of thyroid hormone and precedes any increase in RNA mass. Thus it is possible that the replacement of one population of organelles with another, perhaps more physiologically appropriate, population may be a widespread response to a changing physiological environment.

If one population of mitochondria is being replaced by a more active population the method of this replacement is at present obscure. There is considerable evidence that mitochondria arise by growth and division of pre-existing mitochondria.²⁹ Luck's experiments³⁰ show that *Neurospora* mitochondria evolve from one type to another as a single population, indicating that the "progeny" of a mitochondrial division are identical. A more complex situation may be present in mammalian cells. One may speculate that at some stage in a changing physiological environment mitochondrial division results in two nonidentical progeny, one corresponding to the pre-existing mitochondrion, the other corresponding to the newly synthesized type. The pre-existing moiety is then eliminated by selective degradation. Inherent in this scheme is the idea that qualitative changes in the mitochondria, such as have been reported by Tata et al,²⁰ Roodyn et al,²² and Kadenback,³¹ and are seen in the present data (Table 2) are built into new mitochondria rather than brought about by modification of pre-existing mitochondria.

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A TWO-DIMENSIONAL THIN LAYER CHROMATOGRAPHY SYSTEM FOR PHOSPHOLIPID SEPARATION: THE ANALYSIS OF YEAST PHOSPHOLIPIDS*

By

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Thin layer chromatography has a considerable advantage over other analytic techniques for the rapid and easy analysis of lipid composition, and many such procedures are in use.^{1,2} A comprehensive analysis and the complete resolution of the many species of phospholipids encountered in eucaryotic cells are not easily achieved, however, and for this purpose two-dimensional thin layer chromatography,³⁻⁶ or two-dimensional chromatography on silica gel loaded filter paper^{7,8} are usually employed. In particular, the separation of the acidic phospholipids, phosphatidic acid, phosphatidyl glycerol and diphosphatidyl glycerol (cardiolipin) from one another and from the other major phospholipids, is difficult in a simple thin layer chromatographic procedure. Such preparations are frequently of considerable importance in short term isotope studies of phospholipid metabolism, in which trace quantities of phosphatidic acid, in contradistinction to the other two phospholipids mentioned, are heavily labeled. In this paper we report on a two-dimensional thin layer chromatographic procedure by which the separation of these three phospholipids, as well as of many other phospholipids, can be readily achieved. Our study deals with the use of this procedure for analyzing the phospholipid composition of *Saccharomyces cerevisiae* grown under a variety of conditions. The more specific aspects of the phospholipid composition of this yeast, as they relate to the problem of mitochondrial biogenesis, will be considered in another paper.⁹

MATERIALS AND METHODS

Reagents. The following solvents were used without further purification: methanol - reagent; chloroform - chromat-quality; and methylamine - 40 per cent in water (all Matheson, Coleman, and Bell, Norwood, Ohio); acetic acid - glacial "Baker analyzed," and anhydrous ether (Mallin-krodt Co., Chicago, Ill.). Chromatoplates 20 x 20 cm, 250 μ thickness precoated with silica gel H or H-HR, were obtained from Analtech Co. (Wilmington, Delaware). These plates were stored packed in their original boxes and were not heat activated before use. Silica gel H was used for qualitative work and for quantitative work in which the phospholipids were eluted from the plates prior to analysis. Because of the lower phosphorus blank values obtained, H-HR plates were used for quantitative work entailing direct phospholipid digestion in the presence of the gel.

Chromatographic solvents. The acid solvent run in the first dimension was chloroform/methanol/glacial acetic acid/water, in the proportions 52/20/7/3 (v/v/v/v) and was based on a solvent previously employed.⁴ For the second dimension a basic solvent based upon that of Kuhn

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and Lynen¹⁰ was employed, chloroform/methanol/40 per cent methylamine/water, in the proportions 13/7/1/1 (v/v/v/v).

Phospholipid standards. Egg lecithin, sphingomyelin and cardiolipin were purchased from Sylvana Chemical Co. (Millburn, N. J.). Egg lecithin prepared according to Hanahan et al¹¹ was also employed. Lysolecithin was purchased from Sigma Chemical Co. (St. Louis, Mo.). Phosphatidyl serine was obtained either from Sigma Chemical Co. or Pierce Chemical Co. (Rockford, Ill.). Phosphatidyl inositol was obtained from Pierce Chemical Co. or prepared according to Trevelyan¹² or Colacicco and Rapport.¹³ Synthetic phosphatidyl ethanolamine was either from Mann Research Laboratories (New York), or Sigma Chemical Co. A sample of bacterial phosphatidyl ethanolamine was kindly furnished by Dr. John Law. Dimethylphosphatidylethanolamine was obtained from Mann Research Laboratories. Phosphatidyl glycerol was prepared from Micrococcus lysodeikticus or Escherichia coli by the method of Baumann et al,¹⁴ followed by one-dimensional preparative thin layer chromatography with the acidic solvent used for this two-dimensional procedure, or the method of Skipski et al.¹ Dr. Goldfine also kindly furnished a sample of this compound.

Phosphatidic acid was obtained from Pierce Chemical Co. or was prepared from egg lecithin by enzyme digestion with cabbage phospholipase.¹⁵ Lysophosphatides from phosphatidyl ethanolamine and phosphatidyl serine were prepared from the corresponding diacyl phosphatides by hydrolysis with sodium methoxide at low temperature according to Marinetti.¹⁶ The progress of hydrolysis was followed by one-dimensional thin layer chromatography in the acidic solvent described above, and this was also used for the preparative isolation of the lysophospholipid.

PROCEDURE

Chromatography tanks were lined with filter paper and saturated with the appropriate solvent for at least 60 min before use. Two 2-cm lanes were separated along two intersecting edges of plate and these were used for chromatographing appropriate marker standards in either direction.

After chromatography in the first dimension for 60 to 90 min, the plates were allowed to dry at room temperature for 20 min. Since the presence of trace quantities of acetic acid on the plates interfered with the development of the chromatograms with the methylamine containing solvent, this was removed by washing the plates with diethyl ether. The plate was placed on a stand, with the direction of the first solvent run parallel to the vertical axis of the stand. The area of the plate below the point of sample application was washed down toward the lower edge of the plate. The plate was then turned through 180° and the rest of the plate was washed by gently running about 200 ml of ether over the surface of the silica gel in the direction traversed by the first developing solvent. After the ether wash, the plate was air dried for 20 min before developing it in the second dimension. If the humidity of the laboratory was high, the plates were dried for 20 min in a vacuum oven (with no warming) to prevent condensation of water vapor. The presence of such condensate resulted in all the phospholipids running with the second solvent front. Usually, marker standards for the second dimension were applied to the marker lane of the plate after the ether wash. A second aliquot of the extract being separated was applied to the corner of the plate opposite to that on which the sample was applied. This corner was not traversed by the separating solvents, and the aliquot thus served to check on the quantitative recovery of lipids from the plate. No more than 50 µg of lipid phosphorus was applied to a single plate for separation by the above procedure.

For quantitative determinations, plates were developed in triplicate with the same sample, and a blank plate was also similarly developed.

After development, the plates were air dried, the lipids were visualized with I_2 vapor, and the phospholipids were detected according to Dittmer and Lester.¹⁷ For quantitative determination of the phospholipids, blue spots were outlined, moistened with water, and transferred to digestion tubes for phosphorus analysis.¹⁸ We have no evidence that the presence of silicic acid in the phosphorus samples interfered with the phosphorus analysis. Major phospholipids were analyzed individually from each plate. In analyzing the minor phospholipids it was necessary to combine the appropriate gel from each of the triplicate plates in order to obtain accurate phosphorus estimates. An area of gel that was as closely equivalent as possible in position and size to that of a particular phospholipid spot on the test plate, was taken from the blank plate to serve as gel blank for that phospholipid. This was necessary because there were small variations in the gel blank depending upon the size and location of the spot on the plate.

When the dilutions were necessary for the accurate determination of the phosphorus in major phospholipid spots, these were performed at all stages of the procedure, i.e., all reagents for digestion and color development were doubled or trebled in quantity as appropriate. All tubes were centrifuged briefly to sediment gel immediately prior to spectrophotometry.

Yeast. The yeast analyzed was yeast foam 237. This is a diploid (probably now partially aneuploid) wild type yeast "grande" - ρ^+ - abbreviated in text and tables YF ρ^+ . A cytoplasmic "petite" ρ^- strain (abbreviated YFA ρ^-) derived from the wild type ρ^+ by acriflavin treatment was also employed. The rho factor (ρ) refers to the respiratory status as determined by a cytoplasmic genetic determinant, now thought to be mitochondrial DNA. Thus ρ^+ refers to a cell that has a normal or wild type cytoplasmic determinant and is therefore respiratory sufficient. ρ^- (cytoplasmic "petite") denotes a cell or culture in which the cytoplasmic genetic determinant (mitochondrial DNA) is altered so as to produce respiratory insufficiency. "Petites" are respiratory deficient strains, so named because they form small colonies when grown on medium containing small amounts of fermentable substrate (0.1 per cent glucose) and larger amounts of non-fermentable substrate (2 per cent glycerol). Wild type cells form large ("grande") colonies on this differential medium. Both yeast strains were kindly provided from the collection of Dr. P. P. Slonimski.

Culture media. Cultures were grown aerobically and anaerobically on standard and enriched media. Standard medium (YPG) contained 1 per cent yeast extract, 1 per cent Bactopeptone, 2 per cent glucose. Enriched medium described by Lindenmayer and Estabrook,¹⁹ as modified by Tustanoff and Bartley,²⁰ contained per liter: 10 g Difco yeast extract, 5.0 g acid hydrolyzed casein, 9.0 g KH_2PO_4 , 6.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.3 g $CaCl_2$, 5.0 ml Na lactate (70 per cent w/v), 0.2 g ergosterol, 5.0 ml Tween 80, 0.15 ml wheat germ oil, 5.0 ml ethyl alcohol and 20 g glucose.

Aerobic cultures were grown in 2-liter Bellco baffle shaker flasks and were shaken vigorously in a New Brunswick incubator rotary shaker for 18 to 40 hr at 28°. Anaerobic cultures were grown in 2-liter flasks equipped with a gas-flushing device that had a stopcock at its inlet and a mercury valve at its outlet. Purified nitrogen was passed through two gas wash bottles containing vanadyl sulfate and zinc amalgam in sulfuric acid to remove traces of oxygen, and finally through water to remove sulfuric acid spray. The medium was cooled on ice and gassed with purified nitrogen for 30 min prior to inoculation, and again for 45 min following inoculation of a stationary phase preculture. The stopcock at the inlet was closed and the flasks were shaken at 28°. The

amount of inoculum was varied depending on whether exponential or stationary growth was desired. Cultures were collected either in the early to mid exponential growth phase or in the stationary phase. Stationary cultures were collected 40 hr after inoculation to ensure maximal de-repression of mitochondrial development.

Yeast collection and breakage. The yeast cells were harvested by centrifugation at appropriate periods of the growth cycle, washed twice with ice cold water and either broken or frozen immediately thereafter. For breakage, 5 to 20 g of cells were suspended in 20 to 50 ml of water containing 1 mM EDTA. Cell breakage was performed at 0 to 5° and a pressure of 40,000 pounds per square inch in the Ribi cell fractionator (Sorvall). Small samples of the broken yeast cell suspension were saved for protein determination according to Lowry et al.²¹

Lipid extraction. This was carried out essentially as described by Letters²² at room temperature with constant shaking. Broken yeast cell suspensions were extracted with 20 vols of 95 per cent ethanol for 20 hr, and then, after filtration, the residue was re-extracted twice with 10 vols of chloroform/methanol 2/1 (v/v) for 2 hr each. The yeast residue was finally extracted for 2 hr longer with 10 vols of acidified solvent (chloroform/methanol/HCl 124/65/1 v/v/v). This last extract was neutralized with 2 N sodium hydroxide before combination with the three neutral extracts. The combined extracts were evaporated to dryness in a rotary evaporator at < 40°, and the residue was dissolved in chloroform/methanol 2/1 (v/v) and partitioned against one-fifth volume of 0.6 per cent NaCl according to Folch et al.²³ After removal of the upper non-lipid phase, samples were taken for phosphorus analysis.¹⁸ The extract was stored at 0° for no longer than one week prior to thin-layer chromatographic analysis.

RESULTS

A separation of pure phospholipid standards is illustrated in Figure 1.

Figure 2 shows a similar separation of yeast phospholipids. Variations in the relative mobilities of individual phospholipids on different chromatographic runs are associated with variations in laboratory humidity and are probably also influenced by the fatty acid composition of the particular phospholipids (e.g., yeast versus standards). The yeast phospholipids were identified and characterized in two ways. Single pure phospholipid standards were co-chromatographed with yeast lipid extract and the accentuated spot was readily seen. In addition, a yeast lipid extract was separated on DEAE cellulose prepared according to Rouser et al.²⁴ The basic lipids were eluted with chloroform/methanol 2/1 and 1/1 v/v. Phosphatidyl serine was eluted with glacial acetic acid and the remaining acidic phospholipids were eluted with the chloroform-methanol-water solvent employed by Letters²² for this purpose. The basic phospholipids were further sub-fractionated on two alumina columns as described by Letters,²² into phosphatidyl choline, dimethylphosphatidyl ethanolamine, mono-methylphosphatidyl ethanolamine and phosphatidyl ethanolamine. These separated yeast phospholipids were then chromatographed in this two-dimensional system and the phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and the acidic phospholipids, as well as a similar series of phospholipid standards, were also hydrolyzed in mild alkali according to Wells and Dittmer²⁵ and Dawson et al.²⁶ The water soluble products of alkaline hydrolysis of these phospholipids were further examined by high voltage electrophoresis²⁶ using ninhydrin and a phosphorus detection method²⁷ to locate the derived products. This type of characterization was necessitated by the difficulty we have experienced in obtaining reasonable results from mild alkaline hydrolysis of phospholipids eluted directly

Figure 1. On the left is a photographic reproduction of an actual two-dimensional thin-layer chromatogram. On the back of the glass plate, the area corresponding to the stained spot was blacked with magic marker to accentuate the spots for photographic purposes. On the right is a tracing of the chromatogram, with the individual spots numbered. The plate was a 20 x 20 cm glass plate precoated (Analtech, Wilmington, Delaware) with neutral HHR silica gel (250 μ thick). A sample of mixed phospholipids containing about 30 μ g of lipid phosphorus was spotted. The first solvent developed in the vertical direction was chloroform:methanol:acetic acid:H₂O (52:20:7:3 v/v/v/v). This was followed, after the plates were washed with diethyl ether, by chloroform:methanol:40% aqueous methylamine:H₂O (13:7:1:1 v/v/v/v) in the horizontal dimension. The spots were visualized by spraying with 6 N sulfuric acid and charring. All numbered spots were also stained with the acid molybdate (Dittmer and Lester¹⁷) spray. Spot numbers are as follows: 1, diphosphatidyl glycerol or cardiolipin; 2, phosphatidic acid; 3, phosphatidyl glycerol; 4, phosphatidyl ethanolamine; 5, phosphatidyl serine; 6, phosphatidyl inositol; 7, lecithin or phosphatidyl choline; 8, lysophosphatidyl ethanolamine; 9, sphingomyelin; 10, lysophosphatidyl serine; 11, lysolecithin or lysophosphatidyl choline. The region LL was used for the application of an aliquot of the sample used to check the quantitative recovery.

Figure 2. Reproduction of a thin-layer chromatogram of yeast phospholipids from YF ρ^+ aerobic yeast. For conditions of chromatography and visualization see text and legend to Figure 1. In this case the back of the plate was not marked with magic marker. All spots, including some of the minor unidentified spots, were also stained with the acid molybdate spray, except for Spot 1. The numbered spots on this chromatogram were as follows: 1, neutral lipid; 2, diphosphatidyl glycerol or cardiolipin; 3, phosphatidic acid; 4, phosphatidyl ethanolamine; 5, phosphatidyl glycerol; 6, dimethyl phosphatidylethanolamine; 7, lecithin or phosphatidyl choline; 8, lysophosphatidyl ethanolamine; 9, phosphatidyl serine; 10, phosphatidyl inositol; 11, lysolecithin or lysophosphatidyl choline.

from plates run in this two-dimensional chromatographic system.

Quantitative determination of yeast phospholipid composition. Separate analyses of at least two cultures of a given strain of yeast grown under particular culture conditions were made in most cases. Except where otherwise indicated, the data are all presented as the means of several analyses of different lipid extracts. Table 1 presents a typical triplicate thin-layer chromatographic analysis of a single lipid extract. The variation is shown only for the major phospho-

Table 1
REPRODUCIBILITY OF THIN-LAYER CHROMATOGRAPHIC ANALYSIS
Sample analyzed here is YFA ρ^- grown to stationary phase in standard medium. Since minor spots were pooled, these are not given for separate analysis.

	$\mu\text{g P}$	$\mu\text{g P}$	$\mu\text{g P}$	Mean $\mu\text{g P}$	S.D.*	% total PLP [†]
DPG [†]	0.82	0.82	0.87	0.84	0.02	2.9
PE	5.00	5.70	6.20	5.63	0.49	19.0
PC	11.25	13.50	13.80	12.85	1.09	43.5
DMPE	trace			trace		trace
PG				0.08		0.3
PA				0.29		1.0
PS	2.10	2.30	2.40	2.27	0.13	7.6
LPE				0.31		1.1
PI	5.10	5.80	5.80	5.57	0.33	19.0
LPC				0.53		1.8
?				0.34		1.2
?				0.81		2.7
SUM	26.63	30.48	31.23	29.51	1.91	
TOTAL P	33.30	32.85	34.20	33.45	0.56	
% Recovery	80.00	92.8	91.3	88.2	5.72	

* Standard deviation.

[†] Abbreviations: DPG = diphosphatidyl glycerol; PE = phosphatidyl ethanolamine; PC = phosphatidyl choline or lecithin; DMPE = dimethylphosphatidyl ethanolamine; PG = phosphatidyl glycerol; PA = phosphatidic acid; PS = phosphatidyl serine; LPE = lysophosphatidyl-ethanolamine; PI = phosphatidyl inositol; LPC = lysophosphatidyl choline or lysolecithin; PLP = phospholipid phosphorus.

lipids. It is seen that for the one plate, from which the recovery was only 80 per cent, there was uniformly less phosphorus in each of the major spots, suggesting that the deviation was probably due to a difference in the size of sample spotted. The phospholipid percentage composition calculated from the plate yielding a low recovery is very similar to that calculated from the other two plates. This indicates that the low recovery from some plates is related either to the size of sample spotted or some other problem which does not differentiate between individual phospholipids, so that closely comparable percentage compositional data can be obtained as shown in Table 2.

Table 2 shows that complete duplicate analyses of yeast grown on two separate occasions

Table 2

REPRODUCIBILITY OF PHOSPHOLIPID COMPOSITION ON TWO YEAST FOAM
CULTURES GROWN UNDER SIMILAR CONDITIONS (AEROBIC STATIONARY)

Yeast and medium	YF ρ^+ enriched*		YF ρ^+ standard		YFA ρ^- enriched	
	% PLP [†]		% PLP		% PLP	
	11-6	11-22	11-6	11-22	11-6	11-22
DPG [‡]	6.3	8.7	8.8	9.0	4.3	3.9
PE	16.4	13.4	19.1	18.7	24.0	24.4
PC	44.9	48.0	35.2	35.3	36.9	34.7
DMPE	0.4		trace	trace	0.7	3.2
PG	1.7	1.5	trace	trace	0.2	0.6
PA	2.0	1.2	2.6	trace	1.5	2.1
PS	8.3	10.6	8.5	10.2	9.9	4.5
LPE	1.1	trace	0.2	trace	0.1	0.5
PI	13.5	11.9	21.2	22.6	21.7	21.7
LPC	1.8	trace	0.2	0.2	0.6	1.4
?	2.7	3.2	1.7	trace		0.6
?	1.0	1.6	2.4	4.0	1.5	2.2
PLP μ g/gm protein	2865	2698	4173	2758	1960	

* Enriched medium is that of Tustanoff and Bartley¹⁸ as described in Methods. Standard medium is 1% yeast extract, 1% Bactopeptone, 2% glucose (YPG).

[†] PLP refers to the percentage of phospholipid phosphorus recovered from the respective thin-layer chromatograms.

[‡] For abbreviations, see footnote to Table 1.

under closely similar conditions, yield results that are very closely similar. This also indicates the measure of reliability of the observed differences between the phospholipid composition of the same yeast grown to the same phase of growth in dissimilar media.

Effect of growth medium on phospholipid composition. The nature of the medium influenced the distribution of phosphorus between various phospholipids, as illustrated in Table 3. These effects appear to be strain dependent. Thus, enrichment of the medium increased the lecithin content of the diploid YF ρ^+ but decreased its content of phosphatidyl inositol and phosphatidyl ethanolamine. The reverse changes were found in YFA ρ^- cells grown on the same two media. In addition, YFA ρ^- cells grown in enriched medium had relatively more phosphatidyl inositol than YF ρ^+ yeast in the same medium. The changes in phospholipid composition are quite reproducible (see Table 2) but they cannot be readily rationalized at this time. These reproducible effects offer an opportunity for the investigation of nutritional influences on regulation of phospholipid metabolism.

Influence of growth phase and cytoplasmic mutation on phospholipid composition.

Aerobic yeast. As shown in Table 4, exponential yeast usually contained more phospholipid than did stationary yeast. This was not so for the total phospholipid, lecithin, and phosphatidyl serine of the late stationary phase yeast foam "grande" (YF ρ^+), but the exponential wild type (YF ρ^+) and "petite" (YFA ρ^-) yeast contained more of these lipids as well as the other major

Table 3

EFFECT OF GROWTH MEDIUM ON PHOSPHOLIPID COMPOSITION OF
AEROBIC STATIONARY YEAST

Yeast strain	YF ρ^+ *		YFA ρ^-	
	Enriched	Standard (YPG)	Enriched	Standard (YPG)
DPG*	7.5 [†]	8.9	3.7	2.9
PE	14.9	18.9	24.7	19.0
PC	46.4	35.2	34.7	43.5
DMPE	0.2	trace	2.0	trace
PG	1.6	trace	0.3	0.3
PA	1.6	1.3	1.8	1.0
PS	9.5	9.4	7.6	7.6
LPE	0.6	0.1	0.6	1.1
PI	12.7	21.9	22.1	19.0
LPC	0.9	0.2	1.0	1.8
?	3.0	0.9	0.3	1.2
?	1.3	3.2	1.6	2.7
PLP μ g/gm protein [‡]	2782	3466	1823	2231

* For abbreviations, see footnote to Table 1.

[†] The data in this table are presented as the percentage of total phospholipid phosphorus recovered from the respective thin layer chromatograms.

[‡] Total phospholipid phosphorus recovered in the whole extract for each gram of yeast protein extracted.

phospholipids than the early stationary wild type (YF ρ^+) and late stationary "petite" (YFA ρ^-) yeast. The yeast foam "grande" also had more phosphatidyl inositol and phosphatidyl ethanolamine in the log phase cells than in the stationary culture. Cardiolipin content was, however, higher in the stationary than in the exponential yeast. Also of considerable significance is the comparison of phospholipid composition between the wild type YF ρ^+ and the cytoplasmic mutant YFA ρ^- . In the stationary yeast, larger amounts of lecithin and phosphatidyl serine were present in the wild type than in mutant cells. There was a higher relative cardiolipin content in the YF ρ^+ yeast, in both stationary and log phase cultures, although the difference was more pronounced in the stationary yeast. In all other respects, log phase cells of the wild type (YF ρ^+) and petite (YFA ρ^-) yeast had almost identical phospholipid contents. Among the minor phospholipids, it is perhaps worth noting that dimethyl phosphatidyl ethanolamine appears to be more prominent in log phase YF ρ^+ yeast.

Anaerobic yeast. Table 5 shows the results of phospholipid compositional analysis of several yeasts grown to different stages under anaerobic conditions. As with aerobic yeast, log phase cultures of YF ρ^+ yeast contained more phospholipid than the corresponding stationary cultures. In contrast to the aerobic situation, however, late stationary YF ρ^+ anaerobic yeast contained relatively less lecithin and relatively more phosphatidyl inositol than either the exponential

Table 4

PHOSPHOLIPID COMPOSITION OF WILD TYPE AND MUTANT YEAST AT
DIFFERENT PHASES OF AEROBIC GROWTH

Growth was in enriched medium. For abbreviations, see footnote to Table 1,
and for explanation of mode of expression of data see footnote to Table 3.

Growth phase	Stationary			Exponential	
	Early [*] YP ρ^+ (2) [†]	Late YP ρ^+ (2)	Late YFA ρ^- (3)	YF ρ^+ (3)	YFA ρ^- (2)
	% PLP	% PLP	% PLP	% PLP	% PLP
DPG	6.6	7.5	3.7	3.3	1.3
PE	18.1	14.9	24.7	21.6	21.8
PC	41.3	46.4	34.4	41.6	39.5
DMPE	2.1	0.2	1.9	1.4	1.0
PG	0.5	1.6	0.3	0.3	0.6
PA	1.2	1.6	1.8	0.8	1.8
PS	7.3	9.5	7.6	7.5	7.7
LPE	1.3	0.6	0.5	1.0	0.2
PI	20.4	12.7	22.1	20.3	21.8
LPC	1.1	0.9	1.0	0.3	2.2
?		3.0	0.3	0.4	0.7
?		1.3	1.6	0.9	1.7
Recovery of PLP from thin-layer chromatograms		89.0	95.8	107	100
Total PLP $\mu\text{g/gm}$ protein	1800	2782	1823	2800	2840

^{*} This yeast was grown to early stationary phase, i.e., for 18 hr, while all other stationary yeast was grown to late stationary phase, i.e., 40 hr.

[†] Numbers in parentheses indicate the number of separate cultures employed for the derivation of the data presented.

anaerobic yeast or the aerobic stationary yeast. The stationary wild type yeast also had much more cardiolipin than the cells of the cytoplasmic mutant cultures at this stage of growth. The difference between the cardiolipin content of stationary and exponential YF ρ^+ yeast were also evident in anaerobic yeast. The anaerobic cells had less cardiolipin than aerobic cells of all strains harvested at corresponding periods of the growth cycle. As with aerobic yeast the phospholipid composition of the wild type (YF ρ^+) and "petite" (YFA ρ^-) in the exponential growth phase were very similar to one another and to similarly grown aerobic yeast.

Of the minor phospholipids, dimethyl phosphatidyl ethanolamine was more prominent in most of the anaerobic yeast cultures, especially in those harvested in stationary phase, than in aerobic yeast. No notable difference in lysophospholipids was observed.

Table 5

PHOSPHOLIPID COMPOSITION OF WILD TYPE AND MUTANT YEAST AT
DIFFERENT PHASES OF ANAEROBIC GROWTH

Growth was in enriched medium. For abbreviations, see footnote to Table 1,
and for explanation of mode of expression of data see footnote to Table 3.

Growth phase	Stationary			Exponential	
	Early [*] YF ⁺ (1) [†]	Late YF ⁺ (2)	Late YFA ⁻ (1)	YF ⁺ (2)	YFA ⁻ (2)
DPG	3.6	4.7	0.4	2.5	1.5
PE	19.4	13.5	19.5	15.4	17.8
PC	41.4	20.5	38.0	43.3	46.5
DMPE	4.9	3.7	2.3	2.8	1.5
PG	0.8	1.4	0.2	0.5	trace
PA	2.7	1.5	2.0	0.9	0.8
PS	8.9	9.5	10.9	6.9	5.6
LPE		0.4	1.5	0.5	2.0
PI	16.6	32.8	18.7	25.3	21.6
LPC	2.4	0.6	1.5	0.1	0.3
?		1.7	0.7	0.6	0.6
?		3.6	3.9	0.8	2.0
% recovery of PLP from thin-layer chromatogram		117	82.0	88.0	85.0
Total PLP μ g/gm protein	1302	1050	2260	1790	2210

^{*} This yeast was grown to early stationary phase, i.e., 18 hr, while all other stationary cultures in this table were grown to late stationary phase, i.e., 40 hr.

[†] Numbers in parentheses refer to the number of separate cultures analyzed to provide data presented here.

DISCUSSION

Our results for total phospholipid content of yeast agree fairly closely with those reported by Letters,²² Jollow et al.²⁸ and Kovac et al.²⁹ The methods employed in this study have facilitated a fairly comprehensive analysis of the phospholipid of a number of yeast strains and their mutants grown under a variety of conditions. Our results on aerobic exponential yeast are in broad general agreement with those of Letters.²² The results of Deterkauf and Booi³⁰ for the phospholipid composition of a galactokinaseless mutant of *Saccharomyces cerevisiae* grown to late stationary phase are in excellent agreement with our data for yeast foam "grande." The phospholipid composition of early stationary anaerobic yeast grown in a medium enriched with Tween 80 and ergosterol reported by Jollow et al.²⁸ agree fairly closely with our analysis of similarly cultured yeast. The results obtained by these authors for early stationary aerobic yeast grown in standard medium differ somewhat from our analyses of yeast grown aerobically in this medium to late stationary phase, or in enriched medium to early stationary phase. In each case

we found more lecithin, phosphatidyl inositol, and phosphatidyl serine, and less phosphatidyl ethanolamine than did these investigators. These variations may be due to a composite of strain and culture differences.

Our values for dimethylphosphatidyl ethanolamine are somewhat lower than those reported by Letters²² and Lester and White³¹ who used different methods of analysis.

The detailed differences in phospholipid composition induced by alterations in the nature of the medium and cytoplasmic mutation and at different phases of growth cycle have already been discussed. In brief, it is clear that in most cases exponential yeast had more phospholipid than stationary yeast, and that this affected primarily the major phospholipids, phosphatidyl inositol, phosphatidyl ethanolamine, and lecithin. Cytoplasmic mutation did not result in any substantial differences in these major phospholipids. The effects of medium enrichment were variable and depended on the strain, but were most pronounced in the yeast foam "grande" (YF_ρ⁺), in which the rich medium increased the lecithin content mainly at the expense of phosphatidyl inositol. The phospholipid composition of log phase yeast cells were very similar, irrespective of the strain and of the presence or absence of oxygen during growth. The effects of anaerobiosis were evident in the late stationary phase of growth, especially in the wild type yeast YF_ρ⁺, in which the anaerobic cells contained much less lecithin, cardiolipin, and phosphatidyl ethanolamine and relatively more phosphatidyl inositol than aerobic cells.

The most striking difference in the phospholipid composition of various yeasts under several growth conditions was in their cardiolipin content, which will be discussed more fully in another paper (Jakovcic et al⁹).

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SYNTHESIS OF COLLAGEN, MYOSIN, NON-COLLAGENOUS PROTEIN, AND DNA DURING EXPERIMENTAL MYOCARDIAL HYPERTROPHY IN THE RAT*

By

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During and following the development of cardiac hypertrophy, a number of changes have been observed in the composition and metabolism of rodent hearts,¹ including changes in ventricular DNA.[§] Grove, Nair, and Zak,² for example, noted an increase in the total DNA of the left ventricle following the induction of cardiac hypertrophy in rats by constriction of the thoracic aorta. The DNA increased in proportion to ventricular weight, so that there was no change in the DNA concentration. Histological studies suggested that the increase in DNA might have resulted from proliferation of connective tissue cells, since no mitotic figures were observed in muscle cells, and increased polyploidy of muscle cells could account for only a minute fraction of the increase. Following banding, the mitotic indices of connective tissue cells in hearts increased to values ten times greater than control, and autoradiography after labeling with tritiated thymidine demonstrated that new DNA synthesis occurred almost exclusively in connective tissue cells.³

It is believed that collagen is synthesized only by connective tissue cells. We have undertaken studies of the metabolism of collagen in correlation with cardiac hypertrophy as an index of the changes in the activity of connective tissue cells. Changes in the synthesis of non-collagenous protein (NCP), myosin, and DNA have also been estimated and compared with estimates of collagen synthesis during hypertrophy.

Collagen is, for practical purposes, the only protein in mammalian tissue which contains the imino acid hydroxyproline (hypro). Hypro is derived from proline, which is hydroxylated after it is incorporated into protocollagen, the polypeptide precursor of the collagen peptide chain.⁴ Collagen can be extracted quantitatively from tissues into hot trichloroacetic acid (TCA).⁵ Thus, heart collagen content and radioactivity were estimated by measuring the content and radioactivity of hypro in hydrolysates of hot TCA extracts of heart.⁶

We have confirmed the findings of Grove et al.³ and Bartosova et al.⁷ The collagen content of the heart does not change during the early phases of hypertrophy induced by aortic banding and, consequently, the concentration falls. Later, the heart collagen content and concentration both increase (Figure 1).

In a series of preliminary experiments, we measured the incorporation of radiolabeled proline into collagen hypro at times ranging from 2 to 30 days after induction of cardiac hypertrophy

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[§] Abbreviations used: DNA, deoxyribonucleic acid; NCP, non-collagenous protein; TCA, trichloroacetic acid; hypro, hydroxyproline.

Figure 1. Effect of banding upon total heart collagen and collagen concentration. Open circles represent means from sham-operated animals; closed circles means from banded animals.

by aortic banding of 205-210 g female Sprague-Dawley rats.⁸ Radiolabeled proline was injected intraperitoneally. Three hours later, heart collagen was extracted into hot TCA and its radioactive hypro content measured. Incorporation of radioactivity into NCP (the residue remaining after TCA extraction) was also measured (Figure 2). Collagen hypro radioactivity was increased at each time studied. Enhanced labeling of heart components following aortic constriction could result from an increased delivery of radioactive precursor to the tissue, and as a result there would be an increased specific activity of precursor pool and, subsequently, product. The percentage increase in labeling of collagen hypro exceeded the increase in labeling of NCP (Figure 2). Thus, it is unlikely that enhanced incorporation of radioactivity into collagen hypro resulted solely from an increase in the specific activity of the precursor pool.

The increase in heart weight resulting from aortic banding varies within a given banding procedure, and between experiments. The extent of collagen labeling varied with heart weight (Figure 3). We wished to study the time course of labeling of collagen and NCP. In order to minimize the variations in the hypertrophic response, a large group of animals was banded on a single day. At varying times after banding, animals were injected with tritiated proline as before, and incorporation into collagen and NCP was measured. Collagen labeling reached a peak 4 days after banding (Figure 4); NCP labeling was maximal on about the second day, and remained elevated.

We also explored differences between the metabolism of muscle and connective tissue cells in a group of banded animals. Two, 4 and 7 days after banding, members of the group were injected with a mixture containing tritiated thymidine (to label nuclear DNA), ¹⁴C-lysine (to label myo-

Figure 2. Effect of banding upon recovery of radioactive proline as collagen hypro and in NCP. Each animal was injected with 10 μ Ci proline- $U-^{14}C$ on day 7 after banding, and with 50 μ Ci proline-2,3- 3H on all other days. The height of the bars represents mean radioactivity recovered in hearts from sham-operated (white bars) and banded (black bars) animals. The vertical lines represent 2 S.E. of the means. The number in each group is given at the base of the bars. Each difference is significant by Student's t test with a p value of 0.01 or less.

Figure 3. Correlation of heart weight with collagen synthesis. Individual data from experiments in Figure 2 are plotted. Open circles represent observations in sham-operated animals; closed circles, banded animals.

Figure 4. Time course of collagen and NCP synthesis after banding. The mean radioactivity recovered in sham-operated (open circles) and banded (closed circles) animals is plotted. The vertical lines represent 2 S.E. of the means. Where comparisons between banded and sham-operated animals are possible, the differences are significant by Student's *t* test with a *p* value of 0.02 or less.

Figure 5. Time course of synthesis of collagen, myosin, NCP, and DNA following aortic banding. Animals were injected with a mixture of ³H-proline, ¹⁴C-lysine, and ³H-thymidine 2, 4 and 7 days after banding. Hearts were weighed and pooled according to whether they exhibited gross hypertrophy (solid triangles), moderate hypertrophy (solid squares) or no hypertrophy (sham-operated, open circles). The result from each pool of 2-3 hearts is plotted.

sin* and non-collagenous protein), and tritiated proline (to label collagen). The hearts were removed, weighed, and pooled according to whether they exhibited gross hypertrophy, moderate hypertrophy, or were from sham-operated animals. DNA labeling reached a peak in both groups of experimental animals one week after banding (Figure 5). The curves of myosin and non-collagenous protein synthesis were very similar to each other, reaching a plateau and remaining elevated. Collagen synthesis by the moderately hypertrophied hearts reached a peak on the fourth day after banding. Collagen synthesis in the grossly hypertrophied hearts reached a maximum on or before the second day after banding. The time course of labeling of myosin, which is formed in the muscle cells, is distinctly different from that of the labeling of nuclear DNA and collagen, which are formed by the connective tissue cells. Furthermore, changes in collagen labeling by the connective tissue cells in response to banding occur with a different time course than the changes in labeling of DNA.

We can only speculate about the effects of an increased collagen concentration in the hypertrophied heart. It has been suggested that the collagen of blood vessels prevents excessive distension when pressure in them is increased.⁹ Collagen in the heart might similarly prevent excessive distension which would result in decreased efficiency of contraction processes (cf. Sonnenblick¹⁰).

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*Myosin was extracted by a procedure to be published.

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