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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1970

ALEXANDER GOTTSCHALK, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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ACCIDENTAL RADIOGOLD (^{198}Au) LIVER SCAN OVERDOSE WITH FATAL OUTCOME*

By

J. M. Baron, S. Yachnin, R. Polcyn, F. W. Fitch, and W. Q. Sturmer†

The use of radioisotopes for scanning has added significantly to the clinician's ability to detect and interpret disease manifestations. As with other useful materials administered intravenously to patients, radioisotopes must be most carefully monitored to prevent the type of accidental overdosage described below.

CASE HISTORY

A 73-year-old white housewife was transferred to the Argonne Cancer Research Hospital in August, 1968. She had accidentally received 200 mCi instead of the anticipated 200 μCi dose of radioactive colloidal gold (^{198}Au) intravenously for a liver scan. She expired 69 days later of hemorrhagic complications related to thrombocytopenia.

The patient had been in good general health, except for congenital deaf-mutism, until 1961. At that time she was first noted to have a hemoglobin of 9.0 g per cent. The anemic condition was found to be refractory to multiple hematinics over the next seven years. She required blood transfusion on one occasion in 1963. In March, 1968 peripheral blood and bone marrow findings established the diagnosis of chronic erythremic myelosis.¹⁻³ Figure 1 illustrates two fields from a bone marrow aspirate done at that time. The megaloblastoid maturation with bizarre nuclear configurations in the red cell line and atypical megakaryocytic forms are to be noted. A peripheral blood smear showed many nucleated red blood cells and early myeloid forms, including 5 per cent myelocytes. No therapy was instituted for her blood condition at that time.

Early in August, 1968 the patient was readmitted to her local hospital for evaluation of abdominal swelling and hepatomegaly. Repeat bone marrow aspirate confirmed the earlier findings. Evaluation of the hepatomegaly included the radioactive colloidal gold (^{198}Au) scanning procedure which is the subject of this report. As soon as the overdosage was realized the patient was placed in protective isolation and arrangements were made for her transfer to Argonne Cancer Research Hospital.

Upon arrival at the emergency room some 30 h after the radiogold injection, the patient was monitored externally to check the reported overdose and to estimate her potential hazard to bedside personnel. The exposure dose rate at that time was 35 milliRoentgens/h at one meter distance, which was felt to be consistent with the reported dose of 200 mCi. From the finding of 0.04 $\mu\text{Ci/ml}$ of whole blood, it was calculated that less than 0.1 per cent of the injected dose remained in the total blood volume at 30 h after injection.

A whole body scan was performed without administering additional isotope (Figure 2). The

* This report is taken from a paper that was presented at an IAEA Symposium on Handling of Radiation Accidents, IAEA-SM-119/39, p. 399, 1969.

† Department of Pathology, University of Chicago.

Figure 1. Bone marrow smear made prior to accidental radiogold (^{198}Au) colloid overdosage. Note red cell and megakaryocyte changes of erythremic myelosis. (Wright's stain x180)

total body distribution of detected counts was felt to be typical for uptake of colloidal material by an abnormally extensive reticuloendothelial marrow. A similar result has been noted in patients who have polycythemia vera.⁴ A scan of the upper abdomen done at the same time shows the liver and spleen contours (Figure 3). The unusually large amount of radioactivity in these sites was corroborated by the sizeable count numbers accumulated very rapidly with a pinhole aperture on the detector.

Estimated dosages of radiation to be received by the several key target organs, namely, liver and spleen, bone marrow, and intestine were calculated by standard beta and gamma dose formulas.⁵ Assumptions made for purposes of these calculations were: (1) that liver and spleen combined retained 95 per cent of the total administered isotope;⁴ (2) that their weights were 1700 g and 300 g, respectively; (3) that the effective half-life in these organs of the radiogold-198 is equal to its physical half-life of 2.7 days; and (4) that the geometric factor for gamma dose is equal to that defined for a cylinder of radius 10 cm and length 5 cm. The resulting estimates are given in Table 1. The sizeable liver and bone marrow doses predicted clinical difficulties with these organs. No significant intestinal dysfunction was anticipated or observed.⁶

On arrival at the Argonne Cancer Research Hospital the patient was alert, cooperative, and in no acute distress. Pertinent physical findings included a firm nontender liver edge palpable 5 cm below the right costal margin and spleen palpable 3 cm below the left costal margin. Several small inguinal nodes were present bilaterally. Blood counts on admission were: hemoglobin - 9.7 g per cent; white blood cell count - 5,450 per cu mm; differential - 42 per cent polymorphonuclears, 19 per cent stab forms, 3 per cent metamyelocytes, 28 per cent lymphocytes, and 8 per cent monocytes. Five normoblasts were present for every 100 white blood

Figure 2. Whole body scan made with a gamma scintillation camera (Anger) using a pin-hole collimator at 30 hours after intravenous dose of 200 mCi of radiogold (^{198}Au) colloid. This figure is a composite of three images made with equal exposure time. Note the abnormally extensive distribution of reticulo-endothelial marrow.

Figure 3. Anterior liver scan made in same manner as Figure 2 with a one minute exposure time (728,000 counts). Midpoint of liver was 31 cm from the pin-hole aperture. Liver and spleen appear adjacent with no marked difference between activity in the spleen and the left lobe of the liver.

Table 1

Organ	Estimated absorbed dose*
Liver	7,300 rads
Spleen	7,300 rads
Intestine adjacent to liver and spleen	600 rads
Red bone marrow	400 rads
Intestine of mid abdomen	60 rads

* Estimated absorbed doses after 200 millicuries radiogold (^{198}Au) colloid intravenously. Figures are based on standard beta and gamma dose formulae (Johns, H. E., *The Physics of Radiology*, pp. 562, 565, Second Edition, C. C. Thomas, publisher).

cells. The reticulocyte count was 6.5 per cent. Other laboratory results included an elevated serum uric acid of 16.3 mg per cent, a slightly increased serum alkaline phosphatase (68 International Units), blood urea nitrogen of 23 mg per cent, and normal serum bilirubin and bleeding and clotting workup. The serum lactic dehydrogenase level was approximately 10 times the upper normal value, but this finding had been noted prior to administration of the radiogold.

It seemed clear that the first major management problem would be support of the patient's hematopoietic system.⁶ Bone marrow transplantation was considered, but not attempted because of the lack of a closely compatible donor and the uncertain value of such a procedure in the 400-500 R bone marrow dose range in humans.⁷ The patient was initially placed in protective isolation with strict limitation of exposure time for personnel at the bedside. By the end of the first week, when her absolute granulocyte count had fallen below 500 cells per cu mm, she was in a special two-room isolation suite designed to provide surgical asepsis in the patient's half of the area. Clothing, food, water, utensils, and all other items entering the patient's room were pre-sterilized. The air in the room was kept as dust-free as possible by filtration through laminar flow air hoods (Abbott Clean Air Centers manufactured by Air Control, Inc., Norristown, Pennsylvania, and kindly loaned to us by Abbott Laboratories, North Chicago, Illinois). Oral Neomycin sulfate and Nystatin were started on admission in an effort to reduce the possibility of bacteremia from endogenous intestinal flora. Vitamin K₁, pyridoxine, and folic acid were other oral medications given throughout the hospital course to replace substances normally made by intestinal flora and/or destroyed by food sterilization. Allopurinol was started soon after admission, and the serum uric acid had returned to normal by the tenth day.

The patient was given intermittent transfusions of fresh whole blood, but the number of compatible units available was limited by her A negative blood type. During the first three to four weeks daily infusions of buffy coat concentrates containing granulocytes and platelets⁸ were given, but later platelet concentrates were substituted because of febrile reactions noted after administration of buffy coat preparations.

Daily peripheral blood counts were performed and are illustrated in Figure 4. As expected, the absolute lymphocyte count fell most rapidly and eventually showed some recovery. The granulocyte level dropped somewhat later than the lymphocyte count and showed sufficient improvement by the 9th week to permit discontinuation of the isolation procedures. There was never any significant difficulty with infection. The platelet count reached lowest levels by the third week, but never could be raised above 8,000 per cu mm thereafter despite the infusions of

Figure 4. Peripheral blood leukocyte and platelet counts following intravenous radiogold (^{198}Au) colloid (200 mCi) given on day zero (arrow). Note early fall of absolute lymphocyte and granulocyte counts with later partial recovery. Platelet levels fall later without evidence for recovery by day 70 (patient's demise).

platelet concentrates. Intermittent injections of androgen were given in hopes of stimulating marrow function,⁹ and, later, corticosteroids were started in an effort to reduce capillary fragility and possibly prolong platelet lifespan.

Despite these measures aimed at increasing platelet levels the patient had persistent marked thrombocytopenia and petechiae, intermittent hematuria, and subconjunctival hemorrhage. As the hospital course progressed, the liver and spleen were noted to decrease in size. There was no significant deterioration of the liver function tests. The patient remained alert and ambulatory within her room until the terminal event. On the 68th hospital day she complained of sudden dizziness and fell to the floor. She complained of severe headache, and became progressively obtunded. Consciousness was not regained, and she expired the following day.

POST MORTEM FINDINGS

Post mortem findings of special interest were in the bone marrow, liver, lymph nodes, and brain (Figure 5). Femoral marrow was generally hypocellular, but there were readily identifiable islands of erythroid and granulocyte precursors. Megakaryocytes were notably scarce. Rib, sternal and vertebral marrow specimens were similar. The liver weighed 2090 g. The spleen weighed 490 g. Microscopic changes in the liver were non-specific—a number of hepatocytes were seen to have hyperchromatic and/or double nuclei. Special stains failed to reveal more than a very slight increase in the amount of collagen in the liver. Lymph nodes were somewhat atrophic with prominent extramedullary hematopoiesis both within the medullary regions of the

Figure 5. Post mortem microscopic changes in femoral bone marrow (a, b), liver (c), and lymph node (d). Note moderate hypocellularity with islands of erythroid activity and absence of megakaryocytes in marrow areas (a-Azure-eosin x 45; b-Azure-eosin x 72), focal nuclear atypia and congestion in liver (c-Hematoxylin and eosin x 45), and lymphoid depletion and hematopoiesis in pericapsular fat of lymph node (d Hematoxylin and eosin x 72).

nodes and in the pericapsular fat. Intracerebral, subdural, and subarachnoid hemorrhage were identified.

DISCUSSION

The pattern of radiation injury expected from overdosage of intravenous radiogold colloid is predictable from our knowledge of the fate of injected colloidal particles and the radioactive decay parameters of the specific isotope. The metallic gold particles in colloidal form are 25 to 50 millimicra in size and are avidly phagocytosed by the reticuloendothelial system.¹⁰ These particles are almost completely cleared from the blood in 10 to 30 minutes. The gold particles are metabolically inert and chemically nontoxic in the human body. They are irreversibly trapped in the macrophages until the cells degenerate and die. As a result of this effective sequestration the colloidal material is not excreted to any significant extent in feces or urine. Our patient, for example, excreted only 2.2 μ Ci of radiogold or .001 per cent of the injected dose in the stools over the first 4 days of hospitalization. Urinary radioactivity during this period was also minimal. Attempts at mobilization of the particles with two, three dimercapto-propanol (British anti-Lewisite, BAL) and other chelating agents have been unsuccessful.¹¹ Thus, once cleared by the reticuloendothelial system, the particles are strategically fixed adjacent to proliferating radiosensitive cells in bone marrow, liver, and spleen.

The time course and type of radiation injury sustained by these sensitive cells is dependent on certain physical properties of the gold isotope, which are indicated in Table 2. The major damage from radioactive decay is due to the beta emission. The gamma emission is useful for external monitoring and thus for scanning and dosage estimates. It is also responsible for the exposure hazard to bedside personnel in circumstances of overdosage. The physical half-life of 2.7 days permitted some time to attempt removal or neutralization of the particles before the vast majority of the radiation dose would be delivered, but no effective method of preventing the inevitable radioactive decay at the sites of particle localization is known at present.

Table 2
PHYSICAL CHARACTERISTICS OF RADIOGOLD (^{198}Au)

Physical half life	2.7 days
Average beta energy	0.328 meV
Primary gamma energy	0.411 meV
Dose rate constant (Roentgens per hour one cm from a one millicurie point source)	2.7 <u>R</u> per hr

It was clear from the initial dosage estimates, from the considerations just mentioned, from numerous studies in animals, and from the subsequent clinical course that serious hematopoietic insufficiency is the major early problem. We also suspected that even if this phase could be weathered with supportive care later hepatic damage was likely. Our expectations in this regard were confirmed by the as yet unreported experience of Brodsky¹¹ with a similar overdose accident in which the patient survived a prolonged period of hematopoietic insufficiency with thrombocytopenia as a major feature only to expire of hepatic insufficiency months later. It must be pointed out, however, that the exact nature of the hepatic pathology was not clear in Brodsky's case, and that hepatitis secondary to extensive blood product replacement during the hematopoietic failure phase may have been an important contributing factor.

In contrast to the paucity of information about the toxicity of intravenous radiogold in humans,¹² considerable data are available from animal studies. Blood dyscrasias¹³ and several types of delayed hepatic pathology including hepatoma formation,¹⁴ cystic changes, fibrosis, necrosis, and cirrhosis have been noted.¹⁵

A very interesting aspect of our patient's story, which complicated interpretation of her clinical course and pathologic findings, was her pre-existing hematologic disorder. We were uncertain how the presence of this condition might modify several aspects of the body's ability to handle the radiation injury and recover. It was clear at the outset from the scan of the whole body (Figure 2) and the whole blood count rate at 30 hours, that there was no significant impairment of trapping of the radioactive particles. In fact, the more extensive reticuloendothelial system may have accelerated clearance from the blood. It is known that considerably smaller dosages of intravenous radiogold (approximately 40 mCi) may be beneficial in the therapy of patients with related myeloproliferative disorders.¹⁶ It occurred to us that the overdose might have inadvertently represented "curative" treatment for the patient's condition if she could be supported long enough and if her marrow had the ability to repopulate with normal cell lines. We speculated

further that the liver and spleen which presumably were enlarged as a feature of the erythremic myelosis might have intensified the depression of peripheral blood counts by acting as irradiators of the blood elements circulating through them.

The patient's post mortem findings, viewed in light of the blood count changes after the overdose, seem consistent with attempted regeneration in the hematopoietic tissues with additional foci of blood formation in certain extramedullary sites such as lymph nodes. It is not clear whether the extramedullary hematopoiesis represents a residue of the pre-existing blood condition or an attempt at compensation for the suppressive effect of the approximately LD₅₀ dose to the marrow. In either case it is interesting that in the autopsy material the process is seen in lymph nodes, but not in more likely sites such as liver and spleen.² This suggested that the lymph nodes, despite their significant reticular cell content, may have had access to fewer gold particles than had the liver and spleen because of the very effective irreversible trapping of the particles in these enlarged organs. As a result, the beta radiation dose to the nodes might have been considerably less than to liver and spleen, and extramedullary blood formation might have either continued throughout the course of the overdosage, if it was present before, or have been permitted to begin anew during recovery more readily than in liver or spleen. Consistent with this interpretation is the clinical observation of the necessity for giving intralymphatic rather than intravenous injections of radiogold to effect significant changes in pathologic lymph nodes.^{8,16}

In conclusion, it is important to point out that overdosage with intravenous radiogold colloid is a model type of radiation exposure. The pattern of retention and the chemical inertness of the colloidal particles are most similar to those seen with thorotrast.¹⁷ Several important points of difference are to be noted, however. Radiogold is a beta and gamma emitter rather than an alpha emitter. In addition, the very much shorter physical half-life of radiogold permits the more rapid radiation dose delivery which promotes the acute as well as chronic sequelae of overdosage. Until it is possible to devise a means of mobilizing retained particles or a method of internal shielding, perhaps by giving followup doses of nonradioactive gold colloid, our strongest protection against radiogold intoxication is prevention of overdose through scrupulous monitoring technique before intravenous administration.

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FLUORESCENCE DETECTION: APPLICATION TO THE STUDY OF CEREBRAL BLOOD FLOW*

By

P. B. Hoffer,[†] R. E. Polcyn,[‡] R. Moody,[§] H. J. Lowe,[¶] and A. Gottschalk

The feasibility of imaging thyroidal iodine in patients who have received no radionuclide has been established.^{1,2} In this procedure K-shell fluorescence of the stable iodine within the thyroid gland is induced by exposure to an external source of americium-241 (gamma energy approximately 60 keV). The resulting K_{α} characteristic radiation (x-ray energy, 28.5 keV) is detected with a high resolution lithium-drifted silicon detector which permits good scatter discrimination even at this low photon energy. Thyroid scans produced by this method now supplement standard iodine-125 and iodine-131 scans at our institution.

It has been suggested by Tinney,³ Ter-Pogossian⁴ and one of us⁵ that a similar system could be used for dynamic transit time and blood flow determinations within the brain and other organs. The principal advantage of such a system over current isotopic methods is that where the irradiation from the collimated exciting source (americium-241) and the detector field of view overlap, a tomographic effect is created defining a discrete tissue volume from which data are collected. When the region of interest is adjusted to below the scalp and calvarium, blood flow within the brain can be measured without interference from activity in superficial structures. A nonradioactive "tracer" must be introduced into the blood if regional flow is to be measured. This "tracer" should ideally be of medium-to-high atomic number and just sufficient in quantity to give statistically reliable results. Iodine and xenon are the only elements currently available for such vascular studies.

The applicability of such a system to cerebral transit time and blood flow determinations was demonstrated in the following experiments.

EXPERIMENT I

A rhesus monkey weighing approximately 7 kg was anesthetized, and a cut-down was performed on the left common carotid artery. The external carotid artery was occluded and a No. 20 Cournand needle was inserted into the left internal carotid artery. The animal was then placed beneath our fluorescent detector (3.3 Curie americium-241, annular source, 200 mm² lithium-drifted silicon detector). The region of interest (a volume of approximately 3 cm³) was adjusted so that it was below the level of the calvarium on the left side (see Figure 1). A 3 per cent window was set on the pulse height analyzer (Canberra Model No. 1413) bracketing the 28.5 keV K_{α} characteristic x-ray peak of iodine (window width 28 keV to 29 keV). The count infor-

* This report is taken from a paper that appears in J. Nucl. Med., 10:651, 1969.

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mation was then routed to a multichannel analyzer (RIDL Model No. 34-12B) operating in a time sequence scaling mode. Test injections of renografin-76 (meglumine diatrizoate, 37 per cent iodine) were made into the left carotid artery and counts were collected over a 0.2-sec interval per channel for a period of about 30 sec (see Figure 2). The maximum radiation dose to the brain per study was estimated at 50 millirads. The curves obtained correspond to those that would be anticipated with radionuclide or dye dilution techniques.

Figure 1. Diagrammatic representation of the experimental set-up. The "region of interest" in the monkey's brain represents the region in which the radiation from the americium-241 source and the detector collimation overlap. The actual configuration of this region is more complicated than indicated in the diagram. The region does, however, approximate an ovoid 3 cm in height and 1.5 cm maximum diameter. The radiation dose to the irradiated brain was estimated at 50 millirads per study.

The quantity of iodinated contrast material injected into the carotid artery for this study is in excess of the amount that would be advisable in man. Preliminary human studies carried out in association with carotid arteriography, however, indicate that doses of as little as 3 cc of renografin (meglumine diatrizoate, 29 per cent iodine) can be used for this purpose although statistical reliability of the data obtained is marginal.

It would be helpful if the problem of intraarterial injection of the "tracer" could be eliminated. The inherent tomographic nature of the technique avoids interference from "tracer" in tissues outside of the region of interest. Intravenous injection of the iodinated contrast material has so far proven inadequate because it has been impossible to produce a discrete bolus with

Figure 2. Curves produced after intracarotid injection (internal carotid artery) of 1 cc and 2 cc of renografin-76 respectively. The points on the curve represent total fluorescent counts of iodine within the region of interest at 0.2 sec intervals following injection.

the necessary volume of contrast material. A clearance technique was therefore considered⁶ and is described in Experiment II.

EXPERIMENT II

A rhesus monkey weighing approximately 7 kg was anesthetized and intubated with a cuffed endotracheal tube. The endotracheal tube was attached to a closed rebreathing system with CO₂ absorber. The animal was then placed beneath the fluorescent detector. The apparatus was adjusted so that the region of interest (a volume of approximately 3 cm³) was below the level of the calvarium in the midline. A 3 per cent window bracketing the 29.8 keV K_a characteristic

peak of xenon was set on the pulse height analyzer. The count information was again routed to a multichannel analyzer operating in the time sequence scaling mode. One hundred per cent O_2 was initially administered to the animal and baseline background readings were obtained. A mixture of 80 per cent xenon, 20 per cent oxygen was then administered to the animal for six minutes. At the end of this period, the xenon-oxygen mixture was abruptly flushed from the system by 100 per cent oxygen and the animal was maintained for the remainder of the clearance period on 100 per cent O_2 with frequent flushing of the rebreathing system to prevent accumulation of xenon. The clearance curve obtained (see Figure 3) is apparently monophasic. The radiation dose to the animal was estimated to be less than 3 rads for the entire 30 minute period.

Figure 3. Xenon clearance curve. The animal was initially equilibrated on 100% oxygen. 80% xenon and 20% oxygen was then administered for 6 minutes (period between arrow marked "on" and arrow marked "off"). The rebreathing system was then flushed with oxygen prior to the clearance phase. Counts were collected at 5 second intervals per channel.

Xenon is an inert gas which is mildly anesthetic in 80 per cent concentration at atmospheric pressure. An inert xenon clearance study could be done on a reasonably cooperative patient. This would do away with the need for intracarotid or intravenous injection. The patient would simply breathe a xenon-oxygen mixture. It is unnecessary for the xenon to be in equilibrium with the compartment prior to the start of the clearance study. We have not concerned ourselves with the problem of xenon recirculation in this preliminary experiment. The fact that the clearance curve is monophasic is reasonable when it is considered that the study deals only

with a selected "region of interest" which, in this case, is principally the white matter of the brain.

CONCLUSION

Two methods of combining a nonradioactive "tracer" and fluorescent detector* for the determination of cerebral transit time (Experiment I) and blood flow are demonstrated. Since both methods detect fluorescent activity exclusively in a predetermined volume of brain, it is possible to avoid recording "tracer" activity from overlying bone and soft tissue. The need for selective arterial injection can be eliminated by utilizing the stable xenon clearance technique. The only drawback is the expense of the xenon gas.

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*The fluorescent detector used in these experiments consists of a lithium-drifted silicon detector of 200 mm² manufactured by the Nuclear Equipment Corporation, and an annular americium-241 source of 3.3 curies manufactured by Monsanto Research Corporation.

OPTICAL PROCESSING OF RADIONUCLIDE IMAGES*

By

R. N. Beck, D. B. Charleston, P. V. Harper, Jr., and N. J. Yasillo

In recent years, radionuclides have been used in a variety of medical diagnostic tests.^{1,2} For example, brain lesions may be detected if they perturb the normal distribution of radioactive material administered to the patient. In the interest of a low radiation dosage to the patient, only a small quantity of radioactivity can be used in such tests. As a consequence, the need for high sensitivity places a severe limit on the spatial resolution of radiation detectors designed for radioisotope imaging devices. At best, the resulting images exhibit noticeable imperfections in the form of noise (resulting from random fluctuation in the number of detected γ -rays recorded per unit area), and distortion (mostly smoothing, due to imperfect spatial resolution), scanning artifacts (line structure, scalloping, etc.).

To increase the perceptibility of low contrast, unsharp, structures that are relevant to an accurate diagnosis, a variety of analog³⁻⁵ and digital⁶⁻¹⁰ techniques of image recording and manipulation have been employed with some success. Here we present the results of our preliminary studies of optical spatial filtering techniques for image manipulation.

ESSENTIAL ELEMENTS OF THE THEORY

The basic physical theory underlying optical spatial filtering extends at least as far back as Abbé's theory of the microscope,¹¹ but has been developed and applied extensively in recent years, as intense sources of coherent light have become available. Briefly, this development has made use of the concepts of communication theory in the analysis of optical systems, objects and images being described in terms of their spatial frequency components, and optical systems in terms of their spatial frequency response. The theory has been presented in elegant mathematical form in numerous publications.¹²⁻¹⁷ Here, we shall only recall a familiar observation concerning diffraction gratings which suggests the plausibility of the theory, and state the general theorem on which the spatial filtering procedure is based.

It is well known that a beam of monochromatic coherent light falling on a plane periodic structure, such as the square-wave transmission grating, shown in Figure 1, is diffracted at angles $\pm \theta_m$ such that

$$\sin \theta_m = \frac{m \lambda}{d}, \quad (1)$$

where $m = 1, 2, 3, \dots$ is the diffraction order, λ is the wave length of the light, and d is the grating spacing. As a result, the distribution of light in the back focal plane of a lens having focal length f is a Fraunhofer diffraction pattern with peaks at distances y'_m from the axis, given by

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$$y'_m = \pm f \tan \theta_m \cong \pm f \sin \theta_m = \pm \frac{f m \lambda}{d} \quad (2)$$

for small angles θ_m .

What is crucial to the spatial filtering procedure is the fact that the Fraunhofer diffraction pattern is proportional to the Fourier spectrum, or spatial frequency spectrum, of the diffraction grating. In this particular case, the one-dimensional square-wave transmission grating has

Figure 1. Coherent light with wave length λ is diffracted by a periodic transparency at discrete angles $\pm \theta_m$ that are integral multiples of $\theta \propto 1/d = \nu$, the fundamental frequency of the periodic transparency. The resulting Fraunhofer diffraction pattern is the Fourier spectrum of the transparency. (See also Figure 7.)

a discrete Fourier spectrum that consists of an average, or DC component, a fundamental AC component $\nu = 1/d$, and integral multiples $\nu_m = m\nu$ of this fundamental frequency. That is, the sinusoidal plane wave frequency components comprising the diffraction grating give rise to a light amplitude distribution in the back focal plane, each component ν_m giving rise to the two light spots at

$$y'_m \cong \pm f \lambda \nu_m \quad (3)$$

This observation lends plausibility to two more general statements. First, the frequency spectrum of any one-dimensional transparency in the object plane (replacing the square-wave grating) appears in the back focal plane (also called the frequency plane) with the sinusoidal plane wave component having frequency ν in the object giving rise to light at two small regions with centers at

$$y' \cong \pm f \lambda \nu \quad (4)$$

Second, the two-dimensional spatial frequency spectrum of any two-dimensional transparency in the object plane appears in the frequency plane. This generalization covers the case of photoscans produced by radionuclide imaging systems, and is therefore the theorem underlying this study. The essential mathematical relations are summarized in the Appendix.

Since the spatial frequency components of any such object transparency are separated spa-

tially in the frequency plane, we can place a filter there, which is more nearly opaque* for those components we wish to attenuate than for those we wish to accentuate. The filtered image is formed from those components allowed to pass the frequency plane.

A great variety of filters can be imagined. Some of the simplest, sharp cutoff types are shown in Figure 2. In each case the outer circle with radius R_c corresponds to the maximum aperture in the frequency plane, and this corresponds to a certain high frequency cutoff point, given by $\nu_{\max} = R_c / f\lambda$. Thus, the optical system is always band limited, and can form potentially perfect images of only those objects with spectra lying within this band.

SPATIAL FILTERS

Figure 2. Sharp cutoff filters are masks placed in the frequency plane to block unwanted sinusoidal components.

Within this aperture, all lower frequency components appear, with zero frequency, or the DC component, on the axis. A low-pass filter consists simply of an aperture smaller than R_c , while a high-pass filter is opaque on and near the axis. Combining these types, a band pass filter can be achieved. Finally, a line-stop filter for frequency ν can be approximated by an opaque ring with radius $r = f\lambda\nu$ placed in the frequency plane.

PROCEDURE AND RESULTS

The system used in these studies is shown in Figure 3. This configuration has been described in detail by Cutrona et al.¹⁴ An Optics Technology Model 170 HeNe laser was used as a source of monochromatic coherent light with wave length $\lambda = 6328\text{\AA}$. A double concave lens L_1 , with a short focal length, was used to expand the laser beam to about 1.5 cm diameter. All ob-

*In addition to amplitude filters described here, phase filters may be used for image manipulation.^{15,17}

Figure 3. A sinusoidal plane wave component of the object gives rise to light spots centered at $y = \pm f \lambda \nu$ in the frequency plane. Thus, the frequency components of any two-dimensional transparency are separated spatially in the frequency plane.

ject transparencies were copied from original photoscans using Polaroid P/N film, and reduced to a maximum diameter of ~ 1 cm. This is necessary if the laser beam is to cover the entire object to be filtered. In addition, this reduction increases the effective frequency ν in the object and magnifies displacements y' in the frequency plane in accordance with equation (4). The lenses L_2 , L_3 , and L_4 are identical simple plano-convex uncoated lenses with a focal length of 483 mm. L_2 collimates the laser beam onto the object transparency to be filtered; L_3 focuses the diffracted beam onto the frequency plane where the object spectrum appears; and L_4 re-forms the filtered image on the image plane. A single-lens reflex camera was used to record the filtered images on Kodak Plus-X film.

Figure 4A shows the unfiltered image of a square wave sunburst pattern projected through the system. Figure 4B shows the effect of placing a low-pass filter (a hole about 0.7 mm in diameter in black construction paper) in the frequency plane. In this case, the low frequency sinusoidal components below $\nu = 0.72$ cycles per cm were passed by the filter while all components with higher frequency were eliminated. The sharp cutoff characteristic of this filter is very evident in Figure 4B.

The effect of this filter on a photscan is shown in Figure 5. The unfiltered image of the head and neck regions of a total body scan appears on the left in Figure 5A. This image was made by recording a tiny dot on film for each detected γ -ray.¹⁸ The high frequency components of these dots were eliminated by the low-pass filter; this resulted in a smoothing effect which may be seen in the filtered image in Figure 5B. This technique can be used to remove details that are regarded as too small to be diagnostically meaningful.

On the other hand, we would like to sharpen the somewhat larger details that might be meaningful. To accomplish this, a filter of the high-pass type, which attenuates low frequencies, was used.

For this purpose, a quartz fiber was drawn down to a diameter of 0.006" or 0.15 mm; a 90°

Figure 4. A low-pass filter eliminates the high frequency components with $\nu > 0.72$ cycles/cm in a sunburst pattern. Left—unfiltered. Right—filtered.

Figure 5. The low-pass filter smooths a photoscan. Left—unfiltered. Right—filtered.

bend was made about 0.5" from the tip, and the entire structure was blackened with a spray graphite film. When mounted on the optical axis in the frequency plane, this tip blocked the DC component and all low frequencies up to $\nu = 0.16$ cycles per cm.

Figure 6C shows the result of using this filter to sharpen a brain scan of marginal quality, which was originally produced in a scanning time of just 90 seconds.¹⁹ In this case the number of counts per unit area was quite small and the use of a large step width between scan lines resulted in a relatively poor image, which nevertheless shows a lesion. Filtering a scan of this type to enhance the high frequency components does sharpen the small structures, including the lesion; however, this process introduces the danger of enhancing small structures that are not

Figure 6. A lateral brain scan obtained in 90 seconds of scanning time contains artifacts due to noise, line structure, and distortion.

- A) anatomical structures.
- B) unfiltered photoscan.
- C) sharpened with a high pass filter, eliminating low frequencies $\nu < 0.16$ cycles/cm.
- D) the line structure has been removed with the filter shown in Figure 7 (lower).

statistically significant, and fragmenting integral structures, such as the tumor. This is the principal limitation of the technique.

A quite different type of filtering operation can be performed on such a brain scan, which results in the elimination of the line structure. For this purpose, the filter was made by placing in the object plane a uniform line pattern having the same spacing as the line structure in the brain scan. The filter, consisting of the frequency components of this periodic structure, is obtained by placing a piece of film in the frequency plane (see Figure 7). When this film is developed and put back in the frequency plane, it filters out the line structure. Figure 6D shows this effect; that is, the photoscan in Figure 6B yields the image in 6D when the line structure is filtered out. Note that other structures are neither sharpened nor appreciably smoothed, while the line structure has been eliminated.

DISCUSSION

This study was undertaken to gain some familiarity with the effects of coherent optical

Figure 7. The line pattern (upper) has the same spacing (lower) as the scan raster in Figure 6B. Its frequency spectrum (lower), was recorded on film and used to block the line structure in the brain scan to produce Figure 6D.

spatial filtering techniques on radionuclide images. As such, the study reveals some of the image manipulations that are very easily achieved, and should not be interpreted as indicating the limitations of the technique. Indeed, the apparatus and procedures could readily be improved in the following ways:

- 1) The technique is based on the assumption that the light source is spatially coherent. This condition could be achieved to a much higher degree by passing the laser beam directly through a tiny aperture with a diameter of 25μ to 50μ . In that case, a laser with a higher output power could be used to obtain a suitably short exposure time.

- 2) The lenses used were very inexpensive and of correspondingly limited quality. Better results could doubtless be obtained with better lenses.

- 3) The components of the system were not mounted on a vibration-free optical bench, which might also have afforded the improvements associated with proper alignment of the optical axis.

- 4) No special precautions were taken in copying the original photoscans on a reduced scale for filtering. In fact, the photographic technique should be regarded as marginal at every step in the procedure. If spatial filtering were to be used as a routine procedure, it would be desirable to eliminate the photographic reduction step by recording a minified image which could be filtered directly.

5) To reduce the effects of phase shifts that result from different optical path lengths through developed film, the object to be filtered and the filter itself should be immersed in "liquid gates" having optically flat surfaces and containing a liquid with an index of refraction matched to the film base.²⁰ Such gates were not used.

6) The filters used were of the sharp amplitude cutoff type. Additional control of image manipulation could be achieved by utilizing continuous complex filters having both amplitude transmission and phase shift characteristics.^{15,21}

7) Ultimately, the desired filtering process might be performed by the recording device itself. The rationale for this approach is suggested by the following considerations.

The collimated scintillation detectors used in radionuclide imaging systems can be regarded as low-pass filters, with a cutoff frequency ν_D (typically below 1 or 2 cycles per cm), which is determined by the shape (especially the width) of the detector point-spread function.²² Similarly, the recording devices used in these systems can also be described as low-pass filters with a cutoff frequency ν_R that is determined by the shape (especially the width) of the mark or spot recorded for each detected γ -ray. For example, if very tiny dots [$\delta(x,y)$ functions, in the limit] are recorded, then ν_R may be very high [$\nu_R \rightarrow \infty$, in the limit of $\delta(x,y)$ functions] and may easily exceed ν_D .

Since meaningful structures in the recorded image are band-limited by ν_D of the detector, any spectral component with $\nu > \nu_D$ appearing in the image is due to the recording device. It would be desirable to eliminate such components from the recorded image, since they contribute only to the noise power spectrum,²³ and there is no reason to believe that their presence increases the perceptibility²⁴ of structures to which the detector may have responded.

To eliminate all noise above the detector band pass, the spot shape of the recording device should be designed so that $\nu_R \leq \nu_D$.

This condition could be achieved by a variety of recorded spots; e.g.:

- a) A spot with the same size and shape as the point spread function of the detector would result in a recording device with the same spatial frequency response as the detector; however, this recording device would degrade the image contrast at frequencies $\nu < \nu_D = \nu_R$ just as the detector does. (To prevent this degradation, while eliminating some of the high frequency noise, a bell-shaped spot that is smaller than the detector point spread function is used to record brain scans,¹⁹ such as the one shown in Figure 6B.)
- b) A spot with the shape of $J_1(r)/r$ could provide flat frequency response over the detector band pass, and no response at higher frequencies; however, this function has negative lobes that may require a DC off-set if negative inputs to the recorder are not feasible.
- c) A spot with a shape that would provide a recorder frequency response that was the inverse of the detector frequency response would precisely compensate for the smoothing effects introduced by the detector, so that the overall response would be flat. The utilization of such an equalization filter (or apodizing filter¹⁵) is limited to the frequency band over which the signal-to-noise ratio is regarded as adequate.
- d) An "optimum" spot shape would in general depend on the choice of a criterion for optimization. For example, we may choose to minimize the rms deviation between object and image, where deviations may be both random (due to noise) and deterministic (due

to distortion).²⁵ Linfoot²⁶ has discussed alternative criteria, based on the correlation between object and image, and relative structural content.

- e) If the size and shape of structures relevant to a correct diagnosis were known, the spot shape might be designed to enhance these structures.
- f) If more were known about parameters affecting the perceptibility of structures in a complex field, these factors might be taken into account in designing the spot shape.

Until it is demonstrated that some particular manipulation of the image (or set of manipulations) increases the likelihood of a correct diagnosis, the safest procedure will probably be to record faithfully the detector output for non-destructive post-scan manipulation. Optical spatial filtering techniques appear to provide a simple means for carrying out these manipulations, so that their effects on diagnosis may be studied.

CONCLUSIONS

Coherent optical systems provide a very versatile technique for photo-scan image manipulation. In practice, the technique may have advantages over digital computer manipulation, so long as the diagnosis is based primarily on a visual examination of the image, rather than on quantitative data extracted from it.

Whether or not any such technique of image manipulation will significantly aid in diagnosis remains to be seen. If, however, it could be demonstrated that some particular manipulation was helpful, attention should probably be directed toward designing recording devices that would introduce this manipulation in the recorded image.

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APPENDIX

If the two-dimensional object transparency has transmittance $O(x,y)$, then the light amplitude in the frequency plane is the object spectrum, given by the Fourier transform of the object

$$F(\nu_x, \nu_y) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} O(x,y) \exp [-2\pi i(x\nu_x + y\nu_y)] dx dy \quad (A1)$$

The spatial coordinates (x', y') in the frequency plane are related to the spatial frequency variables (ν_x, ν_y) by

$$x' = \lambda f \nu_x \text{ and } y' = \lambda f \nu_y \quad (A2)$$

If a filter with transmittance $H(\nu_x, \nu_y)$ is placed in the frequency plane, the filtered image is

$$I(x,y) \cong \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} F(\nu_x, \nu_y) H(\nu_x, \nu_y) \exp [2\pi i(x\nu_x + y\nu_y)] d\nu_x d\nu_y \quad (A3)$$

When $H(\nu_x, \nu_y) = 1$, the image is just the inverse Fourier transform of the (truncated) object spectrum; that is,

$$I(x, y) \cong O(-x, -y) \quad (A4)$$

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POTENTIATION OF LYMPHOCYTE TRANSFORMATION BY MEMBRANE-MEMBRANE INTERACTION*

By

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In the search for a better understanding of the mitogenic activity of phytohemagglutinin (PHA) derived from *Phaseolus vulgaris*, our laboratory has developed a method for the purification of the mitogenic proteins present in PHAP, a commonly employed commercial preparation obtained from Difco Laboratories. By a combination of CM Sephadex chromatography, followed by molecular sieving over Sephadex G-150, we have isolated two distinct mitogenic proteins from such preparations.¹ Figure 1 illustrates the final step of the purification procedure, and shows an analysis of the purified mitogens subjected to acrylamide gel electrophoresis, as compared with the starting material. These mitogenic proteins have been designated high titer

Figure 1. Sephadex G-150 chromatography of L-PHAP and H-PHAP pools derived from CM Sephadex fractionation of PHAP. From 2 - 40 μ l aliquots were used in determining the specific activity of transformation for L-PHAP fractions across the elution peak. The L-PHAP and H-PHAP preparations were pooled and concentrated, and compared with the starting material by acrylamide gel electrophoresis (shown on right). The Sephadex G-150 elution peaks of human gamma globulin (γ G) and bovine serum albumin (BSA) are also shown.

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hemagglutinating PHAP (or H-PHAP) and low titer hemagglutinating PHAP (or L-PHAP). Table 1 summarizes the serologic and mitogenic properties of these two proteins. Both are potent mitogens and are also capable of leuko- and lympho-agglutination. H-PHAP, however, is a much more potent red cell agglutinin than L-PHAP, and is also capable of producing mixed red cell-lymphocyte agglutination at very high dilutions. L-PHAP has a molecular weight of 115,000 and contains 4 per cent carbohydrate by weight. H-PHAP is a somewhat larger molecule as determined by molecular sieving and contains approximately twice as much carbohydrate.

Table 1

SEROLOGIC AND MITOGENIC POTENCY OF L- AND H-PHAP

	L-PHAP	H-PHAP
1. Hemagglutination unit μg/ml	227	1.05
2. Leukoagglutination unit μg/ml		
a. Mixed leukocytes	5.6	69.5
b. Lymphocytes	11.3	304
3. Mixed agglutination unit (lymphocyte-erythrocyte) μg/ml	40	0.3
4. Transformation unit μg/ml	0.3	1.05

Figure 2. Red cells were incubated with either [125 I] H- or L-PHAP in the amounts shown at 25° for 60 minutes. Following centrifugation and washing 3X, the red cell buttons were lysed with 2 ml of 1% Triton X-100 and counted in a NaI crystal gamma detector. Both L- and H-PHAP retained full mitogenic capacity after radioiodination.

Both L- and H-PHAP were labeled with [125 I]. Figure 2 shows that over 90 per cent of H-PHAP can be adsorbed by red blood cells while only 3-4 per cent of L-PHAP can be adsorbed in the same fashion. Table 2 reveals that the mitogenic capacity of H-PHAP is almost totally removed by prior red cell adsorption, while the mitogenic capacity of L-PHAP is unaffected by prior exposure to red cells.

Table 2
ADSORPTION OF MITOGENIC ACTIVITY BY RED BLOOD CELLS

PHA type	Packed RBC added (ml)	[14 C]thymidine incorporation (CPM)	% inhibition
L-PHAP*	-	23,925	
	0.05	23,643	1
	0.1	23,377	2.3
H-PHAP†	-	38,396	-
	0.05	2,163	95.7
	0.1	1,182	98.3

Following adsorption of mitogen with the indicated volume of RBC for 30' at 25°, the red cells were removed by centrifugation and the supernatant was added to 2×10^6 lymphocytes in 4 ml MEM containing 12-1/2% autologous plasma. [14 C]thymidine incorporation was determined as outlined in Reference 1.

* 21 μ g.

† 17 μ g.

During our early studies on the comparative mitogenic potency of both H- and L-PHAP we noted that although L-PHAP was a more potent mitogen in the lower regions of the dose response curve, the plateau value of peak response was always higher with H-PHAP. In view of the marked interaction of H-PHAP with red blood cells and its potency in mixed lymphocyte-erythrocyte agglutination, and since our early experiments were done with lymphocytes that were contaminated with 3-5 times their number of erythrocytes, we decided to investigate the effects of the presence of red blood cells on lymphocyte transformation. Pure lymphocytes, entirely free of red cells or red cell stroma, and containing only 20-40 platelets/100 lymphocytes, were obtained by means of gravity sedimentation of whole blood, nylon column passage, osmotic lysis of red blood cells, and centrifugation through a saline-plasma gradient.¹ We used these lymphocytes to study the ability of autologous red blood cells to influence human lymphocyte transformation by both the L- and H-PHAP mitogens, and found that although red blood cells had no effect on lymphocyte transformation by L-PHAP, they markedly potentiated the effects of H-PHAP.

Figure 3 illustrates an experiment with varying doses of mitogen upon the transformation of 2 million lymphocytes with and without the addition of 20 million erythrocytes. The presence of red cells does not substantially affect the transformation of lymphocytes when L-PHAP is the mitogen. The ability of H-PHAP to stimulate DNA synthesis in lymphocytes, however, is markedly enhanced by the presence of red cells. This effect is most marked at low and intermediate points on the dose response curve and diminishes as the plateau of stimulation is approached. Figure 4 illustrates the effect of varying numbers of red blood cells on the transfor-

Figure 3. The potentiation of lymphocyte transformation by red cells. Lymphocytes (2×10^6) were transformed by varying doses of L- or H-PHAP in the presence or absence of 20×10^6 erythrocytes. One-tenth microcurie [^{14}C]thymidine was added to each culture at 72 hours, and the cells were harvested for liquid scintillation counting 16 hours later. See Reference 1 for details of the procedure. Stimulation Index = $\frac{\text{CPM} + \text{RBC}}{\text{CPM} - \text{RBC}} \times 100$.

mation of 2 million lymphocytes in the presence of a constant amount of mitogen. The dose of mitogen was calculated to give from 1/3 to 1/4 of the peak response obtainable in the absence of red blood cells. Here again the presence of red blood cells did not alter the ability of L-PHAP to transform lymphocytes, although a ratio of red cells to lymphocytes as low as 1:1 increased lymphocyte transformation by almost 300 per cent when H-PHAP was employed. The plateau of stimulation by red cells was approached at a red cell:lymphocyte ratio of 10:1. In

other experiments the presence of as few as 1 red cell for every 10 lymphocytes resulted in a 2-fold increase of lymphocyte transformation (Table 3).

Figure 4. The potentiation of lymphocyte transformation by red blood cells. In this experiment the dose of H- or L-PHAP was kept constant and varying numbers of red cells were added to 2×10^6 lymphocytes.

As shown in Table 4, the ability of red cells to potentiate the transformation of lymphocytes by H-PHAP was limited to red cell stromal particles derived from osmotic lysis of red cells; stroma free hemolysate displayed no potentiating capacity. It is noteworthy that the potentiation efficiency of red cell stromal particles was only 1/40 that of intact red cells, since 84 million stromal particles were required to achieve the same potentiation as two million intact red cells. Studies with [^{32}DFP] labeled red cell stroma, however, disclosed that a maximum of 1/12 of the added stromal particles became attached to or sedimented with lymphocytes; the remainder were found free in the supernatant medium (Table 5). Other studies with [^{51}Cr] labeled red blood cells have shown that the potentiation of lymphocyte transformation by H-PHAP is associated with erythrocyte injury as measured by [^{51}Cr] release; red cell injury does not occur if lymphocyte transformation in the presence of red cells is effected by L-PHAP (Table 6).

These observations on the potentiation of lymphocyte transformation by red cells solely in the presence of that mitogen capable of bringing red cells and lymphocytes into closely fixed apposition suggested to us that membrane-membrane interaction between lymphocytes and red

Table 3

ENHANCEMENT OF LYMPHOCYTE TRANSFORMATION BY RED CELLS

A. Constant red cells (10×10^6); varying H-PHAP dose			
PHA dose μg	CPM* Red cells present	CPM* Red cells absent	Stimulation index
2.7	6,166	416	1482
5.4	32,373	4,974	651
13.4	38,909	17,003	229
26.8	39,377	25,377	155
40.2	39,899	29,858	133.6
B. Constant H-PHAP dose (5.4 μg); varying red cells			
Red cells (millions)	CPM*	Stimulation index [†]	
0	4,974	-	
10.0	32,373	651	
5.0	22,618	455	
2.0	18,917	380	
1.0	13,989	281	
0.5	14,817	298	
0.2	10,681	215	

Human lymphocytes (2×10^6) were cultured with and without red cells.

*DNA synthesis as measured by [^{14}C]thymidine incorporation.

[†] $\frac{\text{CPM red cells present}}{\text{CPM red cells absent}} \times 100$.

blood cells, as well as between lymphocytes and lymphocytes, played an important role in the stimulation of DNA synthesis and transformation by mitogen. We reasoned that if this were a general feature of lymphocyte transformation by mitogen, then it should be possible to demonstrate a similar phenomenon with the L-PHAP mitogen, providing that a suitable membrane-bearing particle was used as stimulant and providing also that such a particle, like the red cell, was incapable of responding to L-PHAP by DNA synthesis. The particle chosen for this experiment was the autologous x-irradiated lymphocyte. Figure 5 shows the effect of x-irradiation of lymphocytes on the subsequent capacity of these cells to respond to mitogen as measured by DNA synthesis. This curve, which represents the average of 3 separate experiments, determined the use of a dose of 5000 R in order to reduce DNA synthesis to less than 3 per cent of the control value in the "nonresponding" lymphocyte particles. Table 7 summarizes the results obtained when 1 million normal lymphocytes were exposed to varying doses of both L- and H-PHAP mitogens in the absence of, and in the presence of, an equal number of nonresponding autologous irradiated lymphocytes. The results clearly demonstrate that the presence of these inert lymphocytes markedly potentiated the mitogenic capacity of both L- and H-PHAP. This potentiation resembled that obtained with red cells in the presence of H-PHAP in that it was

Table 4
**ENHANCEMENT OF LYMPHOCYTE TRANSFORMATION BY
 RED CELLS AND RED CELL STROMA**

RBC or RBC equivalent added	Number of red cells added (millions)	H-PHAP dose μ g	[14 C]thymidine incorporation CPM	Stimulation index
A. RBC	20	-	320	-
B. None	-	5.4	9,447	-
C. RBC	20	5.4	52,284	553.4
RBC	2	5.4	33,387	353.4
D. RBC stroma	84	5.4	33,465	354.2
RBC stroma	42	5.4	26,578	281.3
RBC stroma	21	5.4	18,712	198.1
RBC stroma	10.5	5.4	13,688	144.9
RBC stroma	5.25	5.4	10,039	106.3
RBC stroma	2.6	5.4	8,633	91.4
E. RBC hemolysate	74	5.4	8,376	88.7
RBC hemolysate	37	5.4	7,421	78.6
RBC hemolysate	18.5	5.4	6,988	74

Hemoglobin-poor stroma was prepared by osmotic lysis of red cells in dilute phosphate buffer. The stromal particles were harvested and washed 3X by ultracentrifugation prior to use. The supernatant from the first centrifugation was used as hemolysate. Stromal particles were enumerated in a Coulter Counter. Two million lymphocytes were employed in each culture.

Table 5
**CORRELATION BETWEEN RBC STROMA ENHANCEMENT OF LYMPHOCYTE
 TRANSFORMATION AND SEDIMENTABLE [32 DFP] STROMA**

PHAP type	Lymphs (2×10^6)	Stromata (264×10^6)	[14 C]thymidine incorporation	Stimulation index	Sedimentable stromal particles	
					%	No. ($\times 10^6$)
None	+	+	-	-	0.6	
	-	+	-	-	0.4	
H-PHAP (4 μ l)	+	-	16,086	-	-	21.9
	+	+	41,960	261	8.3	
	-	+	-	-	7.3	
L-PHAP (2 μ l)	+	-	13,302	-	-	
	+	+	12,965	97.5	0.4	
L-PHAP (20 μ l)	-	+	-	-	0.5	

Red cells were labeled in vitro with [32 DFP] and stromal particles were prepared and added to replicate cultures with and without PHAP and/or lymphocytes. At the conclusion of the experiment, the cultures were centrifuged at $300 \times g$ for 10 minutes and the supernatants were discarded. The cell buttons were washed 3X in saline, and then assayed for [32 P] and [14 C] in a liquid scintillation counter.

Table 6

CORRELATION BETWEEN RBC POTENTIATION OF LYMPHOCYTE TRANSFORMATION
AND RBC INJURY ($[^{51}\text{Cr}]$ RELEASE)

PHAP type	PHAP μl	$[^{14}\text{C}]$ thymidine incorporation (CPM)		Stimulation index	$[^{51}\text{Cr}]$ release (%)	
		Lymphs	Lymphs + RBC		RBC	RBC + lymphs
None	-	-	-	-	7.3	7.4
H-PHAP	4	8,686	57,259	659	8.0	14.1
	20	43,723	54,608	125	7.7	18.0
L-PHAP	2	5,174	5,130	99	7.4	7.0
	10	31,713	28,922	91	7.3	7.1

After the period of culture, the supernatant fluid was removed and assayed for $[^{51}\text{Cr}]$ content in a NaI crystal gamma counter.

Figure 5. The effect of in vitro irradiation of human lymphocytes on their susceptibility to transformation by phytohemagglutinin.

most marked at low and intermediate points on the dose response curve and diminished as the plateau peak response was approached.

DISCUSSION

In summary, we have shown that membrane-membrane interaction brought about by phytohemagglutinin molecules acting as intercellular ligands is an important mechanism for the potentiation of lymphocyte transformation, and that such potentiation occurs even with the cooperation of a mitotically inert particle lacking genetic information such as the red cell or red cell stromal particle. In all probability the increase in DNA synthesis results not from increasing the extent to which any single cell synthesizes DNA, which remains an all or none phenomenon,

Table 7
POTENTIATION OF LYMPHOCYTE TRANSFORMATION BY
X-RAY KILLED LYMPHOCYTES

PHAP type	PHAP μ l	Normal lymphs	[¹⁴ C]thymidine incorporation (CPM)		Stimulation index [§]
			Normal lymphs + x-ray lymphs (theoretical) [†]	Normal lymphs + x-ray lymphs (observed)	
H-PHAP [*]	4	6,041	6,597	25,036	380
	10	19,556	20,509	29,612	144
	40	31,464	32,417	34,617	107
L-PHAP [†]	1	1,094	1,835	8,062	439
	2	3,734	4,475	17,817	398
	10	21,620	22,514	24,166	107

Lymphocytes (1×10^6) were cultured with and without the addition of an equal number of autologous lymphocytes that had been exposed to a dose of 5000 R.

^{*} 1.34 mg/ml.

[†] 1.54 mg/ml.

[‡] This figure accounts for the small amount of residual DNA synthesis observed when X-irradiated lymphocytes were cultured separately.

[§] Stimulation index = $\frac{\text{CPM observed}}{\text{CPM theoretical}} \times 100$.

but rather through an increase in the efficiency with which individual cells are recruited into the transformation process by a mechanism involving cell-cell, or more specifically, membrane-membrane interaction. These observations may have some bearing on the experiments of Fisher and Mueller which indicate that the lymphocyte membrane undergoes significant modulation of membrane phospholipids as one of the earliest steps in the process of transformation by phytohemagglutinin.² They also fit nicely into the growing body of evidence which indicates that the initiation and perpetuation of the immune response by lymphocytes requires the interaction of two or three varieties of macrophage and/or lymphocytes brought into close and specific contact by antigen and/or antibody molecules acting as ligand.³⁻⁷ In addition to the possibility of information transfer, such cell-cell interaction may act to provoke the derepression of the lymphocyte, and thus enable it to synthesize macromolecules and divide. When the ligands and cell receptors are specific, as in the immune response, the result is specific, namely antibody synthesis. When, however, the ligand and receptor are immunologically nonspecific, as in the case of phytohemagglutinin-induced transformation, the result is abortive.

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ON THE MECHANISM OF ERYTHROPOIETIN-INDUCED DIFFERENTIATION
VI: INDUCED ACCUMULATION OF IRON BY MARROW CELLS*

By

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Previous reports from this laboratory have shown that erythropoietin, which induces erythrocyte formation in vivo,¹ has a similar effect on marrow cells in culture.²⁻⁷ It has now been established that exposure to erythropoietin stimulates synthesis of specific RNA components of marrow cells,^{5,6} this effect being manifest as early as 10-15 minutes after its addition to the cells. Several hours later the cells show increased hemoglobin² and cell stroma synthesis⁷ both of which continue for more than 24 hours. These latter effects are inhibited by actinomycin D, as is the early one, suggesting that erythropoietin action as the primary inducer of hematopoietic stem cell differentiation is mediated via DNA-dependent RNA synthesis.

It has been known for some time that the rate of hemoglobin synthesis in the developing red cell reaches a maximum while the immature cells are still nucleated^{8,9} and declines during maturation of the reticulocyte. Although a large fraction of the iron entering the cell is eventually utilized for hemoglobin synthesis, the presence of stores of non-heme iron (including ferritin) in immature cells¹⁰ indicates that cellular iron uptake is not dependent on hemoglobin synthesis.

The role of transferrin in plasma transport of iron and its delivery to erythroid cells is clear,¹¹ but the path of iron between the cell surface and its utilization in heme synthesis have not been established. It seems likely however, that specific intracellular intermediates are involved in this movement.^{12,13}

In this paper we will describe the effect of erythropoietin on induction of ferritin formation and on other proteins concerned with the uptake of iron by rat marrow cells. A preliminary report of these findings has been published.¹⁴

EXPERIMENTAL PROCEDURES

Culture methods. Stationary cultures were incubated in 35 x 10 or 60 x 15 mm plastic Petri dishes (Falcon Plastics) as previously described.²⁻⁷ Modifications of the original method are summarized for individual experiments. Although a few stationary culture experiments were carried out in triplicate, most of them consisted of four or five replicate samples for each experimental group.

Spinner flask experiments were performed in fifty ml flasks (Bellco Glass). Vigorous stirring was necessary to keep the cells dispersed. All experiments were gassed continuously with 5 per cent CO₂, 95 per cent air.

Marrow cells were removed from the femurs and tibiae of male Sprague-Dawley rats (200-

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300 g) by flushing medium through the bones several times with a 19 g needle and syringe. Large, visible aggregates were broken up by ejection first through a 21 g needle, then through a 26 g needle.

Fetal calf serum and newborn calf serum were purchased from Colorado Serum Company; NCTC-109 from Microbiological Associates. Penicillin G (200 units per ml) was added to the NCTC-109 prior to use.

⁵⁹Fe transferrin sources. Rat serum was dialyzed against a slurry of Chelex-100 resin (Bio-Rad) in 0.15 M NaCl buffered with 0.01 M sodium phosphate at pH 7.0 for forty-eight hours to remove iron. Enough ⁵⁹FeCl₃ was added to bring the transferrin to at least 0.8 saturated.

A generous supply of Cohn fraction IV-4 of human serum was provided by Dr. R. Painter of the Connaught Laboratories, University of Toronto. It was dissolved in NCTC-109 (10-20 mg/ml) and the pH lowered to 4.5-4.6 with 0.5 N HCl. Sufficient ⁵⁹FeCl₃ and unlabeled ferric chloride were added to insure 100 per cent saturation of the transferrin with iron. The amount of iron necessary was determined in advance by following the absorbancy change at 570 nm. The pH of the iron-transferrin solution was then brought to 7.2-7.4 by adding 7.5 per cent sodium bicarbonate. We found that some preparations of fraction IV-4 were not suitable sources of transferrin for rat marrow cells, and that each sample should be tested individually.

Unless otherwise specified, the concentration of radioiron in the final incubation medium was 1 μ Ci/ml.

Termination of cultures. Cultures were terminated by quantitative transfer into three to four volumes of buffered saline; for rate studies all solutions were kept in an ice bath. After centrifugation, the cells were resuspended and washed twice more with buffered saline. The buffers used were: 0.14 M NaCl, 0.01 M sodium phosphate pH 7.2, and 0.14 M NaCl, 0.005 M NaH₂PO₄, 0.005 M K₂HPO₄ adjusted to pH 7.2 with KOH.

Isolation of heme. Routine lysis of the marrow cells was as previously described.² Extraction of heme was as follows: a 1.0 ml sample of lysate was acidified with 0.2 ml of 1.0 N HCl and extracted with 2.0 ml of cyclohexanone (Eastman). After separation of the phases by centrifugation, aliquots of the organic phase were counted in a well-type scintillation counter with a maximum counting error of 3 per cent.

Preparation of lysates with detergent. Marrow cells were suspended in 0.5 ml of a buffer suggested to us by Dr. T. Borun which is composed of 0.067 M Tris, 0.086 M KCl, and 0.0015 M MgCl₂ at a pH of 7.2 (TKM) before addition of 0.5 ml of 1 per cent NP 40 (Nonidet P 40, Shell Chemicals) solution in TKM. At ice-bath temperatures this procedure produces nearly instantaneous lysis of the cell membrane.^{15,16} This method yields almost quantitative (90-95 per cent) removal of hemoglobin from the stroma, to which it tends to bind when the ionic strength is low.

Gel-filtration of marrow cell lysates. After lysis with NP 40 insoluble material was removed by centrifugation, and the residue was washed once with 0.25 ml of TKM. The combined supernatants and washes from all replicates were pooled, and gel filtration on Biogel P-150 was performed as recommended by the manufacturer (Bio-Rad Laboratories). Absorbancy readings of the column effluent were not made because of interference by the detergent at 280 nm.

Isolation of hemoglobin. Hemoglobin was isolated from lysates by a two column, chromatographic method using DEAE cellulose in 5×10^{-3} M sodium acetate pH 5.5, before and after

formation of the specific hemoglobin-haptoglobin complex. Details of this method are to be reported elsewhere.¹⁷

Isolation of transferrin. Carrier transferrin in the form of serum previously saturated with ferric chloride, was added to the lysates, and the resultant solution was precipitated with Rivanol (6,9 diamino-2-ethoxy-acridine lactate).¹⁸ The supernatant was treated with potato starch to remove the excess Rivanol and chromatographed on DEAE Sephadex A-50^{19,20} to isolate transferrin. In estimating the final yield, it was assumed that the yield of the carrier transferrin from serum was identical with that from the lysate.

Determination of ferritin. Rabbit anti-serum against horse spleen ferritin was bought from Pentex Inc. Lyophilized horse spleen ferritin (5x crystallized, low Cd) containing at least 20 per cent iron, purchased from Mann Laboratories, was used to standardize the anti-serum and as carrier in later experiments. The anti-serum had a precipitin titer of 0.8 mg of ferritin per ml of anti-serum at the zone of maximum precipitation. The experimental system contained 0.1 mg of carrier ferritin, 0.2 ml of anti-serum (diluted 1.1/2.0 with 0.15 M NaCl), and 0.5 ml of lysate in TKM in a 1.0 ml conical centrifuge tube. The mixture was incubated at 37° for 30 minutes and stored at 4° overnight after which precipitation was complete. The precipitates were collected, resuspended, and washed with phosphate buffered saline four times, when the supernatant was essentially free of ⁵⁹Fe. Control experiments showed that reducing the amount of carrier, when the amount of anti-serum was constant, increased the precipitated counts slightly, and treatment of the supernatant, after a first precipitation, with fresh carrier and fresh anti-serum yielded a precipitate with only a trace of radioactivity (less than 10 per cent of the first precipitate). This indicated that contamination of the precipitate with adsorbed non-ferritin radioactivity was unlikely and that at least 90 per cent of the rat ferritin was precipitated in the first reaction.

Sources of erythropoietin. Erythropoietin derived from anemic sheep plasma with a potency of 20 units/mg or greater²¹ was used for all experiments.

Additional sources of materials. ⁵⁹Fe was purchased from New England Nuclear Corporation. Specific activities varied from 5-15 mCi/mg Fe. Puromycin was obtained from Nutritional Biochemicals. Actinomycin D was a gift of Merck, Sharp and Dohme, and cycloheximide of Upjohn Pharmaceuticals.

RESULTS

Figure 1 shows the results of experiments testing the effect of graded amounts of erythropoietin on uptake of radio-iron by marrow cells, and on its incorporation into heme. The solid lines show results after 24 hours of incubation; both total uptake and incorporation into heme increased linearly with the logarithm of the amount of erythropoietin. The slope of the dose-response curve for iron-uptake (4.1) was greater than that for heme formation (1.9). The difference between the stimulated total uptake and the radioactivity found in heme is labeled "residue," and it also was affected by erythropoietin. The broken line in Figure 1, illustrating the effect of erythropoietin on total uptake in a four hour incubation, shows that the slope under these conditions (5.2) is not very different from that found for the longer time.

Studies of the rates of ⁵⁹Fe uptake and of heme synthesis by marrow cells have shown that the stimulated total uptake precedes stimulated heme synthesis (Figure 2). In this experiment the increase in iron uptake began about two hours after exposure to erythropoietin, no increase

Figure 1. Incorporation of ^{59}Fe into erythropoietin-stimulated marrow cells. Conditions of incubation: medium—45% fetal calf serum, 49% NCTC-109, 6% rat serum; volume 1.0 ml/dish. Cell concentration was 8.9×10^6 nucleated cells per ml. Control incorporations were 14.6% uptake into total cell fraction, and 4.9% incorporation into heme. Broken line is dose response for total ^{59}Fe uptake at four hours of incubation. Solid lines for 24 hour incubation.

in the rate of heme synthesis being seen for another two to three hours. The large apparent uptake of iron by the cells at zero time, which we attribute to nonspecific binding of labeled transferrin by the cells, has been eliminated in subsequent experiments by using a slightly hypertonic buffered saline to wash the cells (0.145 M NaCl, 0.013 M potassium phosphate pH 7.3).

The effect of erythropoietin on iron uptake by marrow cells is dependent on protein synthesis. When the cells were preincubated with inhibitors, both puromycin and cycloheximide eliminated the erythropoietin-stimulated response. Simultaneous addition of the inhibitor and the inducer, however, failed to abolish the effect of erythropoietin completely (Table 2), although we found that protein synthesis under these conditions was 99 per cent inhibited. There was a significant depression of control iron uptake (about 50 per cent with both inhibitors), but inhibition of the response to erythropoietin was about 20 per cent with puromycin, and about 48 per cent with cycloheximide. When the cells were pre-incubated with puromycin for ten minutes before addition of erythropoietin and ^{59}Fe , the stimulation was about 93 per cent eliminated, while thirty minutes of pre-incubation with the inhibitor resulted in complete loss of response (Table 3).

Stimulated iron uptake is probably dependent on the prior synthesis of RNA since it was inhibited by actinomycin D (Table 4). In the same experiment, the addition of dinitrophenol at a concentration of 3×10^{-4} M did not inhibit this effect of erythropoietin. Five hours of incubation

Figure 2. Time course of ^{59}Fe uptake and incorporation into heme by marrow cells in Spinner flasks. Conditions of incubation: medium—47.5% newborn calf serum, 47.5% NCTC-109, 5% rat serum; volume 30 ml per flask. Cell concentration was 1.4×10^7 nucleated cells per ml. ^{59}Fe was bound to rat serum ($2 \mu\text{Ci}$ per ml of medium). Erythropoietin concentration was 0.10 units/ml. Cells were added as 3 ml of concentrated suspension to 27 ml of medium at 37° containing all additions. Broken lines represent erythropoietin stimulated cells; solid lines, control cells.

Table 1
UPTAKE OF ^{59}Fe INTO MARROW CELLS DURING FOUR HOURS
OF EXPOSURE TO ERYTHROPOIETIN

Experimental group	Net CPM ^{59}Fe	Δcpm	P
Control	25200 ± 480		
0.05 units erythropoietin	28100 ± 800	2900 ± 900	.02
0.10 units erythropoietin	30800 ± 450	4600 ± 680	< .01

Conditions of incubation: Medium—44.5% newborn calf serum, 55.5% NCTC-109, volume 1.12 ml per dish. Cell concentration was 2.10×10^7 nucleated cells per ml. ^{59}Fe was bound to 2 mg of fraction IV-4 ($0.5 \mu\text{c}/\text{dish}$). Uptake of iron is expressed as the mean and the standard error of the mean. P is the probability that experimental values are identical to the controls; values were obtained from a t test of the raw data.

Table 2

UPTAKE OF ^{59}Fe INTO MARROW CELLS DURING SEVEN HOURS OF INCUBATION
IN THE ABSENCE OF PROTEIN SYNTHESIS

Experimental group	Control	% inhibition	Erythropoietin	% inhibition	Δ	% inhibition
Control	23100 $\pm$ 650	0	26200 $\pm$ 170	0	3100 $\pm$ 670	0
75 $\mu\text{g/ml}$ puromycin	12800 $\pm$ 160	45	15300 $\pm$ 260	42	2500 $\pm$ 310	20
50 $\mu\text{g/ml}$ cycloheximide	12100 $\pm$ 200	48	13700 $\pm$ 490	46	1600 $\pm$ 530	48

Conditions of incubation: Medium—46% newborn calf serum, 54% NCTC-109, volume 1.0 ml per dish. Cell concentration was 1.0×10^7 nucleated cells per ml. ^{59}Fe was bound to 1 mg of fraction IV-4 (0.25 $\mu\text{Ci/dish}$). Erythropoietin concentration was 0.125 units/ml. All additions were at zero time.

Table 3

EFFECT OF PRE-INCUBATION WITH PUROMYCIN ON THE INHIBITION
OF ^{59}Fe UPTAKE

Time of pre-incubation minutes	CPM incorporated			% inhibition induced uptake
	Control	Erythropoietin	Δ	
10 Control	13600	16300	2700	-
Puromycin	10000	10200	200	93
30 Control	21200	23700	2500	-
Puromycin	12400	12000	400	100

Conditions of incubation: Medium—22.5% newborn calf serum, 77.5% NCTC-109, total volume 1.0 ml per dish. Cell concentration was 1.2×10^7 nucleated cells per ml. ^{59}Fe was bound to 2 mg of fraction IV-4 (0.5 $\mu\text{Ci/dish}$). Cells were pre-incubated with 75 μg of Puromycin in 0.9 ml then ^{59}Fe and 0.055 units of erythropoietin in 0.1 ml were added. Incubations were stopped after 310 minutes.

Table 4

EFFECT OF INHIBITORS ON ^{59}Fe UPTAKE

Experimental group	Control cpm	Erythropoietin cpm	Δ cpm	% inhibition of induced uptake
Control	49000	58100	9150	-
Puromycin 75 $\mu\text{g/ml}$	34800	39800	5000	45
Actinomycin D 2.5 $\mu\text{g/ml}$	48000	48100	100	89
Dinitrophenol $3 \times 10^{-4}\text{M}$	48700	57600	8900	8

Conditions of incubation: Medium—45% newborn calf serum, 45% NCTC-109, 10% rat serum, volume 1.0 ml per dish. Cell concentration was 1.9×10^7 nucleated cells per ml. Cells were pre-incubated with the inhibitors for 5 minutes before the additions of 0.18 units of erythropoietin. Incubation was terminated at five hours.

in the presence of KCN at 3×10^{-4} M caused a pronounced depression of the total uptake and abolished the erythropoietin effect (Table 5); but at shorter times the inhibition was not always seen.

Table 5
EFFECT OF CYANIDE ON ^{59}Fe UPTAKE BY MARROW CELLS

Experimental group	CPM ^{59}Fe	Δ cpm
Control	11600 $\pm$ 190	
0.18 units erythropoietin	12500 $\pm$ 220	900 $\pm$ 250
KCN (3×10^{-4} M)	2600 $\pm$ 120	
KCN (3×10^{-4} M) + erythropoietin	2600 $\pm$ 80	0 $\pm$ 100

Conditions of incubation: Medium—45% newborn calf serum, 45% NCTC-109, 10% rat serum, volume 1.0 ml per dish. Cell concentration was 1.3×10^7 nucleated cells per ml. Incubation was terminated at five hours.

Intracellular distribution of ^{59}Fe in erythropoietin-stimulated marrow cells. The iron-containing component in the stimulated marrow cells which was not hemoglobin, seemed likely to be ferritin, which has been described as an iron storage protein in erythroid cells.¹⁰ Ferritin has a minimum molecular size that is nearly four times that of hemoglobin, so they should be separable by gel filtration. Figure 3 compares elution profiles on Biogel P-150 of ^{59}Fe -containing material present in the soluble portion of lysates from marrow cells that had been incubated with and without erythropoietin. These elution profiles represent pooled lysates from the control and erythropoietin (0.10 units/ml) groups listed in Table 1, and show the presence of at least two fractions of ^{59}Fe labeled material. The first fraction was eluted with the void volume and should contain ferritin; the second fraction contained all the hemoglobin in the soluble portion of the lysate. There was no indication of radioactivity in the cells as ionic iron.

The peaks were normalized to 100 per cent recovery and were integrated using the minimum in the profile of the erythropoietin-treated material as an arbitrary separation. A comparison of the data obtained in this manner, shown in Table 6, makes it clear that the heavy fraction, which would contain ferritin, also contained a component stimulated by erythropoietin.

In a similar experiment we measured iron incorporation into marrow cell ferritin directly by precipitation with the specific antibody (Table 7). After 5 hours of exposure to erythropoietin, incorporation of ^{59}Fe into ferritin was increased by about 70 per cent whereas hemoglobin synthesis was only slightly stimulated (about 10 per cent).

This experiment also shows, however, that there was a large amount of material in the lysates (labeled residue in Table 7) which had incorporated ^{59}Fe but could not be identified as either hemoglobin or ferritin. This unidentified, labeled, material was not transferrin. We isolated transferrin from lysates of cells which had been incubated for 5 hours with and without erythropoietin after the addition of carrier transferrin. Less than 10 per cent of the nonhemoglobin, nonferritin counts were identified as transferrin when corrections were made for losses during isolation, but both total counts and specific activity of the isolated transferrin were affected by erythropoietin.

Figure 3. Gel filtration of soluble fraction of lysates from control and erythropoietin-treated cells. Column size: 1.8 x 15.0 cm. The column was eluted with 0.14 M NaCl, 0.01 M NaCl, 0.01 M phosphate at pH 7.2. Fractionation: Thirty drops per fraction were collected. Because of the presence of detergent in the lysates, the drop size is considerably reduced in the fraction representing the volume of the column. The broken line represents the fraction from erythropoietin-stimulated cells; the solid line represents controls.

DISCUSSION

Among the events that occur in marrow cells exposed to erythropoietin in vitro, is an increase in the uptake of iron in a form that is not heme. In the culture system described, non-heme iron accounts for about 77 per cent of cellular uptake after 5 hours of incubation. Only 20 per cent of the non-heme iron at this time is ferritin; the remainder contains a small amount of transferrin but most of it is as yet unidentified. The increased uptake of iron into these non-heme fractions begins one to two hours after administration of erythropoietin whereas the erythropoietin-stimulated synthesis of heme can only be detected three to five hours after exposure (Figure 2). The per cent stimulation at these times is generally small, but highly significant and reproducible. Control iron-uptake is due in part to the presence of already differentiated cells;

Table 6
NORMALIZED RATIOS OF ^{59}Fe LABELED FRACTIONS OBTAINED
BY GEL FILTRATION OF MARROW CELL LYSATES

	Heavy fraction	Light fraction
Ratios of integrated peaks (stimulated/control)	1.5	1.1
Net change in CPM	9600	6800

Table 7
DISTRIBUTION OF ^{59}Fe AFTER 5 HOURS OF INCUBATION
WITH ERYTHROPOIETIN

Fraction	cpm			% of Δ cpm found in soluble fraction
	Control	Erythropoietin	Δ	
Soluble	42800	57400	14600	100
Hemoglobin	26500	30400	3900	27
Ferritin	3670	5890	2220	15
Residual	12630	21110	8480	58

Conditions of incubation: Medium 45% fetal calf serum, 50% NCTC-109, 5% rat serum, total volume 3.0 ml, 60 x 15 mm dishes used; ^{59}Fe was bound to rat serum (1 $\mu\text{C}/\text{ml}$ of medium), cell concentration was 1.2×10^7 nucleated cells per ml; 0.18 units of erythropoietin. Hemoglobin counts were determined from the cyclohexanone extract. The fraction marked "residual" refers to the non-heme, non-ferritin bound ^{59}Fe .

cells as mature as the reticulocyte take up labeled iron in our culture system but do not respond to erythropoietin.

The erythropoietin-stimulated fraction of iron incorporation, and to a lesser extent the control level of iron incorporation, requires the synthesis of one or more protein components (Tables 3, 4). This suggests that certain proteins are necessary for either the entry of iron into the cell and/or the sequestration of intracellular iron and that synthesis of these proteins is, in part, a consequence of erythropoietin-induced differentiation. A mechanism for cellular iron-uptake has been suggested by other workers.^{12,13} In these two papers three separate pools of radioiron, in addition to hemoglobin, were identified in marrow cells. These three pools were ferritin, "stromal bound iron," and an unidentified protein referred to by Greenough et al¹² as "fraction I." Since we find that the stroma fraction from marrow cells lysed with water contains a significant amount of hemoglobin, there may be some doubt whether a pool of iron bound to the stroma actually exists independent of hemoglobin contamination. By pulse labeling marrow cells Zail et al¹³ demonstrated that radioiron bound to the stroma, or present in "fraction I," served as a precursor to hemoglobin but ferritin apparently did not.

In marrow cells that have been cultured in the presence of erythropoietin just long enough to establish a small but significant increase in iron uptake and hemoglobin synthesis (Table 7) we have found a relatively large increase in the incorporation of iron into ferritin and a still

larger increase into the uncharacterized fraction. Since we routinely lyse labeled cells with detergent in isotonic medium and achieve a high yield of the total counts in a soluble form, it appears likely that our non-hemoglobin, non-ferritin fraction would contain any non-hemoglobin "stromal bound" iron that may be solubilized under these conditions.

Zail et al¹³ have suggested that since ferritin is not an active intermediate in hemoglobin synthesis, it may function only as an emergency form of long term iron storage, and is removed from the maturing cells before they are released into the circulation. Their experiments also suggested that iron was stored in ferritin only when the availability of iron in the intracellular pools was greater than that required for hemoglobin synthesis. We interpret our data as indicating that erythropoietin acts to increase the membrane-bound iron, the soluble iron transfer components, or both, in the earliest erythroid cells. Since there is a temporal lag between the induced influx of iron and subsequent de novo hemoglobin synthesis, the increase in ferritin iron storage after erythropoietin addition may reflect only the excess iron in the intracellular pool. The data in this and other papers suggest that the sequence of events following exposure of sensitive cells to erythropoietin may be the following: (1) increase in some specific RNA synthesis,⁶ (2) increase in the system for moving ionic iron from extracellular transferrin to the inside of the cell, (3) synthesis of "fraction I," (4) synthesis of ferritin or incorporation of iron into apoferritin, and (5) synthesis of hemoglobin. We suggest that events (3) and (4) may be simultaneous and depend on the presence of intracellular iron. They may be dependent upon erythropoietin only inasmuch as erythropoietin causes events (1) and (2) to occur.

The concept of membrane alteration occurring in response to erythropoietin is attractive because of the unusual nature of the inhibition of puromycin (Tables 2, 3). These data suggest that some cellular alteration occurs very rapidly so that an effect of erythropoietin is still noted after five hours of incubation. Inhibition of protein synthesis, of course, stops incorporation of iron into hemoglobin, but since there is essentially no stimulation of hemoglobin formation at these short times of incubation the inhibition of the erythropoietin effect on total cell iron-uptake should be attributed solely to the non-hemoglobin compartment. The inhibition of hemoglobin synthesis under these conditions will be discussed in more detail in a subsequent paper.

We have been able to isolate small amounts of transferrin from lysates of washed cells but these quantities of transferrin are not sufficient to account for a significant amount of the non-heme, non-ferritin radioactivity. Erythropoietin, however, did exert some effect on this small amount of transferrin bound to marrow cells. Since transferrin presumably associates with a specific component in the erythroid cell membrane, this may indicate an erythropoietin effect on that component of marrow cells.

The erythropoietin effect on iron uptake requires the synthesis of new RNA for expression (Table 4) as do the erythropoietin-induced effects on hemoglobin synthesis and glucosamine incorporation into cell stroma. Since erythropoietin appears to act as a primary inducer of erythroid differentiation, many new functions must arise in the course of events after the initial step of induction. It is impossible in the current state of our knowledge to distinguish between those primary events concerned with induction and those arising as a consequence of a temporal program set in motion by an initial event, nor can we determine which events in the program are causally related to each other. The logical necessity of providing a large intracellular pool of

iron before massive hemoglobin synthesis occurs during differentiation, suggests that this is the normal and necessary sequence of events, and data lending support to this hypothesis will be presented elsewhere.

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POSSIBLE NON-RANDOM SELECTION OF D GROUP CHROMOSOMES
INVOLVED IN CENTRIC-FUSION TRANSLOCATIONS*

By

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Evidence from the work of Court-Brown¹ and the newborn surveys of Walzer,² Sergovitch,³ and Lubs⁴ indicates that the centric-fusion translocation involving the long arm of two D group chromosomes, t(DqDq), is the commonest structural abnormality in human populations (incidence about 1/1000). Court-Brown has noted an increased frequency of spontaneous abortions in families with a t(DqDq) although this is not confirmed by an analysis of 26 families reported by Hamerton.⁵

The possibility that translocation chromosomes composed of different D group chromosomes might carry significantly different risks prompted us to examine the data on the ascertainment of individuals carrying a t(DqDq) in which the D chromosomes involved had been identified by autoradiography. A second purpose of this study was to analyze the frequency with which different D group chromosomes were involved in t(DqDq). Previous autoradiographic analyses⁶⁻⁸ of thirty-nine t(Dq21q) indicated that the D group chromosomes did not participate on a random basis; 87 per cent contained chromosome 14 whereas none involved chromosome 13.

This paper provides data to show that the D group chromosomes are not randomly present in t(DqDq) and discusses a number of different hypotheses to account for this nonrandomness.

Case report. L.P. is a 38-month-old, severely retarded Caucasian female who was found to have a t(DqDq) chromosome inherited from the maternal grandfather (Figure 1), as well as 2 marker chromosomes involving B and C group chromosomes (Figure 2). She was the 3rd offspring of a 30-year-old mother (gravida 5 para 3 abortion 2) and a 31-year-old father. The child was well-nourished and her growth was within the normal range for her age. On physical examination, she exhibited hypertelorism, epicanthus, antimongoloid slant to the eyes, low-set ears, and micrognathia (Figure 3). Constant drooling was present. She did not have microcephaly or cleft palate. A "cat cry" was not noted during infancy. The carrying angle of the arms was increased. Pes planus and a shuffling gait were noted. Her local physician has been treating her for hypothyroidism since the age of 7 months. Routine laboratory tests were within normal limits. Bone age was reported to be advanced, 5 years at 3 years 2 months, except for the distal epiphyses of the last four metacarpals which were 4 years 2 months. Dermatoglyphics revealed whorls on all 10 digits (present in 1 per cent of control populations) and the axial triradii were in the t' position. An EEG showed a few (2-3) generalized brief sharp wave bursts, but no clear-cut seizure discharges.

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Figure 1. Pedigree of the W family.

Figure 2. Karyotype of the proband with the $t(DqDq)$ which is morphologically indistinguishable from A3. The two marker chromosomes (mar 1 and mar 2) are derived from B and C group chromosomes (45,XX,4-,C-,13-,14-, $t(13q14q)+$, mar+,mar+).

Figure 3. Proposita showing hypertelorism, anti-mongoloid slant to the eyes and low-set ears.

Cytogenetic analysis. Chromosomal analysis revealed a modal number of 45. There were only four D chromosomes but three A3 chromosomes, one of which was presumed to represent a t(DqDq) chromosome (Figure 2). In addition, chromosomes from the B and C groups were missing and were replaced by 2 marker chromosomes. The nature of this latter structural chromosomal aberration will be discussed in another paper.⁹ The sex chromatin pattern was that of a normal female. The t(DqDq) chromosome was present in the mother, the mother's sister and the mother's father. The father and two siblings had a normal karyotype.

Autoradiographic analyses of chromosomes were made from peripheral blood cultures of the patient and her mother.¹⁰ These indicated that the mother's translocation involved one number 13 and one 14 chromosome and her karyotype was designed, 45,XX,13-,14-,t(13q14q)+. Autoradiographic analysis of the patient's chromosomes revealed only one B4 chromosome and her karyotype was designated 45,XX,4-,C-,13-,14-,t(13q14q)+,mar+,mar+.

Source of t(DqDq) chromosomes identified by autoradiography. We know of a total of thirty-four t(DqDq) chromosomes present in 33 individuals that have been analyzed by autoradiography. These were obtained as follows:

- a) 1 case as described above;
- b) 18 cases were found in reviewing the literature;^{7,11-21}
- c) 14 cases were communicated to us by other investigators through letter and telephone conversation.²²⁻³¹

Composition of the t(DqDq) chromosomes. The results of autoradiographic analysis of

thirty-four t(DqDq) chromosomes are presented in Table 1 along with pertinent data on the probands and their families. Twenty-six translocations involved chromosomes 13 and 14, four involved chromosomes 13 and 15 and only one case involved chromosomes 14 and 15. In three cases only chromosome 13 was involved, either as a t(13q13q) or as a 13 qi (long arm isochromosome). The distribution of the thirty-four t(DqDq) chromosomes differed significantly from the expected equal participation by chromosomes 13, 14, and 15 ($\chi^2 = 47.2$, d.f. = 3; $p < .005$). The χ^2 test was based on four classes, since t(14q14q) and t(15q15q) would lead to trisomy 14 and 15. These trisomies have not been identified and are presumably lethal.

Ascertainment. Only six, and possibly seven, of the centric-fusion translocations were ascertained by the occurrence of trisomy 13 syndrome, whereas all the t(Dq21q) chromosomes were identified because of the occurrence of Down's Syndrome. In four cases, the translocations were sporadic, and in three of these the translocation involved only chromosome 13. The fourth case involved a child who possessed two t(DqDq) chromosomes thought to be t(13q14q) and t(13q15q). In the three familial cases, two phenotypically normal relatives carried a t(13q14q), while in the third case (classified as trisomy 13 because of the presence of arrhinencephaly), a t(13q15q) was present in the family.

Data on the remaining 26 cases indicated that ten of the probands had chromosomal aberrations distinct from a t(DqDq) chromosome. Seven of these had Down's Syndrome of which only two carried a t(13q14q) chromosome,^{19,23} and the other three included a ring D,²³ XXY,¹⁵ and the present case. Although these findings may suggest multiplicity of structural and numerical chromosome aberrations within families and cells, the possibility of biased sampling has not been eliminated.

Karyotypic analysis of 29 pedigrees revealed that the t(DqDq) chromosome was present in 2 or more generations in 25 cases and of these 21 involved t(13q14q). Only 15 of thirty-nine t(Dq21q) chromosomes were known to be familial. The results suggest that familial t(DqDq) chromosomes, associated with a normal phenotype, usually involve chromosomes 13 and 14, while centric-fusion translocations associated with trisomy 13 may involve chromosome 13 with itself or with chromosomes 14 or 15.

In contrast to the distribution of D group chromosomes in centric-fusion translocations, noncentric-fusion translocations can occur between all of the D group chromosomes and any other chromosome group.¹⁶

DISCUSSION

These observations raise the following questions:

- 1) Why is chromosome 14 identified more frequently in centric-fusion translocations than 13 or 15?
- 2) Why are centric-fusion translocations involving chromosomes 14 and 15 rarely observed in the liveborn, while those involving chromosomes 13 and 21 are not observed at all?

Several hypotheses are presented.

Hypothesis 1. Breaks at or near the centromere occur with different frequencies in chromosomes 13, 14, and 15 and thus lead to different rates of production of various translocation chromosomes. This hypothesis may explain the low frequency of involvement of chromosome 15 with both chromosomes 21 and 13, as compared with chromosome 14. The hypothesis, however,

Table 1
DESCRIPTION OF INDIVIDUAL CASES WITH t(DqDq) CHROMOSOMES

Composition of translocation	Propositus	Karyotype	Carriers in family	Ref.
t(13q13q)	Trisomy 13	46,XX,13-,t(13q13q)+	None	12
	Trisomy 13	46,XX,13-,t(13q13q)+	None	16
Normal/ t(13q13q)	Incomplete Trisomy 13	46,X?/46,X?,13-, t(13q13q)+*	None	21
t(13q14q)	Trisomy 21	47,XY,21+	Mother; present 3 generations	11
	Mental retardation	46,XX	Mother and other relatives	13
	Familial mental retardation	45,XY,13-,14-,t(13q14q)+	Siblings and father	14
	Klinefelter's Syndrome	46,XXY,13-,14-,t(13q14q)+	Parents dead; family not studied	15
	Normal newborn	45,XY,13-,14-,t(13q14q)+	Relative (unspecified) (family 818 or 157)?	16
	Normal newborn	45,XY,13-,14-,t(13q14q)+	Relative (unspecified) (family 852 or 2040)?	16
	Trisomy 21	47,XY,21+	Mother and sibling (family #74)	16
	Trisomy 13	46,XX,14-,t(13q14q)+	Mother and grandfather (family #327)	16
	Multiple congenital malformations	45,XY,13-,14-,t(13q14q)+	Mother (family #1539)	16
	Failure to thrive	45,XX,13-,14-,t(13q14q)+	Mother and grandmother	18
	Trisomy 21	46,XY,13-,14-, t(13q14q)+,21+	Mother and grandfather (#926)	19
	Trisomy 21	Male; not karyotyped	Mother and grandfather; also (t13p14p)+ (#1156)	19
	Leukemia	45,XX,13-,14-,t(13q14q)+	No family available	22
	Trisomy 21	46,X?,13-,14-, t(13q14q)+,21+*	Father	23
	Multiple congenital abnormalities	46,XY,Dr	Mother	23
	Trisomy 13	45,XX,13-,14-,t(13q14q)+/ 46,XX,14-,t(13q14q)+	Father (father's sister is 47,XX,21+)	24
				25
				25
				25
	Seizures, mental retardation	45,XX,13-,14-,t(13q14q)+	Mother	26
	Louis-Bar Syndrome	45,XX,13-,14-,t(13q14q)+	Not stated	27
	Multiple abortions and abnormal child	45,X?,13-,14-,t(13q14q)*	Mother and grandmother	28
	Cornelia de Lange Syndrome	45,XY,13-,14-,t(13q14q)+	Mother and other relatives	29, 30
	Trisomy 21	47,XY,21+	Several relatives	31
	Multiple congenital abnormalities	45,XX,B-,C-,13-,14-, t(13q14q)+,mar+,mar+	Mother and grandfather	Pres. report
t(13q14q) t(13q15q)	Trisomy 13	45,XX,13-,14-,15-, t(13q14q)+,t(13q15q)+	None	17
t(13q15q)	Trisomy 21	47,X?,21+*	Mother and grandmother	7
	Short stature, mental retardation	45,XY,13-,14-,t(13q14q)+	Mother	26
	Arrhinencephaly (Trisomy 13?)	Not done		25
t(14q15q)	Multiple abortions	45,XX,14-,15-,t(14q15q)+	Mother and other relatives	20

*X? = sex not stated.

Table 2

DISTRIBUTION OF t(DqDq) CHROMOSOMES AS RELATED TO NATURE OF
TRANSLOCATION AND METHOD OF ASCERTAINMENT

Type of translocation	Ascertainment	t(13q13q)	t(13q14q)	t(13q15q)	t(14q15q)
Familial 2 or more generations	Trisomy 13		2 ^{16,24}	1 ²⁵	
	Trisomy 21		6 ^{11,16,19,23,31}	1 ⁷	
	Cong. abn. +/- or men- tal retard.		7 ^{13,14,16,26,28,} 29, present case	1 ²⁶	
	Newborn survey		2 ¹⁶		1 ²⁰
	Multiple abortions		1 ¹⁸		
	Failure to thrive		3 ²⁵		
	Not stated				
Only one individ- ual examined, or included in re- port	XXY		1 ¹⁵		
	Congenital abnor- mality		2 ^{23,27}		
	Leukemia		1 ²²		
Sporadic (par- ents normal)	Trisomy 13	3 ^{12,16,21}	1 ¹⁷⁺	1 ¹⁷⁺	
Total known to be familial - 25		3(0)	26(21)	4(3)	1(1)
Total known to be sporadic - 4		+ two translocations in same child			
uncertain - 4		() number familial			

fails to explain the absence of t(13q21q), for if chromosome 13 breaks frequently enough to join with chromosome 14, it should also join with chromosome 21.

Hypothesis 2. All the acrocentric chromosomes break with equal frequency but some mechanism leads to preferential association between certain pairs of chromosomes, and the observed nonrandom translocation merely reflects this nonrandom association during meiosis. The association of the terminal chromomeres of the acrocentric chromosomes with the nucleolus³² could provide such a mechanism for maintaining the centromeric regions of some acrocentric chromosomes in close proximity, so that, if simultaneous breaks occur, they result in union of the long arms of the broken acrocentric chromosomes. Such an explanation, however, would have to assume that the terminal chromomeres of chromosomes 13 and 14 and chromosomes 14 and 21, are closely associated, but that chromosomes 13 and 21 are never so associated. There is no evidence for such preferential association of nucleolus-organizing segments in mammalian systems.

Hypothesis 3. The locations of chromosomal breaks are such that they occur only in the short arms of chromosomes 13 and 21 and the long arm of chromosome 14. Thus, a chromosome formed by 13 and 21 would be dicentric and nonviable. Although there is evidence for preferential sites of chromosome breakage, there is no evidence that chromosomes cannot break anywhere along their length.

Hypothesis 4. Segments of the short arm of chromosome 14 are homologous with segments

near the centromere in the long arm of chromosomes 13 and 21. Pairing of homologous segments and crossing over during meiosis would lead to t(13q14q) or t(14q21q) chromosomes. On the other hand, the union of chromosomes 13 and 21 which would be expected to occur merely by chance, has not been observed.

Hypothesis 5. Selection may be responsible: certain combinations of deficiencies may be, with rare exceptions, lethal. The double deficiency of short arm segments of chromosomes 13 and 21 is not sufficient to explain the failure to recover t(13q21q) chromosomes. One would expect to find such translocations in individuals with Down's Syndrome, since the additional 21 producing Down's Syndrome would provide a short arm segment of 21.

Prezygotic selection or meiotic drive has already been proposed to explain the significant excess of balanced translocation carriers over chromosomally normal progeny among the offspring of male carriers of t(DqGq) and t(DqDq) chromosomes.⁵ The nonrandom occurrence of D group chromosomes in these same two translocations may represent another example of selection.

Selection may also be responsible for recovering chromosome 14 six times more frequently than chromosome 15 in t(DqDq) chromosomes as well as seven times more frequently in t(Dq21q) chromosomes. Chromosome 14 shows a pattern of late DNA replication in the region of the short and long arm near the centromere. This late replicating area is presumed to contain heterochromatin, to be relatively genetically inactive, and therefore dispensable.

The single example of a familial t(14q15q) chromosome may represent the rare survival of individuals with chromosomal deficiencies which are usually lethal.²⁰ Of 14 pregnancies in 3 generations, the carriers had 2 chromosomally normal children, 3 carriers, and 9 spontaneous abortions. Whether the large number of abortions and reduced fertility are related to selection because of the combination of deficiencies associated with this translocation, or whether they are related to the occurrence of trisomy 14 or 15, both of which are presumably lethal, or to some other factor is unknown. Since the only third generation carrier of the t(14q15q) chromosome has had six spontaneous abortions and no viable conceptions, this particular translocation has been subjected to effective selection. However, the t(14q15q) chromosome may appear to be rare only because of the small number of t(DqDq) chromosomes which have been analyzed by autoradiography.

Each of the first four hypotheses could explain the observation that chromosome 14 is the D chromosome most frequently identified in centric-fusion translocations (question 1). They suggest various mechanisms leading to preferential entry of this chromosome into such translocations. The deficiency of each hypothesis is that it does not explain the absence of t(13q21q) chromosomes and the rarity of t(14q15q) chromosomes (question 2). The selection hypothesis suggests a mechanism which does account for all the available data.

The D group chromosome(s) involved in all centric-fusion translocations should be identified by autoradiography: first, to obtain more data to determine the relative roles of selection as compared with preferential entry in explaining the nonrandom involvement of the D chromosomes in these translocations. Second, as t(DqDq) chromosomes appear to represent the commonest structural chromosomal anomaly in man, it is important to determine whether carriers of different combinations vary in the incidence of nonviable conceptions and offspring with congenital malformations and/or additional chromosome aberrations.

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ON THE GEOMETRICAL ARRANGEMENT OF THE PROTEIN SUBUNITS OF
HUMAN SERUM LOW-DENSITY LIPOPROTEIN: EVIDENCE
FOR A DODECAHEDRAL MODEL*

By

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Studies on the structure of human serum low-density lipoprotein, LDL, are relevant because this macromolecule (a) is among the better known naturally occurring lipoproteins; (b) plays an important role in fat transport; (c) is probably implicated in atherosclerosis; and (d) serves as a useful model for investigating the possibly general nature of protein-lipid interactions.¹ A major advance in this field has been the demonstration that the protein moiety of LDL, apo LDL, can be solubilized in aqueous media after essentially complete removal of its lipid complement. This has been achieved in the past by either chemical modification of the protein,² the use of surfactants alone,^{3,4} or in addition to dissociating agents such as urea or guanidine HCl.^{5,6} We have recently reported on a new solubilization procedure,⁷ which is carried out under carefully controlled pH titration. The availability of an apo LDL, prepared under rather mild conditions, prompted us to examine its properties with particular reference to defining its role in LDL structure. We wish to report here on those studies leading to the formulation of a model of LDL in which its 20 protein subunits, probably globular, are arranged in a dodecahedral pattern with icosahedral symmetry. The model requires that the LDL surface is occupied by both protein and lipids. In terms of the latter the presence of phospholipids was supported by the results of the hydrolysis of LDL by phospholipases A and C.

MATERIALS AND METHODS

LDL of d 1.019-1.063 was prepared from fresh sera obtained from healthy, male Caucasian donors (20-24 years of age, group A, Rh+) by ultracentrifugation in NaCl³ or in sucrose⁷ and further purified by equilibrium banding on sucrose gradients. The immunologically pure products contained 22 per cent protein, 22 per cent phospholipids, 37 per cent cholesterol esters, 8 per cent free cholesterol and 10 per cent triglycerides. Apo LDL, essentially free of lipids, was prepared by extraction with ethanol-ether² and solubilized as previously described⁷ except that 8 M urea was used to expedite the solubilization of the protein. Apo LDL prepared in urea was indistinguishable, after removal of the dissociating agent, from the protein solubilized by simple pH titration at 11.5 and prompt neutralization to pH 8.5. A water-soluble, neutral lipid-free derivative of LDL was prepared by extraction with ethyl ether at 3°C for 24 hours. Succinylation of

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LDL or apo LDL was carried out by the reaction with succinic anhydride.² The hydrolysis rate of LDL by either phospholipase A* or phospholipase C† was monitored with a Radiometer Automatic Titrator. The experimental conditions for phospholipase A were: 10 mg LDL in 4 ml 0.01 M borate buffer, pH 8.0; 0.006 M CaCl₂; 1 per cent heptane-extracted human serum albumin, titrated with 0.01 M KOH; 25 µg enzyme/ml; 40°C. The experimental conditions for phospholipase C were: 10 mg LDL in 4 ml of 0.05 M glycine-NaOH buffer, pH 7.6-8.2; 0.005 M CaCl₂; 50 µg/ml enzyme (0.01 units), titrated with 0.01 M KOH; 40°C.

Sedimentation equilibrium studies were performed according to Yphantis⁸ at 20°C using interference optics, six chamber Yphantis cells with 12 mm path length, and protein concentrations between 0.15 and 1.00 mg/ml. The buffer for apo LDL was the solubilization medium (0.005 M borate, pH 11.5, 8 M urea). All studies on apo-LDL were carried out within 3-4 hours after solubilization. The partial specific volume ($\bar{v}$ = 0.718) for apo LDL was computed from the amino acid composition³ and corrected for the presence of 8 M urea.⁹ Counterions were omitted from the medium to minimize aggregation.⁹ A correction factor for the electrostatic effect of the high pH on the molecular weight was computed according to Jeffrey.¹⁰ The technique for determining the molecular weight of LDL was as described by Scanu et al.² The results were consistent with those reported previously.

Electron microscopy was performed in an RCA EMU-3 unit using a pointed filament, accelerating voltage of 50 kV and a maximum instrument magnification of 30,000 X. Magnification was determined by reference to a calibration grid (54,864 lines per inch, E. F. Fuller, Inc., Schenectady, N. Y.). Particle sizes were determined directly from negatives on a Nikon micro-comparator with 20 X magnification. Before microscopy, the samples were extensively dialyzed against 0.01 M phosphate buffer, pH 8.6, 0.001 M EDTA. Ultra-thin-carbon coated, fenestrated formvar grids were employed. The best negative stain was 2 per cent phosphotungstic acid in 0.01 M phosphate buffer, pH 7.0. Uranyl acetate and sodium silico-tungstate‡ were also employed. A pre-stain with osmic acid§ did not affect the results and neither did changes of the pH between 7.0 and 11.5.

RESULTS

The sedimentation equilibrium study of apo LDL demonstrated that it was an extensively associating system. At pH 11.5, in 8 M urea, the minimum molecular weight was computed as $27,500 \pm 2,000$. Under milder conditions (0.01 M Tris or phosphate, pH 8.6) apo LDL had a minimum average molecular weight of 80,000-100,000 with maximum average molecular weight of about 500,000. By comparison, LDL was homogenous with a molecular weight of $2.2-2.3 \times 10^6$ (Figure 1). Based upon a protein content of 22-24 per cent, the number of subunits could be computed as 18-22.

By electron microscopy (Figure 2A) LDL was homogenous with spherical particles of an

* Phospholipase A was a gift from Professor L. L. M. van Deenen, Department of Biochemistry, the University of Utrecht, The Netherlands.

† Phospholipase C was a gift from Dr. T. Pastan, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland.

‡ Gift of Dr. J. Schwartz, Department of Microbiology, University of Chicago.

§ Suggested by Dr. B. Lombardi, Department of Pathology, University of Pittsburgh.

Figure 1. Sedimentation equilibrium analyses of LDL and apo LDL.

average diameter of 193 Å. For this computation, only particles that were unaggregated and not obviously broken were selected. The computed molecular weight of the particle, using the density of 1.040, was in the range obtained by sedimentation equilibrium. Each particle had a substructure. The molecular weight of the subunits seen in the electron microscope, computed assuming sphericity and a density of 1.37, was about 30,000. The subunits in LDL were also seen in both the neutral lipid-free LDL and apo LDL. In the case of the neutral lipid-free LDL they were predominantly arranged in strings of up to 500 Å in length (Figure 2B). Apo-LDL was found to consist of beaded strings as well as spherical structures of 50-150 Å in diameter (Figure 2C).

The substructure observed in LDL was not affected by succinylation or by treatment with phospholipase A and C. Phospholipase A-treated LDL (almost complete conversion of lecithin to lysolecithin) had a particle, size, hydrated density and subunit distribution identical to those of native LDL. Succinylation of LDL was necessary either before or after phospholipase-C treatment to prevent precipitation. The hydrated density of this product was identical to that of LDL, though particle aggregation was not eliminated.

The fact that a subunit structure was noted by electron microscopy with LDL prepared in either the presence or absence of lipids, or after the action of lipolytic enzymes, led us to conclude that the observed subunits did represent proteins.

DISCUSSION

The results of the combined application of the techniques of ultracentrifugation and electron microscopy to the study of human serum LDL and some of its derivatives permits several conclusions of structural significance: (1) LDL of d 1.019-1.063 is composed of spherical particles with uniform size (about 200 Å) and weight (2.2 - 2.3×10^6). (2) The protein moiety has a subunit structure with about 20 subunits, most probably very similar or identical in size with a mini-

Figure 2. Electron micrographs of: (A) LDL₂; (B) and (B') ether extracted LDL; (C) and (C') apo-LDL. Magnifications of (A), (B') and (C') are 522,500; (B) and (C) are enlarged 285,000-fold. All preparations were in 0.01 M PO₄ buffer, pH 8.6, 1 mM EDTA, and 2% PTA. Arrows indicate subunits.

num molecular weight of about 27,000. (3) Particle size and subunit structure of LDL are not affected by either succinylation or digestion with phospholipase A. (4) Treatment with phospholipase C does not affect the substructure of individual LDL particles but causes them to aggre-

gate, probably as a consequence of the decrease in net charge density.

Although the data on whole LDL are consonant with previous reports from this laboratory,² the figure for the minimum molecular weight of the protein subunits is significantly smaller than was reported previously.² In the previous report, the studies were carried out on succinylated apo LDL and the value of 36,000 was considered only tentative in view of the numerous assumptions made in the computations. Since more direct analyses could be attained in the apo LDL utilized in our current experiments, we regard the figure of about 27,000 to be the more probable one. It is of interest to note that if the molecular weight data of apo LDL by Shore and Shore⁵ are corrected to anhydrous weight, the figure obtained is of the same order as that found in the present studies.

Previous electron microscopy studies of LDL have been confined to computations of size and molecular weight. Forte et al.¹¹ who studied LDL by negative staining with phosphotungstic acid, observed particles of comparable size to those in our experiments, but described a granular surface. A similar observation was also reported by Gotto et al.⁴ In contrast, our studies have clearly shown a subunit structure in LDL. The model discussed in the following section was in fact the result of efforts to interpret the various views of LDL seen by the negative staining technique.

A model for LDL. An analysis of enlarged electron micrographs of LDL suggested the existence of a dodecahedral arrangement of the protein subunits with characteristic 2, 3, and 5-fold rotation axes as well as 5 and 6 edges (Figure 3). Such a model implies that the 20 protein

Figure 3. Views along the principal rotation axes of LDL particles seen in the electron microscope (top row) and of a scale dodecahedral model (bottom row).

subunits (25-30,000 MW each) occupy the vertices of a dodecahedron with a diameter of about 193 Å. The problem of placing the lipids in this system was somewhat alleviated by an attractive, though strictly theoretical, model for LDL proposed by Oncley and coworkers in 1950.¹² They supposed that protein and phospholipids are distributed over the surface of the LDL molecule in a mosaic pattern with the neutral lipids located in a central core. Our model would be consistent with theirs if the phospholipids occupied the faces of the dodecahedron formed by the protein subunits and the neutral lipids were in the interior of the dodecahedral shell. Although

we can make no statement about the detailed molecular arrangement of the various lipids in LDL, the phospholipase experiments do suggest that the charged groups of the phospholipids (possibly both glycerol- and sphingolipids) are oriented outwards in contact with the external solvent.

To demonstrate that our model was consistent with these suppositions, we constructed a scale model of LDL using spherical styrofoam balls (2.5 cm dia) to represent the protein subunits, and a transparent plastic icosahedron as armature. Photographs of such a model closely resembled the subunit arrangement seen in electron micrographs of LDL. For a dodecahedron with a diameter of 193 Å, the area of 12 faces (exclusive of that occupied by subunits and the area between them) is 36,000 Å². Such a figure compares closely to that of 27,800 Å² computed for the 694 lecithin-equivalents present in LDL assuming a cross-sectional area for lecithin of 40 Å².¹⁶ The volume enclosed by the dodecahedral shell of our model is 5.36 x 10⁶ Å³, compared to that of 3.29 x 10⁶ Å³ required for LDL neutral lipids. It would thus appear that our proposed model is consistent with the concept of LDL structure proposed by Oncley.

The postulate that the neutral lipids form the inner core of LDL, although also proposed by others¹³ does not rest at the moment on a firm experimental basis. The studies of Bruckdorfer¹⁴ indicate that no structural conclusions can be based on results from differential extraction with organic solvents, and this applies, in our experience, to anionic detergents, although a different claim has been made by Gotto et al.¹⁵

The problem of whether the general organization of LDL is directed by protein-protein or protein-lipid interactions, or both, is not resolved by our present studies. Although LDL, like all spherical viruses studied to date, has "icosahedral symmetry," the organization of its protein subunits appears unique in that the subunits are located on the faces of the icosahedron, instead of on the vertices (see photographs of scale model in Figure 3). Consequently, according to our model all subunits would be related to the others by a threefold rotation axis. This may be either dependent upon a direct interaction between protein subunits (in this case, the subunits should be asymmetric, since, if spherical, they would be separated by about 20 Å), or upon a special arrangement between protein and phospholipids.

In the final analysis, the LDL molecule is a spherical structure with icosahedral symmetry in which the protein subunits occupy the vertices of a dodecahedron. The faces of the dodecahedron would then be occupied by phospholipids, possibly in conjunction with some free cholesterol. The interior of the spherical structure would contain the water-insoluble neutral lipids (cholesterol esters and triglycerides). Such a model is inconsistent with that proposed by Vandenheuvel, who postulated the presence of an extended protein layer entirely covering the lipid interior.¹³

Future studies will be needed to define the molecular nature of the protein-protein, lipid-lipid and protein-lipid interactions in LDL. The possibility of preparing the protein moiety in lipid-free form should now permit the resolving of the chemical relationship among the various subunits (i.e., homogeneity vs heterogeneity) and the relative role of their structure on lipid binding.

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SELECTIVE TRANSLATION OF T4 TEMPLATE RNA BY RIBOSOMES FROM T4 INFECTED Escherichia coli*

By

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Recent reports from this laboratory^{1,2} and others³ have provided evidence for the coding of transfer RNA's (tRNA) by the T4 genome. The relevance of phage-specific tRNA's to the viral replicative process is not known. Hattman and Hofschneider⁴ have shown that when Escherichia coli is infected with the RNA phage M12 and then superinfected with T4, progeny M12 phage is strongly inhibited or completely blocked. These investigators further reported that in mixed M12 and T4 infections, M12 protein synthesis is interfered with.⁵ It therefore seemed possible that T4 tRNA's might be involved in the "shut-off" of RNA phage protein synthesis and perhaps of host protein synthesis.

In the course of work to determine the merits of this idea, it was observed that ribosomes from T4 infected cells were much less efficient for translation of MS2 and E. coli template RNA's than were ribosomes from uninfected E. coli, although translation of T4 RNA was similar for both types of ribosomes. This communication describes in vitro studies with normal- and T4 infected-cell ribosomes and compares their translational efficiency with different RNA templates.

MATERIALS AND METHODS

Preparation of ribosomes and supernatant fraction. E. coli B cells, normal or 12 min-infected with T4am61 (a lysozyme amber mutant, 10 PFU/cell) were ground with alumina (Alcoa 305, 2 g/g cells) in the cold, mixed with buffer A (10 mM Tris, pH 7.5, 30 mM KCl and 10 mM magnesium acetate) plus 5 µg/ml of DNase (RNase free) and then sedimented at 30,000 x g for 30 min. The S30 supernatant was adjusted to contain 6 mM 2-mercaptoethanol (MSH), centrifuged again as above, incubated at 37° for 1 hr after adding various reagents (ATP, PEP, PEP kinase, all 20 amino acids) as described by Nirenberg et al,⁶ dialyzed overnight at 4° against two changes of buffer B (buffer A plus 2 mM MSH) and then centrifuged at 49,000 rpm for 2.5 hr in a Spinco No. 50 rotor. The top 3/4 of the clear S165 supernatant was collected and used as the soluble supernatant fraction; the sedimented material was rinsed with buffer B, suspended in the same buffer and centrifuged at 10,000 x g for 10 min. The non-sedimentable material was used as the source of unwashed ribosomes.

Washed ribosomes. Unwashed ribosomes were extracted with buffer B containing 1 M NH₄Cl as described by Ghosh et al.⁷ After overnight mixing (16 hr) at 4° in the high salt-buffer, ribosomes were sedimented at 49,000 rpm as described above. The top 3/4 of the clear extract was saved and processed for initiation factor activity as indicated below; the pellet material was suspended in high salt-buffer B, extracted and sedimented once more as described above. The pellet fraction was rinsed with buffer B, suspended in this same buffer and served as washed ribosomes. Both washed and unwashed ribosomes were stored at 4° and used within one week.

* This report is taken from a paper that appeared in P.N.A.S. (U.S.), 64:345, 1969.

Preparation of initiation factors. The 1 M NH_4Cl ribosomal-extract described above was adjusted to 70 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$ at 4° . The precipitate that formed was collected by centrifugation, suspended in 10 mM Tris (pH 7.5), 1 mM magnesium acetate, 1 mM MSH and 10 per cent glycerol and dialyzed against this buffer. The preparation was clarified by centrifugation at $10,000 \times g$, adjusted to contain 0.25 M KCl and passed over a DEAE-cellulose column previously equilibrated with the same buffered-KCl solution. The A_{280} material that did not adsorb to DEAE was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and then dialyzed as above. This material was stored at 4° and used as the source of initiation factors within 4 days.

Isolation and purification of template RNAs. MS2 RNA was isolated from MS2 phage by repeated phenol extraction and several precipitations with ethanol.⁸ The RNA was dried in vacuo, then dissolved in H_2O and stored at -20° .

E. coli B and T4 template RNAs were prepared from normal and 15 min-intected E. coli B cells (infection was at 37°) using either wild type T4 or T4am61 phage; the procedure for RNA extraction included freeze thawing with lysozyme present, phenol extraction in the presence of 1 per cent sodium dodecyl sulfate and 0.01 per cent bentonite (room temperature), ethanol precipitation, DNase (RNase free) treatment, phenol treatment, several precipitations with ethanol, and then drying, in vacuo. The RNA was dissolved in 10 mM Tris (pH 7.5)-10 mM MgCl_2 and adjusted to contain 1 M NaCl. The precipitate that formed at 4° by standing overnight was collected, dissolved in water, dialyzed and designated crude template RNA. Further purification was accomplished on BD-cellulose by a slightly modified procedure of Sedat et al.⁹ The RNA solution (20 mg) was adjusted to contain 1.2 M NaCl-0.02 M Tris (pH 7.2) in a volume of 100 ml and then passed over a 1 x 4 cm BD-cellulose column, at room temperature. The column was washed with the same buffered-salt solution until the effluent A_{260}/ml was 0.02, or less, and then the fraction eluting from BD-cellulose with 1.5 M NaCl-15 per cent ethanol was concentrated and dialyzed, and was used as purified template RNA.

Poly U was a product of Miles Chemical Company, Elkhart, Indiana.

Assay for amino acid incorporation into proteins. The assay system (0.13 ml) contained the following: 40 mM Tris (pH 7.5), 10 mM magnesium acetate, 80 mM, NH_4Cl , 4 mM MSH, 2 mM ATP, 0.2 mM GTP, 2 mM PEP (Na^+ salt), 8×10^{-3} mM each of 19 amino acids (minus leucine), 4×10^{-3} mM ^3H -L-leucine (200 $\mu\text{c}/\mu\text{mole}$), 15 μg of calcium leucovorin (Lederle Laboratories), 50 μg of E. coli B tRNA, 1 μg of PEP kinase, 75 μg of soluble S165 protein, template RNA as indicated in the text and ribosomes (100 μg of unwashed and 80 μg of washed ribosomal protein estimated by the method of Lowry¹⁰). When polyuridylyate served as template, amino acids and leucovorin were omitted and 8×10^{-2} mM ^3H -L-phenylalanine (100 $\mu\text{c}/\mu\text{mole}$) was substituted for labeled leucine. Incubation was at 37° for 15 min, the reaction was stopped with 1 ml of 10 per cent TCA and the material was then heated for 20 min at 80° . After cooling, the acid-insoluble material was collected on millipore filters, washed with 5 per cent TCA, dissolved in a scintillation mixture of Triton x 100-toluene and counted in a Nuclear Chicago Mark 1 scintillation computer.

^3H -amino acids were purchased from Schwarz BioResearch, Orangeburg, N. J., and New England Nuclear Corporation, Boston, Mass.

T4am61 and T4amE4307 (an amber mutant in DNA polymerase) were generous gifts from Dr. E. P. Geiduschek and Dr. R. Haselkorn.

RESULTS

Table 1 indicates that when poly U is used as a template in a cell-free system containing equivalent amounts of ribosomes from either normal or T4am61-infected *E. coli*, nearly equal quantities of polyphenylalanine are formed. With T4 RNA as template, similar levels of ^3H -leucine are also converted into polypeptide form with both types of ribosomes. With MS2 RNA as template, however, a striking difference is observed; ribosomes from infected cells are only 12 per cent as efficient as normal ribosomes for leucine incorporation into protein.

Table 1

COMPARISON OF PROTEIN SYNTHETIC ACTIVITY OF RIBOSOMES FROM NORMAL AND T4 INFECTED *E. coli* WITH DIFFERENT TEMPLATE RNA's

Ribosome source	RNA template	^3H -amino acid	^3H -incorporation into acid-insoluble fraction (cpm)	Incorporation* relative to <i>E. coli</i> (%)
<i>E. coli</i>	None	L-Phe	361	-
T4am61	None	L-Phe	302	-
<i>E. coli</i>	Poly U (20 μg)	L-Phe	23,139	100
T4am61	Poly U (20 μg)	L-Phe	24,739	107
<i>E. coli</i>	None	L-Leu	252	-
T4am61	None	L-Leu	369	-
<i>E. coli</i>	T4 (250 μg)	L-Leu	1,864	100
T4am61	T4 (250 μg)	L-Leu	1,784	88
<i>E. coli</i>	MS2 (40 μg)	L-Leu	2,170	100
T4am61	MS2 (40 μg)	L-Leu	570	12

The ribosomal assay system (0.13 ml) was as described under Methods. Unwashed ribosomes from normal and 12 min T4 am61 infected cells were used; 100 μg of ribosomal protein for each type. The type and quantity of template RNA is indicated above. *E. coli* S165 supernatant proteins were used in all reactions.

* Corrected for ^3H -incorporation in the absence of template RNA.

The selective translational inhibitory property imparted to *E. coli* ribosomes after phage infection is abolished when chloramphenicol is present just prior to infection. Ribosomes isolated from such cells exhibit essentially normal translational activity with each of the template RNAs tested as compared to ribosomes from uninfected *E. coli* (Table 2). In the absence of chloramphenicol, infected-cell ribosomes demonstrate their translation inhibitory property with MS2 as well as with *E. coli* template RNAs, although translation of the latter is less inhibited than the former. Table 2 also indicates that poly U template activity is similar for all three ribosomal preparations and that similar levels of ^3H -leucine incorporation are obtained with 1/10th the amount of purified as with crude T4 RNA. This makes it unlikely that the selective translational property of infected-cell ribosomes is due to an increased RNase activity.

In all the experiments described above the soluble supernatant factors were derived from

Table 2

EFFECT OF CHLORAMPHENICOL ON T4-INFECTED RIBOSOMAL ACTIVITY

RNA template	³ H-amino acid	³ H-incorporation with ribosomes from normal and infected cells*			Incorporation relative to <u>E. coli</u>	
		<u>E. coli</u> (cpm)	T4 (cpm)	T4(CM) (cpm)	T4 (%)	T4(CM) (%)
Poly U (20 µg)	L-Phe	20,403	26,938	19,417	132	95
T4 crude (250 µg)	L-Leu	4,686	3,900	4,620	83	99
T4 purified (22 µg)	L-Leu	4,784	4,910	3,937	103	82
MS2 (40 µg)	L-Leu	6,117	970	6,517	16	106
<u>E. coli</u> purified (20 µg)	L-Leu	1,660	700	1,360	43	82

The ribosomal assay system (0.13 ml) was as described under Methods. Equivalent amounts of unwashed ribosomes prepared from normal E. coli, from E. coli infected with T4am61 for 12 min either in the absence or presence of 50 µg/ml chloramphenicol (CM), were used as indicated above. The template RNAs employed and their concentrations are shown. E. coli S165 supernatant was used in all reaction mixtures.

*The values shown have been corrected for ³H-incorporation in the absence of template RNA, about 300 cpm.

normal E. coli. When supernatant factors from T4 infected cells were employed, no significant difference was observed in the translation of T4, MS2, and E. coli template RNAs with ribosomes from normal cells. However, restricted translation of MS2 and E. coli templates occurred with ribosomes from infected cells (Table 3).

Figure 1 shows that optimal amino acid incorporation for ribosomes from normal and infected cells occurs between 8-10 mM magnesium ion concentration with MS2 and T4 RNAs as template. Although significant differences in rates of amino acid incorporation occur at different metal ion concentrations, the translational inhibitory effect of ribosomes from infected cells with MS2 RNA is observed over a wide range of magnesium ion concentration (8-12 mM). Over

Table 3

COMPARISON OF EFFECTS OF NORMAL- AND INFECTED-CELL SUPERNATANT FRACTIONS ON TRANSLATION

RNA template	³ H-leucine incorporation		
	EC super plus EC ribosomes (cpm)	T4 super plus EC ribosomes (cpm)	T4 super plus T4 ribosomes (cpm)
T4 purified (22 µg)	4,784	4,549	4,725
MS2 (40 µg)	6,117	7,264	1,341
<u>E. coli</u> purified (20 µg)	1,660	1,574	860

The reactions for the values shown in the last two columns were run simultaneously with and under identical conditions to the reactions in Table 2 except that an equivalent amount of S165 supernatant protein from T4-infected cells replaced S165 supernatant from normal cells. The ³H-leu incorporations in column one are the same as those shown in Table 2, column one, for the respective template RNAs.

Figure 1. Efficiency of T4 and MS2 RNA translation at varying concentrations of magnesium. The assay system (0.13 ml) contained equal amounts of unwashed ribosomes from normal or T4 infected *E. coli* as described under Methods, except that the concentration of magnesium acetate was varied as shown above. Where indicated, 250 μ g of crude T4RNA or 40 μ g of MS2 RNA served as template.

this same range of metal ion concentration, the difference in amino acid incorporation with the two ribosomal preparations for T4 RNA translation is small and fairly constant.

Table 4 shows that translation of T4 template RNA with infected-cell ribosomes, relative to uninfected *E. coli* ribosomes, is essentially the same either early or late after infection with T4 wild type phage or with the DNA negative mutant T4amE4307. On the other hand, ribosomes from 5-min T4 wild type infected cells translate MS2 RNA about half as efficiently as uninfected ribosomes and after 10 min infection the translational restriction is almost maximum. Ribosomes from cells infected with T4amE4307 also show a high degree of translational inhibition with MS2 RNA.

Initiation factors required for protein synthesis can be removed from ribosomes by extraction with high salt.¹¹ These extracted ribosomes (washed ribosomes) are inactive for template RNA translation unless the initiation factors are added simultaneously to the in vitro system.

Table 4
EFFICIENCY OF RIBOSOMAL TRANSLATION EARLY AND LATE AFTER
INFECTION WITH T4 AND WITH A T4 DNA NEGATIVE MUTANT

RNA template	³ H-leucine incorporation relative to uninfected <i>E. coli</i> ribosomes		
	T4 wild (5 min) (%)	T4 wild (10 min) (%)	T4 _{am} E43 (10 min) (%)
T4	96	100	97
MS2	43	19	18

E. coli B were infected with T4 wild type or T4_{am}E4307 for 5 or 10 min and the cells collected after addition of NaN₃ and rapid cooling to 4°. Unwashed ribosomes were prepared from each of these different infected cell preparations, as well as from uninfected *E. coli*, and equivalent amounts assayed in the ribosomal system described under Methods with T4 and MS2 template RNAs. The efficiency of translation by the T4 infected-cell ribosomes is expressed as the percentage of that obtained with uninfected *E. coli* B ribosomes. The levels of ³H-leucine incorporation into protein were similar to that shown in Table 1.

Table 5 shows that with normal *E. coli* washed ribosomes (No. 6-9) the 1 M NH₄Cl extract of ribosomes from either normal or infected cells functions equally well for translation of T4 template RNA. In the presence of T4-ribosomal extract, however, the incorporation of ³H-leucine into protein with MS2 RNA is significantly lower (57 per cent) than with extracts from normal ribosomes. Using washed ribosomes from T4 infected cells (No. 10-13) a similar and more marked inhibition is observed. With T4 template RNA, normal and infected-cell ribosomal extracts serve almost equally well for protein synthesis, but with MS2 RNA, extracts from ribosomes of infected cells show only 26 per cent labeled leucine incorporation compared to extracts from normal ribosomes. These results indicate that some ribosomal-associated factor(s) from infected cells are extractable with high salt and can impart the property of selective template translation to normal ribosomes when mixed with them. Table 5 (No. 8, 9, 12, and 13) also shows that washed ribosomes from infected cells are still more inhibitory for MS2 RNA translation than washed uninfected ribosomes in the presence of either type of ribosomal extract, suggesting that the T4-inhibitory factor has not been completely extracted or that multiple T4-inhibitory factors may be involved.

The inhibitory factor in ribosomal extracts from infected cells adsorbs to DEAE-cellulose and is eluted with 0.25 M KCl-0.05 M Tris (pH 7.5); lower salt concentrations are less effective. Figure 2 shows that in the in vitro assay system containing washed normal ribosomes and saturating amounts of normal initiation factors, translation of MS2 RNA is inhibited with increasing amounts of the T4 DEAE-factor, but translation of T4 RNA is unaffected or slightly increased at factor quantities of 46 µg or less. Higher amounts of factor give increased inhibition of MS2 RNA translation, but inhibition of T4 RNA translation also becomes evident. Figure 2 also shows that when the factor is heated for 8 min at 80°, inhibition of MS2 RNA translation is no longer observed.

DISCUSSION

The studies described in this communication indicate that isolated ribosomes from T4 infected *E. coli* are strongly impaired in their amino acid incorporating activity with MS2 and

Table 5

EFFECT OF E. coli AND T4 RIBOSOMAL EXTRACTS ON TRANSLATION
OF T4 AND MS2 TEMPLATE RNA's

Expt. no.	Source of washed ribosome	Source of ribosomal extract	RNA template	Incorporation of ^3H -leucine (cpm)	Incorporation relative to <u>E. coli</u> ribosomal extract* (%)
1	EC or (T4)	-	-	104 (206)	-
2	EC or (T4)	EC (60 μg)	-	220 (306)	-
3	EC or (T4)	T4 (60 μg)	-	241 (398)	-
4	EC or (T4)	-	T4 (22 μg)	300 (305)	-
5	EC or (T4)	-	MS2 (56 μg)	211 (194)	-
6	EC	EC	T4	7,044	100
7	EC	T4	T4	6,695	95
8	EC	EC	MS2	7,149	100
9	EC	T4	MS2	4,373	57
10	T4	EC	T4	7,916	100
11	T4	T4	T4	6,562	81
12	T4	EC	MS2	3,169	100
13	T4	T4	MS2	1,144	26

The reaction system (0.13 ml) contained equivalent amounts of washed ribosomes and 1 M NH_4Cl ribosomal extract, from normal or 12 min T4am61 infected cells, as indicated under Methods. The amount and source of ribosomal extract as well as the type and amount of template RNA used are indicated in the table. The ^3H -incorporation values outside the brackets are for E. coli washed ribosomes, and the values inside the brackets are for the T4 infected-cell washed ribosomes. Normal E. coli S165 supernatant was used in all reactions.

* Corrected for ^3H -incorporation in the absence of template RNAs.

E. coli template RNAs but appear to function normally with T4 RNA and poly U templates. The inhibition of R17 RNA-directed protein synthesis in extracts of T4-infected cells has been reported by Salser and Gesteland, as cited by Hattman and Hofschneider.⁵ Several observations described here suggest that the selective translational property of infected-cell ribosomes is dependent upon some viral-induced change of host ribosomes. (1) Ribosomes from cells infected with T4 in the presence of chloramphenicol exhibit normal translational properties similar to ribosomes from uninfected cells. (2) The soluble S165 supernatant factors from T4-infected cells have no effect on translation with normal ribosomes, and (3) a heat-labile factor can be extracted from infected-cell ribosomes, which, when presented to normal ribosomes causes restricted translation of MS2 RNA but not of T4 RNA. The nature of the T4-ribosomal factor is unknown but its behavior on DEAE-cellulose and its heat-lability suggest that it is protein. Recently, Smith and Haselkorn¹² reported that several new proteins were found associated with ribosomes after T4 viral infection.

The assays with ribosomes from cells infected with the DNA negative phage mutant and at different times with wild type T4, suggest that the viral-induced modification of ribosomal translational properties occurs prior to the synthesis of viral DNA (probably earlier than 5 min) and

Figure 2. The effect of T4 DEAE-factor on translation of MS2 and T4 RNA. The assay system (0.13 ml) was as described under Methods except that only washed normal *E. coli* ribosomes were used and each reaction contained 50 µg of normal *E. coli* initiation factors. Where indicated above, 250 µg of crude T4 RNA or 40 µg of MS2 RNA served as templates. The amount of T4 DEAE-factor was varied as shown and was prepared from 10-min infected-cell ribosomes by extraction with 1 M NH_4Cl as described for preparation of initiation factors. Further treatment involved adsorption onto DEAE in 0.05 M Tris (pH 7.5), washing with 0.1 M KCl-buffer, elution with 0.25 M KCl-buffer, concentration and dialysis of fractions with the highest A_{280} readings. Where indicated, the T4 DEAE-factor was heated for 8 min at 80°.

continues into the later stages (post-viral DNA synthesis) of phage replication. The phenomenon of selective template translation may play a role in the "shut-off" process of host protein synthesis, although this process appears to be completed within 3 min after T4 infection¹³ and ribosomes from 1-3 min infected cells have shown little or no restricted translation of MS2 or *E. coli* template RNAs. It is possible that the in vitro ribosomal assay does not accurately reflect the functional on-set, in vivo, of selective template translation at very early times after T4 infection.

The property of selective template translation by T4-infected cell ribosomes resembles work done on the mechanism of interferon action.^{14,15} The studies of Marcus and Salb¹⁴ indicate that interferon induces host synthesis of a translational inhibitory protein (TIP) which associates with ribosomes and specifically blocks readout of viral mRNA but not host mRNA. It appears then, that under special conditions ribosomes can be restricted to translation of specific template RNAs in both bacterial and higher cell organisms. This phenomenon implies that control of genetic expression at the translational level by ribosomes may be an important regulatory mechanism during viral and normal cell development.

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SYNTHESIS OF MITOCHONDRIAL AND NUCLEAR DNA IN ANAEROBICALLY
GROWN YEAST DURING THE DEVELOPMENT OF MITOCHONDRIAL
FUNCTION IN RESPONSE TO OXYGEN*

By

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The view, advanced about 80 years ago,^{1,2} that cellular organelles such as mitochondria and chloroplasts are semi-autonomous, has gained much support in the last few years.³⁻⁵ It is now established that mitochondria and chloroplasts contain DNA that may differ in base composition⁶⁻¹² and certainly differ in size and physical structure from nuclear DNA.¹³⁻¹⁸ These organelles also contain their own DNA and RNA polymerases^{9,19-23} and species of ribosomal RNA,^{24,25} sRNA and amino acid activating enzymes²⁶⁻²⁸ which differ from those present extramitochondrially. In synchronized cultures of *Chlamydomonas*, chloroplast DNA replicates in a semi-conservative manner independent of nuclear DNA replication.²⁹ Mitochondrial DNA presumably also replicates by a semi-conservative mechanism, although in *Neurospora*, the transfer experiment has been complicated by the presence of a persistent precursor pool.³⁰

The ease with which mitochondrial development may be altered by simple environmental manipulations, makes yeast a particularly suitable organism for studying aspects of mitochondrial development. The suitability of these organisms is further enhanced by the availability of mutants, both chromosomal and non-chromosomal, affecting mitochondrial function.

Mitochondrial DNA of Baker's yeast, *Saccharomyces cerevisiae*, has a buoyant density considerably lighter than the corresponding nuclear DNA, which facilitates their separation by density gradient centrifugation.^{11,31-34} When yeast is cultured in the absence of oxygen, it grows well in the presence of a fermentable substrate, but mitochondrial function and cytochromes disappear.^{35,36} It has also been suggested that the mitochondria themselves disappear completely.³⁷ When such anaerobic cells are exposed to oxygen, even in a medium that does not support cell growth for lack of an assimilable nitrogen source, mitochondrial functional development proceeds rapidly.^{35,36,38,39} This process of O₂ adaptation may involve the development of incomplete mitochondria into functioning units, and perhaps also the replication of mitochondria and their DNA. Oxygen adaptation is known to be altered by a variety of factors, including the type of substrate and its concentration in the adaptation medium.^{35,36} Inhibitors of protein and nucleic acid synthesis also affect the induction of respiratory activity by O₂.³⁹⁻⁴²

In this communication we examine the effect upon the synthesis of mitochondrial and nuclear DNA of several factors which control and alter respiratory O₂ adaptation in yeast. Our purpose is to determine the relationship between mitochondrial DNA synthesis and the subsequent development of mitochondrial functional activity.

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METHODS AND MATERIALS

Growth conditions and strains. The diploid prototroph Yeast Foam 237 ρ^+ kindly provided by Professor P. P. Slonimski was used in most experiments. Strain D243-2Bg ρ^- , a haploid (α ad^- lys^- $p7^-$ ρ^-) chromosomal and neutral cytoplasmic petite (respiratory deficient) also provided by Professor Slonimski was used to study DNA synthesis in petites. Cultures were grown anaerobically in an enriched medium described by Lindenmayer & Estabrook⁴³ as modified by Tustanoff & Bartley.⁴⁴ Each liter of medium contained 10.0 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), 0.02 g ergosterol, 5.0 ml Tween 80, 0.15 ml wheat germ oil, 5.0 ml ethyl alcohol and glucose 50 g. Cultures were grown in 2-liter Bellco baffle shaker flasks equipped with a gas-flushing device having a stopcock at its inlet and a stopcock and mercury valve at its outlet. Pre-purified nitrogen (Matheson, >99.997 per cent pure) was passed first through 2 gas wash bottles containing vanadyl sulfate and zinc amalgam in sulfuric acid to remove traces of oxygen, and then through water to remove sulfuric acid spray. The medium was cooled on ice and gassed with nitrogen for 30 min prior to the inoculation and again for 45 min following the inoculation of a standard volume (1/200 the volume of medium) of a stationary phase preculture grown in the same medium. The stopcock at the inlet was closed, the flasks were warmed to 28°C and shaken in a New Brunswick incubator rotatory shaker at this temperature for 18-20 hr, by which time the culture was at stationary phase after 7-8 cell divisions. The cultures were cooled to 4° in an ice water bath prior to harvesting. The outlet stopcock was closed to prevent reflux of Hg into the flask. The yeast was collected by centrifugation at 0-4° and washed 2 times in ice-cold H_2O that had been previously gassed with purified nitrogen for 30 min.

Oxygen adaptation conditions. Ten to fifteen g of washed cells (wet weight) were suspended in cold 400 ml of adaptation medium consisting of 0.05 M succinate, 0.05 M phthalate buffer pH 4.9 containing 0.1 per cent glucose, 2 per cent ethanol. The low concentration of glucose was chosen for this medium in order to encourage the development of respiratory capacity under maximally derepressed conditions. Under these conditions this amount of glucose was rapidly consumed. Therefore at 30 min intervals during the aerobic adaptation, 100 mg of glucose was added for each 100 ml adaptation medium. This procedure also resulted in a much more predictable and standardized respiratory adaptation. Oxygen adaptation was achieved by warming the flasks to 28° and vigorously shaking in air using a rotatory shaker. In some cases cells were incubated in adaptation medium under nitrogen for 2 hr prior to aeration. The nitrogen was bubbled through the complete adaptation medium for 10 min at 0° in flasks similar to those employed for anaerobic growth prior to warming to 28°. This procedure is subsequently referred to as nitrogen preincubation.

At appropriate intervals 0.5-0.75 mc of [³H]adenine, specific activity 6 mc/m-mole, was added. When the additions were to anaerobic cultures, the [³H]adenine was first bubbled with N_2 and injected into the flasks through the tubing. In some experiments, [³H]adenine was tipped in from a side-arm on the flask to maintain better anaerobiosis. Pulses of 30 to 90 min were used in different experiments. The pulse was terminated by the addition of 2 mg of adenine for each ml of medium and by rapidly cooling the flasks in an ice-water mixture.

Preparation of protoplasts and mitochondria. Protoplasts were prepared by a modification of the method of Duell, Inoue & Utter.⁴⁵ The walls of aerobically grown and O_2 adapted cells

can be completely digested by snail digestive juice in a single step method. The cells were washed 5 times in water and once in 1.2 M sorbitol. Each g wet weight of cells was then suspended in 3-5 ml of a solution containing 1.2 M sorbitol, 0.1 M citrate KPO_4 , pH 5.8, 0.03 M 2-mercaptoethylamine (Calbiochemical), 1×10^{-3} M EDTA and 0.125 ml snail digestive juice (Glusulase, Endo Laboratories, N. Y.). The suspensions were gassed with nitrogen and stoppered when appropriate. The mixture was gently shaken at 30° for 60 to 90 min. The formation of protoplasts was followed by comparing the cell counts of preparations suspended in 1.2 M sorbitol with preparations suspended in H_2O . The protoplasts were washed 3 times with 1.2 M sorbitol to remove traces of glusulase and suspended in 6 ml/g wet weight of 0.9 M sorbitol, 0.1 per cent bovine serum albumin, 0.001 M EDTA, pH 7.2. The protoplasts were disrupted in a Waring blender at high speed for 30 sec. The nuclear and debris fraction was sedimented at 1,600 g for 10 min at 0° . The supernatant fraction was resedimented in the same way to remove residual nuclear contamination. The mitochondrial fraction was then sedimented at 15,000 g for 10 min at 0° . The mitochondrial pellet could then be frozen prior to DNA extraction.

Isolation of DNA. A method similar to that of Radloff, Bauer & Vinograd⁴⁶ was used for the isolation of DNA. Mitochondrial pellets were suspended in 2.0 ml of 0.05 M Tris, pH 7.5, 0.01 M EDTA, 0.8 per cent sodium dodecyl sulfate. The clarified suspensions were kept at 25° for 30 min. They were then cooled on ice and NaCl was added to a final concentration of 1 M. After 15 min at 0° , the mixture was centrifuged at 20,000 g for 15 min at 0° . The slightly yellow clear supernatant fluid was dialyzed for 2 hr against standard sodium citrate, SSC (0.15 M NaCl, 0.015 M Na citrate). The solution was incubated simultaneously with 100 $\mu\text{g/ml}$ pancreatic RNase (Worthington 5X recrystallized) and 10 units/ml T_1 RNase (Calbiochemical) at 37° for 1 hr. After exhaustive dialysis against 10X SSC followed by SSC the DNA was purified and separated by preparative cesium chloride density gradient centrifugation. The initial density of CsCl was adjusted to 1.710 g/cm^{-3} with an Abbe-Zeiss refractometer. The 2.5 ml columns were centrifuged in the Spinco SW39 rotor at 35-39,000 RPM for 60 hr. Approximately 50 equal fractions were collected after piercing the bottom of the tubes. ^{32}P nuclear DNA ($2-4 \times 10^3$ cts/min) of high specific activity ($1-4 \times 10^4$ cts/min/ μg) was added to each tube prior to centrifugation. The ^{32}P nuclear DNA was isolated from anaerobically grown cells harvested in the exponential phase of growth and was purified by CsCl centrifugation. The optical density at $260 \text{ m}\mu$ of each fraction was measured in the Beckman DU spectrophotometer. An aliquot was hydrolyzed in 0.3 M NaOH at 30° for 18 hr. After neutralization, 30 μg denatured salmon sperm DNA was added as carrier, the DNA was precipitated with cold 5 per cent TCA, and collected on Millipore filters. The filters were washed with 50 ml 5 per cent TCA and with 5 ml 80 per cent ethanol, dried, and counted in 15 ml of toluene scintillation mixture (0.5 per cent, 2,5-diphenyloxazole, 0.05 per cent 1,4-bis-2-(5-phenyloxazolyl)-benzene in toluene) in a Tri-Carb liquid scintillation spectrometer. Corrections were made for the ^{32}P counts that appeared in the ^3H channel (2-3 per cent of ^{32}P cts/min).

Enzyme assays. Oxygen consumption of yeast cells was measured with a Gilson oxygraph. Succinate cytochrome c reductase was measured according to the method of Rabinowitz & DeBernard⁴⁷ but without added serum albumin.

Cytochrome c oxidase was measured by a modification of the methods of Cooperstein & Lazarow⁴⁸ and de Duve, et al.⁴⁹ Horse heart cytochrome c (Sigma type III) in 0.08 M K phosphate buffer pH 7.4 was reduced with sodium dithionite under carefully controlled conditions.

Each preparation was checked for the 550 m μ /565 m μ absorption ratio and was only used when this exceeded 6-10. In addition, each preparation of reduced cytochrome c was checked for any excess of unoxidized dithionite by ascertaining whether any further reduction of an extra aliquot of oxidized cytochrome c took place. Optimal activity of both of the above respiratory enzymes was obtained with unbroken protoplasts which were, therefore, routinely used in these studies. For cytochrome oxidase assay, protoplasts suspended in 1.2 M sorbitol were diluted 1:5 in 0.005 M K phosphate pH 7.4 and kept at room temperature for 5 min. The suspensions were further diluted with 0.08 M K phosphate pH 7.4, and assayed with the Gilford recording spectrophotometer at 37° C. Enzyme preparations were diluted sufficiently to obtain linear initial rates of reduced cytochrome c oxidation. All assays were performed at two enzyme concentrations, under conditions where the reaction rate was linearly related to enzyme concentration. Results are expressed as m μ moles cytochrome c oxidized/min/mg protein.

Materials. Chloramphenicol was a gift of Dr. Loren M. Long, Parke Davis Company.

RESULTS

Properties of the O₂-adaptation system. When *Saccharomyces cerevisiae* is grown under strictly anaerobic conditions, it loses its capacity for aerobic metabolism.^{35,36} As shown in Figure 1, succinate cytochrome c reductase and cytochrome oxidase activities are very low in anaerobically grown cells. After aeration in a minimal medium containing no assimilable nitrogen source, the activity of these enzymes increases in almost linear fashion with time. High glucose concentrations are known to repress mitochondrial development.^{35,36,39} In order to minimize this effect, 0.1 per cent glucose 2 per cent ethanol was used as substrate, and glucose supplements were added as described under Methods.

The activity of the respiratory enzymes continued to rise 9 hr after the onset of aeration (Figure 1). The maximum respiratory activity achieved, however, was only 20-40 per cent of that shown by cells grown aerobically to the stationary phase.

Under O₂-adaptation conditions there was essentially no cell division. The number of cells remained constant, although the RNA, DNA and protein contents of the cell rose by 25-50 per cent (Figure 2).

DNA synthesis during respiratory adaptation. The incorporation of [³H]adenine was selected as a measurement of DNA synthesis because of the very limited entry of thymine into the yeast cell. Since incorporation of [³H]adenine into RNA exceeds its incorporation into DNA by several hundred fold under the conditions of the experiment, each sample was subjected to RNase and alkali digestion before estimation of its tritium content.

The buoyant density of yeast *Saccharomyces cerevisiae* mitochondrial DNA has been shown to be lighter than that of nuclear DNA by about 0.014 g/cm³.^{11,31-34} The difference is great enough to allow effective separation of mitochondrial and nuclear DNA by preparative CsCl centrifugation. The procedure for isolation of mitochondria was adjusted so that almost equal amounts of mitochondrial and contaminating nuclear DNA would be present. In this way, the specific activities of both mitochondrial and nuclear DNA's could be estimated by analysis of a single preparative CsCl gradient.

Independent calculation of specific activities of nuclear and mitochondrial DNA could be made from 2 to 5 samples of the CsCl gradient. The values usually agreed within 5-10 per cent. The characteristic pattern of incorporation of [³H]adenine during 1 hr of O₂-adaptation is shown

Figure 1. Development of respiratory activity after exposure of anaerobically grown yeast to oxygen. Respiratory O₂ adaptation was carried out as described in Materials and Methods. The period of pre-incubation in a nitrogen atmosphere was omitted. The standard O₂ adaptation medium containing 0.1% glucose, 2% ethanol was supplemented with 100 mg glucose powder/100 ml adaptation media every 30 min. Samples were collected every 90 min. Enzyme assays were done on washed protoplasts prepared with snail digestive juice as described in Materials and Methods. —■—■—, succinate cytochrome c reductase; —▲—▲—, cytochrome oxidase; —●—●—, O₂ consumption.

in Figure 3. Marker ³²P nuclear DNA is seen as a sharp, narrow peak. There was essentially no contamination of the mitochondrial DNA peak with nuclear DNA in most experiments. Analysis by CsCl isopycnic centrifugation in the Beckman Model E ultracentrifuge (Figure 4) also demonstrated that nuclear and mitochondrial DNA peaks were essentially free of cross-contamination.

[³H]adenine was predominantly incorporated into the mitochondrial DNA peak. The specific activities of mitochondrial DNA were usually 10- to 25-fold greater than those of nuclear DNA. A possible error in the calculation of the specific activity of nuclear DNA is suggested by the presence of a small γ mitochondrial satellite band with a density of 1.701, somewhat heavier than the density of nuclear DNA ($\rho = 1.698$).^{32,33} The gradients occasionally showed a slightly higher specific activity on the heavier side of the nuclear DNA peak. Gamma satellite DNA was

Figure 2. Changes in cell count, protein, RNA and DNA content during respiratory oxygen adaptation in yeast. The conditions of the experiment were identical to those described in Figure 1, except that samples were collected hourly. Protoplast formation was complete in all samples. Washed protoplasts were precipitated in cold 5% trichloroacetic acid and analyzed for RNA, DNA and protein as described in Materials and Methods. —□—□—, cell count; --○--○--, protein content; —△—△—, DNA content; —x—x—, RNA count.

not detectable at the peak of the nuclear DNA band by CsCl analysis in the ultracentrifuge. The possibility that nuclear DNA may be slightly contaminated with γ satellite DNA must, however, be considered in the evaluation of the results.

The maximal incorporation of [³H]adenine into both mitochondrial and nuclear DNA occurred during the first 15 min of respiratory adaptation (Figure 5). In the experiment illustrated, incorporation fell rapidly thereafter to a level that was about 5 per cent of that seen in the first period. The specific activity of mitochondrial DNA was initially 22-fold higher than that of nuclear DNA; it was 8-9 times greater at later times.

An attempt was made to determine whether the initial rapid rate of DNA synthesis was a requirement for respiratory adaptation or was due to other factors. For example, it could have been secondary to a nutrient step-down effect. The yeast was grown anaerobically in a nutritionally-rich medium and reached a stationary phase of growth because of depletion of glucose. The cells transferred to the nutritionally-poor adaptation medium could have retained sufficient nutrient from the rich growth medium to support the early rapid DNA synthesis on the glucose in the adaptation medium. To test this hypothesis, yeast was collected and suspended in adaptation medium under the most stringent anaerobic conditions. All manipulations were done at 0° in a plastic isotope hood continually flushed with nitrogen, and solutions and centrifuges were flushed with nitrogen. The yeast was suspended in adaptation medium, which was continuously bubbled with nitrogen at 28° for 2 hr prior to aeration.

Figure 3. CsCl density-gradient centrifugation of DNA sample isolated from yeast during respiratory oxygen adaptation. Respiratory O_2 adaptation was carried out in the standard manner. After a 2-hr period of shaking in a nitrogen atmosphere, 1 mc of $[^3H]$ adenine was added to the flask and aeration begun. The yeast was collected in 1 hr and processed as described in Materials and Methods. The DNA was extracted and purified from protoplasts as described in Materials and Methods. ^{32}P -labeled nuclear DNA (4×10^3 cts/min, specific activity 4×10^4 cts/min/ μg) previously purified by CsCl centrifugation was added to the sample before separation and analysis by CsCl density-gradient centrifugation. —x—x—, optical density at 260 m μ ; —○—○—, $[^3H]$ adenine incorporation into DNA cts/min; --Δ--Δ--, ^{32}P -labeled nuclear DNA marker cts/min.

Figure 4. Analysis by isopycnic CsCl density gradient centrifugation in the analytical ultracentrifuge samples of DNA obtained from CsCl density gradients in the Spinco L2-65 ultracentrifuge. Respiratory O_2 adaptation was carried out as described in Figure 3, and the DNA separated by preparative CsCl centrifugation. DNA from tubes at the peaks of the mitochondrial and nuclear DNA bands were analyzed in the Spinco model E centrifuge by CsCl density-gradient centrifugation as described by Vinograd & Hearst.⁵⁶ Densitometric tracings of the ultraviolet light photographs were taken with a Joyce-Loebl densitometer. SPO-1 phage DNA density 1.740 was used as marker.

Figure 5. Time course of incorporation of [3H]adenine into mitochondrial and nuclear DNA during respiratory oxygen adaptation. Respiratory O_2 adaptation was carried out according to the standard procedure without a period of preincubation in a nitrogen atmosphere. Thirty-min pulses of [3H]adenine were given, and yeast and DNA processed as described in Materials and Methods. Each point in the figure is plotted as the mid-point of the time period of sampling. Each determination of specific activity represents the average from 2 to 5 samples of the gradient. — Δ — Δ —, specific activity nuclear DNA; — $\bullet$ — $\bullet$ —, specific activity mitochondrial DNA; — $\square$ — $\square$ —, succinate cytochrome c reductase specific activity.

Despite precautions taken to maintain anaerobiosis, there was evidence of some exposure to oxygen during the manipulations. Cytochrome oxidase, and to a greater extent, succinate cytochrome c reductase, rose significantly above control anaerobic values during the first 60 min of nitrogen preincubation (Figure 6), but fell to control levels after the second hour of nitrogen flushing.

Maximal incorporation into DNA again occurred in the first 15 min and fell off sharply thereafter (Figure 6). However, the specific activity of mitochondrial DNA in the first period was only 6 times greater than that of nuclear DNA, as compared to a 20-fold greater incorporation when the suspensions were aerated directly. Aeration after the period of nitrogen preincubation resulted in a significant stimulation of synthesis of mitochondrial DNA and to a lesser extent of nuclear DNA. The level of incorporation usually was 10-80 per cent of that found in the first anaerobic adaptation period (Figure 6, Tables 1, 4). The specific activity of mitochondrial DNA was 15-20 times greater than that of nuclear DNA. The increased synthesis, especially of mitochondrial DNA, persisted throughout the 120 min aeration of the experiment. Although it has not been proven, the suspicion remains that the initial rapid DNA synthesis in part may be related to a nutrient step-down effect.

Figure 6. Time-course of $[^3\text{H}]$ adenine incorporation into DNA during respiratory oxygen adaptation following a 2-hr period of preincubation in a nitrogen atmosphere. Anaerobically grown yeast was collected, washed and suspended in the standard O_2 adaptation mixture. The yeast was shaken for 2 hr while the flasks were continually bubbled with N_2 before exposure to air. 30-min pulses of $[^3\text{H}]$ adenine (0.8 mc) were given. The adenine solutions were bubbled with N_2 before injection through tubing into the flasks during the anaerobic period. The points in the figure are plotted as the mid-point of the time period. $\text{---}\Delta\text{---}\Delta\text{---}$, specific activity of nuclear DNA; $\text{---}\times\text{---}\times\text{---}$, specific activity of mitochondrial DNA; $\text{---}\circ\text{---}\circ\text{---}$, cytochrome oxidase specific activity.

The stimulation, by aeration, of a synchronized replication of mitochondrial DNA may be another possible explanation for the early DNA synthesis in respiratory adaptation. Another synchronous replication might then be detected at some later time during long periods of adaptation. No evidence for a second burst of mitochondrial DNA synthesis was observed during 9 hr of aeration (Figure 7). Cytochrome oxidase activity rose steadily during the 9-hr adaptation. The specific activity of mitochondrial DNA was highest in the first 90-min period, and fell throughout the remainder of the experiment. Specific activities of nuclear DNA fluctuated somewhat, but had essentially reached a plateau after the first period. The preferential synthesis of mitochondrial DNA in the early period was no longer evident after 4-5 hr of aeration.

Effect of glucose concentration in the adaptation medium. Mitochondrial development in yeast is known to be sensitive to the glucose concentration in the growth medium.^{35,36} When high glucose concentrations are present during the exponential growth phase, mitochondrial function and formation are arrested; if galactose, or nonfermentable substrates such as lactate or ethanol are used instead of glucose as carbon source, mitochondrial development is depressed. The effects of high and low glucose concentrations in the adaptation medium on the development of respiratory activity and on DNA synthesis were, therefore, examined.

Figure 7. [^3H]adenine incorporation of mitochondrial and nuclear DNA during a 9-hr respiratory oxygen adaptation. Respiratory O_2 adaptation was done in the standard way without preliminary nitrogen incubation. 90-min pulses of 0.75 mc [^3H]adenine were given. Samples were collected and DNA was extracted and analyzed as described in Materials and Methods. — Δ — Δ —, specific activity of nuclear DNA; -- $\circ$ -- $\circ$ --, specific activity of mitochondrial DNA; —x—x—, cytochrome oxidase specific activity.

Table 1

OXYGEN ADAPTATION—EFFECT OF GLUCOSE CONCENTRATION ON DNA SYNTHESIS AND CYTOCHROME OXIDASE ACTIVITY

Experimental system	Cytochrome oxidase (spec. act.)*	Spec. act. DNA (cts/min/O.D. 260 m μ)		Ratio spec. act. DNA mitochondrial/nuclear
		Mitochondrial	Nuclear	
Anerobic cells	0.4			
Direct adaptation				
0.1% glucose	19.6	36,000	5,130	7.1
5% glucose	2.9	27,000	6,300	4.4
N_2 pre-incubation				
0.1% glucose	24.9	9,300	820	11.3
5% glucose	11.3	1,240	970	1.3

O_2 adaptation of anaerobically grown cells was carried out for 1 hr with or without a 2-hr period of N_2 pre-incubation as described in Materials and Methods. The incubation medium contained succinate phosphate buffer, pH 4.8, and either 0.1% glucose-2% ethanol, or 5% glucose-2% ethanol. DNA was isolated and purified as described in Materials and Methods. Each determination of specific activity of DNA represents the average of 2 to 4 values from individual tubes of the CsCl density gradient.

* Specific activity, m μ moles cytochrome c oxidized/min/mg protoplast protein.

The results of a characteristic experiment are shown in Table 1. Cytochrome oxidase activity rose to substantially higher values when 0.1 per cent glucose, 2 per cent ethanol rather than 5 per cent glucose, 2 per cent ethanol served as substrate. The effect was noted whether or not nitrogen preincubation was used. The synthesis of mitochondrial DNA was inhibited by high glucose concentrations. The inhibition was discernible after direct O₂-adaptation, but was very much more striking following nitrogen preincubation. Preferential synthesis of mitochondrial DNA was almost eliminated in 5 per cent glucose after nitrogen preincubation; in contrast, a ratio of mitochondrial DNA/nuclear DNA specific activity of 11.3 was observed at low glucose concentration.

Estimation of amount of DNA synthesized. It is extremely difficult to determine accurately the amount of mitochondrial DNA present in yeast cells. Preferential synthesis of mitochondrial DNA under conditions of aerobic adaptation is indicated in the above data, but no estimation of the magnitude of such synthesis can be made from them. In order to estimate this quantity, cells were cultured anaerobically with ³²P to the mid-exponential phase of growth. After adding a chase of cold phosphate anaerobically from a side arm of the growth flask, the growth was continued to stationary phase. The cells were collected and washed in phosphate buffer, and respiratory adaptation was then carried out in 0.1 M phosphate pH 6.8. DNA synthesis was again monitored using [³H]adenine. A comparison of the specific activity of the ³²P-labeled DNA before and after adaptation should provide a measure of the DNA synthesized relative to the DNA content of the cell. During respiratory adaptation in 3 separate experiments, there was a 10 to 30 per cent dilution of the specific activity of mitochondrial DNA, but no significant dilution of the specific activity of nuclear DNA. The results of a typical experiment are shown in Table 2. The usual brisk incorporation of [³H]adenine into mitochondrial DNA was also apparent (Table 2, Figure 8). It may be concluded that a small but appreciable fraction of mitochondrial DNA is replicated during O₂ adaptation, but that little replication of nuclear DNA occurs. The mitochondrial DNA synthesized during O₂ adaptation had the same buoyant density as the mitochondrial DNA present in anaerobic cells. This is shown by the fact that the peak of DNA labeled with [³H]adenine during respiratory adaptation coincides with the peak labeled with ³²P during anaerobic growth (Figure 8).

Table 2
DILUTION OF SPECIFIC ACTIVITY OF DNA PRE-LABELED WITH
³²P DURING OXYGEN ADAPTATION

Experimental system	Spec. act. DNA (cts/min/ μ g DNA)				Cytochrome oxidase (m μ moles/min/ mg protein)
	^{32}P		^3H Adenine		
	Mitochondrial	Nuclear	Mitochondrial	Nuclear	
Control	29.9	30.5	0	0	1.3
Adapted	22.3	30.0	156	9.8	15.8

Yeast was grown anaerobically in an enriched medium containing 1 mc³²P/l. and no added unlabeled phosphate to the early exponential phase of growth. 15 g of K₂HPO₄ was then added anaerobically from a side-arm, and the culture was grown to the stationary phase (2 to 4 generations). The yeast was collected and washed 3 times in 0.1 M-phosphate buffer, pH 6.8. O₂ adaptation was carried out for 1 hr with N₂ pre-incubation in 0.1 M-phosphate buffer, pH 6.8, 0.1% glucose, 2% ethanol. DNA was determined by the method of Burton.⁵⁷

Figure 8. CsCl density gradient of DNA labeled with ^{32}P during anaerobic growth and with $[^3\text{H}]$ adenine during respiratory oxygen adaptation. Yeast was grown anaerobically in an enriched medium containing 1 mc $^{32}\text{P}/\text{l}$ and no added unlabeled inorganic phosphate. During the early exponential phase of growth 15.0 g of K_2HPO_4 was added anaerobically from a side-arm and the culture was grown to the stationary phase. The yeast was collected and washed 3 times in 0.1 M-phosphate buffer, pH 6.8. Adaptation was performed with N_2 preincubation in 0.1 M-phosphate buffer, pH 6.8, 0.1% glucose, 2% ethanol. One mc $[^3\text{H}]$ adenine was added after completion of the period of gassing with nitrogen, and the yeast was collected 1 hr later. DNA was extracted and analyzed by CsCl density-gradient centrifugation. —x—x—, optical density 260 $\text{m}\mu$; --Δ--Δ--, ^{32}P cts/min; —O—O—, ^3H cts/min.

Oxygen adaptation in respiratory deficient mutants. Although respiratory deficient yeast, "petites," are incapable of oxidative metabolism it was of interest to determine whether they were still capable of a limited response to oxygen. The haploid strain D243-2Bg ρ^- a neutral cytoplasmic and chromosomal mutant was chosen because Mounolou and colleagues had shown that its mitochondrial DNA was lighter even than normal yeast mitochondrial DNA.^{31,39} Therefore, it could be easily separated from nuclear DNA. Anaerobically grown D243-2Bg cells were aerated, with and without nitrogen preincubation, in the presence of low (0.25-0.5 per cent) and high (5 per cent) glucose concentrations in the standard adaptation medium. The results obtained were essentially similar to those with yeast foam ρ^+ . There was an early incorporation of $[^3\text{H}]$ adenine into the mitochondrial and nuclear DNA of D243-2Bg (Table 3). Preferential incorporation of radioactivity into mitochondrial DNA was observed. High glucose concentration in the adaptation medium again inhibited mitochondrial DNA synthesis after the period of nitrogen preincubation; the effect of high glucose was not apparent when direct adaptation was employed. The same pattern of response was observed in 3 experiments. It therefore appears that respiratory-deficient yeast still responds to O_2 by stimulation of mitochondrial DNA synthesis

Table 3

DNA SYNTHESIS IN A CYTOPLASMIC PETITE DURING OXYGEN ADAPTATION IN HIGH AND LOW GLUCOSE MEDIUM

Experimental system	Specific activity DNA (cts/min/O.D. 260 m μ)	
	Mitochondrial	Nuclear
Direct adaptation		
N ₂ control 0.25% glucose	2000	500
Aeration, 0.25% glucose	71,000	15,500
Aeration, 5.0% glucose	73,000	19,000
N ₂ pre-incubation		
N ₂ control 0.5% glucose	9,900	2,900
Aeration, 0.5% glucose	99,800	19,300
Aeration, 5% glucose	11,900	11,500

Strain D243-2Bg was grown anaerobically, and O₂ adaptation was carried out for 1 hr in the standard way except that the initial glucose concentration of the adaptation medium was 0.25% for direct adaptation and 0.5% when N₂ pre-incubation was used. In the latter case, 250 mg glucose/100 ml of medium was added after the 2-hr period of N₂ pre-incubation. N₂ controls are samples to which [³H]adenine and glucose were added anaerobically and gassing with nitrogen was continued during the 1-hr experimental period. It was confirmed that no cytochrome oxidase activity had developed at the end of the adaptation.

which is most readily demonstrated when low glucose concentrations are employed in the adaptation medium. That Mounolou³⁹ was not able to demonstrate the preferential synthesis of mitochondrial DNA during aerobic adaptation in petites may be due to the fact that his experiments were performed in the presence of high glucose concentrations.

The effects of inhibition of macromolecular synthesis.

(i) Cycloheximide. Cycloheximide is thought to affect protein synthesis occurring on cytoplasmic ribosomes and to have no effect on mitochondrial protein synthesis.⁵⁰ Respiratory adaptation, whether carried out with or without a 2-hr nitrogen preincubation, was inhibited markedly by cycloheximide in the concentration range of 1 to 25 μ g/ml (Table 4). Similar results were obtained by Fukuhara.^{40,41} Yeast growth is inhibited at still lower concentration (0.1 to 0.25 μ g/ml). The development of cytochrome oxidase activity after aeration of anaerobically grown yeast was inhibited by 20 to 60 per cent at 1 μ g/ml cycloheximide, and by over 95 per cent at 25 μ g/ml. There was no inhibition of DNA synthesis even at the highest concentrations of cycloheximide employed in any of the 6 experiments done. A typical result is shown in Table 4. There were indications in several experiments that [³H]adenine incorporation into mitochondrial DNA might rather have been stimulated by high concentrations of cycloheximide. Specific activities of mitochondrial DNA were 6 to 20 times greater than nuclear DNA both in the presence and in the absence of cycloheximide. It is clear that inhibition of cytoplasmic protein synthesis by cycloheximide results in complete abolition of O₂ induction of respiratory enzyme activity, although there is no discernible inhibition of mitochondrial DNA synthesis.

(ii) Chloramphenicol. Chloramphenicol is thought to be a relatively specific inhibitor of mitochondrial protein synthesis in yeast.^{51,52} If mitochondrial DNA polymerase is induced by O₂ and is made by the mitochondrial apparatus responsible for protein synthesis, chloram-

Table 4

**EFFECT OF CYCLOHEXIMIDE ON DNA SYNTHESIS AND RESPIRATORY
ENZYME FORMATION DURING OXYGEN ADAPTATION**

Experimental system		Specific activity of DNA (cts/min/O.D. 260 m μ)		Cytochrome oxidase (m μ moles/min/mg protein)	Control (%)
		Mitochondrial	Nuclear		
Anerobic cells				2.3	
Direct adaptation	0	53,000	8,300	14.2	-
	1	47,850	8,800	12.4	85
	25	61,070	8,840	2.9	5
N ₂ pre-incubation	0	7,350	840	15.2	-
	1	9,500	770	6.6	33
	25	14,400	340	2.8	4

O₂ adaptation was carried out for 1-hr periods in the standard manner.

phenicol might be expected to inhibit mitochondrial DNA synthesis. Respiratory adaptation was inhibited 10 to 40 per cent by chloramphenicol in concentrations of 0.5 mg/ml, and 65 to 80 per cent by concentrations of 4 mg/ml (Table 5). Chloramphenicol did not inhibit the large incorporation of [³H]adenine into DNA present in the first 30 min aeration immediately after harvesting anaerobically-grown yeast. The results were similar to those seen with cycloheximide; a slight stimulation of DNA synthesis was observed in several experiments. On the other hand, DNA synthesis was affected by chloramphenicol 1 to 2 hr after the beginning of aeration, or after 2 hr of nitrogen preincubation. There was a parallel decline in nuclear and mitochondrial DNA specific activity. Under the conditions of these experiments, no indication for a preferential effect on mitochondrial DNA synthesis was obtained in any of the 3 separate experiments performed.

Table 5

**EFFECT OF CHLORAMPHENICOL ON DNA SYNTHESIS AND RESPIRATORY
ENZYME FORMATION DURING OXYGEN ADAPTATION**

Experimental system	Chloramphenicol (mg/ml)	Mito- chondrial	Specific activity DNA (cts/min/O.D. 260 m μ)		Control (%)	Cytochrome oxidase	
			Control %	Nuclear		Spec. act. *	Control %
Anerobic cells						1.1	
Direct adaptation	0	60,000		16,500		9.1	-
	0.5	49,300	82	11,900	72	8.0	87
	4.0	67,000	111	18,100	109	4.0	36
N ₂ pre-incubation	0	9,100		1,000		15.1	-
	0.5	6,900	76	310	31	9.3	59
	4.0	3,200	35	300	33	3.7	19

One-hour periods of O₂ adaptation were carried out in the standard manner.

* Specific activity of cytochrome oxidase in m μ moles/min/mg protoplast protein.

DISCUSSION

Yeast cells grown under strictly anaerobic conditions completely lose their oxidative capacity but retain their ability to form functioning mitochondria upon reintroduction of oxygen.^{35,36} Double membrane-limited vesicles containing osmiophilic fibrils of DNA have been demonstrated in anaerobic yeast, by suitable electron microscopic techniques.^{53,54} DNA with a buoyant density characteristic of mitochondrial DNA may be isolated from anaerobically-grown cells.^{4,53-55} As was shown in Figure 8, the material labeled with ³²P anaerobically had the same buoyant density as the ³H-labeled mitochondrial DNA synthesized during adaptation.

Upon exposing cells grown in an anaerobic environment to oxygen in a medium low in glucose and lacking a nitrogen source to support growth, there is brisk synthesis of mitochondrial DNA. This synthesis is maximal within 15 to 30 min after aeration and falls off rapidly thereafter. Mitochondrial DNA synthesis becomes negligible after 60 to 120 min of oxygenation. Respiratory activity by the mitochondria, however, continues to develop for at least 6 to 9 hr after aeration. Only a very small fraction of the cytochrome oxidase activity which ultimately develops has appeared during the period of maximal mitochondrial DNA synthesis. It is necessary to point out that the above estimates of mitochondrial DNA synthesis are based solely upon the level of incorporation of adenine into DNA, without regard to changes of the pool size and specific radioactivity of adenine precursors. Such changes could complicate the interpretation of our data. However, our conclusions are based largely upon the preferential incorporation into mitochondrial DNA over that into nuclear DNA, which serves as an internal control unless the adenine precursor pools for these two syntheses are vastly different in size and metabolic behavior. Furthermore, our data do not permit us to differentiate between the complete de novo replication of whole mitochondrial DNA molecules or the repair or completion of incomplete molecules. These questions are considered further in the succeeding discussion.

The amount of mitochondrial DNA synthesized during O₂ adaptation, as estimated by the isotopic dilution technique, appears to be small compared to the amount already present in the cell. The dilution of the labeled mitochondrial DNA during respiratory adaptation suggests that there has been a 10 to 30 per cent increase in DNA content. These experiments provide confirmation of the fact that adenine incorporation into mitochondrial DNA measures a small but real net synthesis of DNA. If it is assumed that a unit of the mitochondrion contains a fixed amount of mitochondrial DNA, it can be concluded that the O₂ adaptation process represents primarily the functional and structural development of incompletely formed mitochondria already present in the anaerobic cell. The relatively small DNA synthesis may also represent an aspect of true replication of mitochondrial units with the ultimate formation of new ones. It is of interest that the number of mitochondria observed by electron microscopy appears to remain constant during the first 6 hr of O₂ adaptation, but appears to increase by division into a larger number of smaller mitochondria thereafter.^{53,58} This occurs in the absence of a significant increment in mitochondrial DNA synthesis at that time.

The data presented in this paper support the premise that the early stimulation of mitochondrial DNA synthesis by the introduction of oxygen to anaerobically-grown cells is a necessary but not sufficient requirement for the development of respiratory activity. This is based upon the following considerations which are discussed in detail elsewhere in this section. (a) Mitochondrial DNA synthesis precedes the maximum development of respiratory capacity. (b) It occurs after

nitrogen preincubation in the adaptation medium and is therefore dependent on the presence of oxygen and independent of other chemical changes in the incubation medium. (c) High glucose concentrations inhibit the development of respiratory capacity, and also inhibit mitochondrial DNA synthesis. This hypothesis is further supported by Mounolou & Slonimski's observations^{39,55} that nalidixic acid, an inhibitor of DNA inhibits respiratory adaptation. Conversely, we find that inhibitors of respiratory adaptation, e.g., cycloheximide, do not necessarily inhibit mitochondrial DNA synthesis.

Mounolou & Slonimski have also described a maximal synthesis of mitochondrial DNA in the first 30 min of O₂ adaptation.^{39,55} The ratios of specific activity of mitochondrial DNA/nuclear DNA which they observed (4 to 6 times) were considerably lower than those obtained in our experiments. This may be explained by two factors: (1) they employed a less effective method for the separation of mitochondrial and nuclear DNA; and (2) O₂ adaptation was carried out in the presence of a high glucose concentration. Our observations indicate that high concentrations of glucose inhibit both the synthesis of mitochondrial DNA and the development of respiratory enzymes during adaptation. This is particularly true for the cytoplasmic petite yeast, which also responds, under appropriate conditions, to aerobic "adaptation" with a preferential synthesis of mitochondrial DNA. Mounolou and Slonimski postulate that the early DNA synthesis may be essential for the subsequent development of mitochondrial function.^{39,55} They suggest that the phenomenon may be analogous to the maturation of phage DNA during T₄ infection of Escherichia coli. It is also possible, however, that the early DNA synthesis, especially in direct adaptation experiments, may be secondary to a nutrient step-down effect. The synthesis of mitochondrial DNA stimulated by exposure to O₂ may be maximal early because the nutrients from the anaerobic culture media are still available to the cell at this time. Experiments to test this possibility were not completely effective. Preincubation in a nitrogenated adaptation medium for 2 hr prior to aeration did not prevent the early DNA incorporation. It appears, however, that completely anaerobic manipulation during transfer to the adaptation medium cannot be attained. A small rise in succinate cytochrome c reductase activity indicated that minimal exposure to oxygen did occur during the collection and washing procedure. This could have been sufficient to stimulate DNA synthesis. Aeration following nitrogen preincubation resulted in a smaller stimulation in mitochondrial DNA synthesis. In some experiments this latter stimulation was considerably greater. This strongly suggests that the rate of mitochondrial DNA synthesis is more directly responsive to the O₂ stimulus and that the nutrient step-down was not the only, and perhaps not the primary factor in the early DNA synthesis.

It is well established that the glucose concentration of the medium may influence the degree of mitochondrial development.^{35,36} High glucose concentration represses mitochondrial development. The relative concentration of mitochondrial DNA to nuclear DNA in cells harvested in the exponential phase of growth in a glucose medium is also decreased.³² We now find that a high glucose concentration in the O₂ adaptation medium markedly depresses the O₂ stimulated synthesis of mitochondrial DNA. The effect is more apparent after a 2-hr period of preincubation in a nitrogen atmosphere. The smaller inhibition by glucose on the early mitochondrial DNA synthesis may be due to the time required for the intracellular accumulation of metabolites of glucose which may be the true repressor, or to the fact that some of this early mitochondrial DNA synthesis is not specifically associated with the replication of mitochondrial units. Physiologically, mitochondrial DNA synthesis, as well as synthesis of respiratory enzymes, is therefore,

inhibited by high glucose levels and by the anaerobic state, and is stimulated by the presence of oxygen. We have evidence that phospholipid synthesis during respiratory adaptation also responds similarly to high and low levels of glucose in the medium. This information will be presented in a separate publication.

The synthesis of mitochondrial DNA is not necessarily closely coupled to the synthesis of respiratory enzymes. Inhibitors of protein synthesis such as cycloheximide and chloramphenicol, which markedly diminish O_2 -induced respiratory activity, may have no effect on mitochondrial DNA synthesis. Replication of mitochondrial DNA evidently does not depend on the functional development of mitochondria, but appears to be stimulated by the level of oxygen or glucose independently of the level of respiratory function. The fact that potent inhibitors of cytoplasmic and mitochondrial protein synthesis fail to decrease DNA synthesis suggests that induction of new DNA polymerase activity may not be involved in the process. This conclusion is very tenuous since these inhibitors are known to lead to an increase in certain enzyme levels under special conditions. A complex interaction between synthetic and degradation systems may contribute to the level of enzyme activity and may be affected in varying ways by inhibitors. Although cycloheximide does not inhibit amino acid incorporation into isolated mitochondria,⁵⁰ it is an extremely effective inhibitor of mitochondrial development in response to oxygen. This emphasizes the principle that essential mitochondrial proteins are synthesized in extra-mitochondrial ribosomal systems.

It is also of note that O_2 induces mitochondrial DNA synthesis in respiratory-deficient petites and high glucose inhibits this synthesis. A strain that had mitochondrial DNA of abnormally light buoyant density was selected in order to facilitate separation from nuclear DNA. This strain is a neutral cytoplasmic petite derived from a chromosomal petite. It is apparent that the two mutations do not affect one aspect of the integrated response to oxygen which results in the formation of new mitochondria. As in the normal cell, mitochondrial DNA synthesis is stimulated by oxygen and repressed by high glucose levels, even though the development of respiratory activity cannot be detected. This further underscores the independence of the response of mitochondrial DNA synthesis to the introduction of O_2 from the functional respiratory state of the mitochondria.

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PARTIAL PURIFICATION OF MITOCHONDRIAL AND SUPERNATANT DNA
POLYMERASE FROM Saccharomyces cerevisiae*

By

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It is now firmly established that mitochondria contain species of DNA which differ from that present in the nuclei.¹⁻³ Mitochondrial DNA appears to be synthesized in a semiconservative manner⁴ independent of synthesis of nuclear DNA.⁵ DNA polymerase activity has been reported in mitochondria isolated from yeast⁶ and from rat liver.^{7,8} Kalf and Ch'ih⁹ and Meyer and Simpson¹⁰ recently described the partial purification of mitochondrial DNA polymerase from rat liver. Their studies suggest that the enzymes differ from nuclear DNA polymerase in physical and enzymatic properties. This paper describes the solubilization and partial purification of DNA polymerase from mitochondrial and supernatant fractions of Saccharomyces cerevisiae. The cell supernatant fraction contains most of the DNA polymerase derived from the nucleus. The enzymatic properties of the enzymes are compared. The data suggest that the mitochondrial and supernatant enzymes may differ in molecular size.

METHODS

Strain D243-R1 (a,ad, ρ^+), a haploid "grande," kindly provided by Dr. P. P. Slonimski, Laboratoire de Génétique Moléculaire, Gif-sur-Yvette, France, was used in the study. Cultures were grown aerobically at 28° in a New Brunswick rotary shaker in a medium consisting of 1 per cent yeast extract, 1 per cent bactopectone, and 2 per cent glucose. Cultures used in the isolation of supernatant DNA polymerase were collected in the mid-exponential phase of growth. Mitochondrial DNA polymerase was purified from yeast harvested in the stationary growth phase. Yeast was washed 3 times with cold distilled water prior to disruption.

Preparation of Supernatant Enzyme

One hundred g (wet wt) of cells were suspended in 300 ml of 0.02 M glycyl glycine buffer (pH 7.0) containing 0.65 M sorbitol. All procedures were carried out at 4°. The cells were disrupted in a Ribi-Cell fractionator (Model RF-1 Sorvall) at 20,000 lb/square in at 2-6°. The suspension was centrifuged at 12,000 x g for 15 min and the slightly turbid supernatant was collected (Fraction I).

One-tenth volume of 2 per cent protamine sulfate, Nutritional Biochemical Corp., Cleveland, Ohio, was slowly added to the supernatant. After standing for 10 min the precipitate was collected by centrifugation at 12,000 x g for 10 min. The precipitate was extracted for 10 min with 150 ml 0.1 M K phosphate (pH 7.4) 0.01 M 2-mercaptoethanol, 0.001 M EDTA. The suspension was centrifuged at 46,000 x g for 20 min and the supernatant was collected (Fraction II). To each

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100 ml of Fraction II, 31.4 g of $(\text{NH}_4)_2\text{SO}_4$ was added and the precipitate was collected by centrifugation at 12,000 x g for 10 min. The precipitate was dissolved in 65 ml of 0.01 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA (Fraction III). Fraction III was diluted with 2 vol of 0.01 M K phosphate (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA, and absorbed on C_γ alumina gel (final concentration 5 mg/ml). After stirring for 10 min, the gel was separated by centrifugation and eluted twice with 100 ml of 0.3 M K phosphate (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA. The protein was precipitated with 43 g/100 ml of $(\text{NH}_4)_2\text{SO}_4$, the precipitate separated by centrifugation at 12,000 x g for 10 min and dissolved in 10 ml 0.05 M K phosphate (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA (Fraction IV).

After dialysis against 0.02 M phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA the dialysate was applied to a column (3.18 cm) of DEAE cellulose equilibrated with the same buffer. The column was washed with 200 ml of 0.02 M K phosphate buffer (pH 7.4), 300 ml 0.05 M 2-mercaptoethanol and 200 ml 0.1 M K phosphate buffer (pH 7.4) containing 2-mercaptoethanol and EDTA. The enzyme was eluted with the last buffer (Fraction V).

Fraction V was applied directly to a column (1.5 x 3.0 cm) of hydroxylapatite (hypatite), Clarkson Chemical Co., Williamsport, Pa., which had been equilibrated with 0.02 M K phosphate, and the enzyme was eluted at 0.3 ml/min with 0.2 M K phosphate (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA (Fraction VI). An equal volume of glycerol was added and the enzyme was stored at -20° . In the presence of glycerol, enzyme activity was stable for at least a month.

Purification of Mitochondrial DNA Polymerase

Formation of protoplasts. Protoplasts were prepared by modification of the methods of Duell, et al.¹¹ and Kovac, et al.¹² Two hundred g of cells (wet wt) harvested in the stationary phase of growth were incubated in 600 ml of 0.5 M mercaptoethylamine (Calbiochem Corp., Los Angeles, Calif.), Grade B, 0.1 M Tris (pH 9.3) for 30 min at 30° . The cells were then washed twice with 400 ml 0.9 M sorbitol, 0.01 M citrate phosphate buffer (pH 5.8), 0.001 M EDTA, and suspended in 400 ml of the same buffer. Twenty-five ml of glucalase, Endo Laboratories, Garden City, New York, was added and the suspension was incubated with shaking at 30° for 90 to 120 min. When protoplast formation had reached over 80 per cent completion as indicated by lysis of the cell in H_2O , the suspension was centrifuged at 10,000 x g for 10 min. The protoplasts were washed twice with 300 ml of 0.9 M sorbitol, 0.002 M K phosphate (pH 7.0), 0.001 M EDTA.

Isolation of mitochondria. Protoplasts were suspended in 600 ml of 0.8 M sorbitol, 0.02 M glycyl glycine buffer (pH 7.0), 0.001 M EDTA, disrupted in a Waring blender for 15 sec at top speed, and centrifuged at 1,200 x g for 5 min. The supernatant was centrifuged again at 1,200 x g for 5 min. The supernatant was centrifuged at 10,000 x g for 10 min and the crude mitochondrial pellet was suspended in the same volume of buffer. The suspension was centrifuged twice more at 1,200 x g for 5 min to remove unbroken protoplasts and cells with fragments. The mitochondria were sedimented again from the supernatant at 12,000 x g for 10 min, and washed twice more in the same way. Further purification of mitochondria was carried out when indicated by discontinuous sorbitol density gradient centrifugation according to the method of Jayaraman, et al.¹³

Solubilization and purification of mitochondrial DNA polymerase. Mitochondria (Fraction I) (218 mg protein) were suspended in 10 ml of 0.02 M glycyl glycine buffer (pH 7.0) containing 1 M

KCl, 0.01 M 2-mercaptoethanol and 0.001 M EDTA, and stirred for 20 min. The suspension was centrifuged at 50,000 x g for 15 min and the pellet was extracted twice more with 10 ml portions of the same buffer. The combined supernatant was centrifuged at 55,000 x g in the Spinco Model L centrifuge (rotor 30) for 1 h, and the clear amber supernatant (Fraction II) was collected.

To 30 ml of Fraction II was added 10.5 g of ammonium sulfate. After 20 min the precipitate was collected by centrifugation at 12,000 x g for 10 min and dissolved in 5 ml of 0.05 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA (Fraction III).

Fraction III was dialyzed against 1 liter of 0.05 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA with 2 changes of buffer. The dialysate was applied to a DEAE cellulose column (2 cm · 11 cm) that had been previously equilibrated with 0.05 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA. The column was washed with 100 ml of the same buffer and the enzyme was eluted with 70 ml of 0.1 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA (Fraction IV). The flow rate was 1 ml/min, and 5 ml fractions were collected.

Fifteen ml of combined Fraction IV was applied to a column of hydroxylapatite (1.5 cm · 3 cm) which had been washed with 70 ml of 0.02 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA, and then washed with 80 ml of 0.1 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA. The enzyme was eluted with 0.2 M potassium phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA. The flow rate was 0.3 ml/min and 2 ml fractions were collected. An equal volume of glycerol was added to fractions containing enzyme activity and these were then stored at -20° (Fraction V).

DNA polymerase assay. Enzyme activity was assayed in a manner similar to the procedure of Kornberg, et al.¹⁴ The 0.3 ml incubation mixture contained 0.05 M Tris (pH 7.5), 0.01 M MgCl₂, 0.001 M ME, 0.167 mM dATP, dGTP, dCTP and [³H]TTP (specific activity 1.4-2.7 · 10⁷ counts/min/μmole), 25 μg native salmon sperm DNA, and enzyme. The mixtures were incubated at 37° for 15 min. After adding 500 μg of salmon sperm DNA as carrier, the DNA was precipitated with cold 0.5 N perchloric acid and collected by centrifugation. The DNA was dissolved in 0.3 ml of 0.2 N NaOH and again precipitated with 0.5 N perchloric acid. The DNA was washed three times in this manner. Finally, the precipitate was dissolved in 0.5 ml of 0.2 N NH₄OH and the radioactivity was measured in Bray's solution with a Tricarb liquid scintillation counter. A unit of enzyme activity is defined as the incorporation of 1 mμmole of tritiated deoxynucleotide into acid-insoluble product during the 15 min incubation period. DNA polymerase activity in mitochondria was assayed in the same manner except that salmon sperm DNA was not included in the reaction.

Protein was determined by the method of Lowry et al.¹⁵ using bovine serum albumin as standard. Protein concentration in eluates from DEAE cellulose and hydroxylapatite columns were measured spectrophotometrically by the method of Warburg and Christian.¹⁶

In order to characterize the product formed by mitochondria, DNA was isolated and preparative CsCl density gradient centrifugation was carried out as previously described.¹⁷

Unlabeled deoxyribonucleoside triphosphates and [³H]TTP were purchased from Schwarz BioResearch, Inc., Orangeburg, New Jersey. Salmon sperm DNA was obtained from Calbiochem Corp., Los Angeles, Calif., and calf thymus DNA from Sigma Chemical Co., St. Louis, Mo. E. coli (K 12) DNA was a gift from Dr. S. Liao. Denatured DNA was prepared by heating DNA dis-

solved in 0.01 M Tris (pH 7.5), 0.01 M NaCl, in a boiling water bath for 10 min and quickly cooling in ice.

RESULTS

DNA Polymerase in Intact Mitochondria

Intact yeast mitochondria incorporate [³H]thymidylic acid into DNA (Table 1). The reaction requires divalent cations, and is almost completely dependent on the presence of 4 deoxynucleoside triphosphates. The addition of DNA is not required and does not stimulate DNA synthesis,

Table 1

REQUIREMENTS FOR CYTOPLASMIC AND MITOCHONDRIAL DNA POLYMERASE

	Mitochondria*	Mitochondrial polymerase*	Supernatant polymerase*
Complete +added DNA	107	25.5	95.1
-added DNA	105	0.2	1.0
-MgCl ₂	3.2	0.5	0.0
-2 mercaptoethanol	103	25.3	4.8
-dATP	3.4	5.9	4.8
-dGTP	16.4	3.3	14.1
-dCTP	6.4	3.3	6.9
-dATP, dGTP, dCTP	1.0	2.8	
+30 µg deoxyribonuclease	88	0.0	0.0

Mitochondria were assayed using 1.0 mg of material purified by sorbitol gradient. Six µg of Fraction V was used to assay partially purified mitochondrial DNA polymerase and 6 µg of Fraction VI to assay partially purified supernatant DNA polymerase.

* Activity is expressed as pmoles [³H]TMP incorporated into DNA in 15 min.

presumably because mitochondrial DNA is already available to the polymerase. The addition of 30 µg of pancreatic deoxyribonuclease to the reaction vessel results in only a small decrease in incorporation of [³H]thymidylic acid into DNA. The mitochondrial membrane appears to protect the DNA template and product from the action of deoxyribonuclease. The small inhibition of incorporation by deoxyribonuclease may be due to disruption of some mitochondrial membranes or to the presence of small amounts of cytoplasmic polymerase.

DNA synthesized by intact mitochondria has a buoyant density identical to that of yeast mitochondrial DNA when analyzed by CsCl density gradient centrifugation (Figure 1). The [³H]DNA bands almost exclusively with carrier mitochondrial DNA ($\rho = 1.685$); while less than 5 per cent of the radioactivity bands with carrier nuclear DNA ($\rho = 1.700$). The mitochondria therefore appear to be almost free of contaminating nuclear DNA polymerase and template.

DNA Polymerase Activity in Different Phases of Growth

Mitochondrial development is repressed by high glucose concentration in the culture medium.^{18,19} When glucose is used as an energy source, oxidative enzyme activity is greatly diminished during the exponential phase of growth, but is markedly increased in the stationary phase

Figure 1. CsCl density gradient analysis of [^3H]DNA synthesized by isolated yeast mitochondria. The reaction system was that described in the methods except that no DNA was added. Unlabeled yeast mitochondrial and nuclear DNA and ^{32}P yeast nuclear DNA were added prior to centrifugation. O—O [^3H]DNA synthesized by mitochondria; □---□ marker nuclear and mitochondrial DNA; x-.-.-x marker ^{32}P nuclear DNA specific activity $2 \cdot 10^4$ counts/min/ μg DNA.

of growth. Table 2 shows the DNA polymerase activity of mitochondria and of the cell supernatant in yeast harvested in the exponential and stationary growth phases. Mitochondrial polymerase activity was maximal during stationary growth, when it might be expected that mitochondrial replication was taking place, whereas supernatant DNA polymerase activity was maximal during the exponential phase of growth.

Table 2
DNA POLYMERASE ACTIVITY IN SUPERNATANT AND
MITOCHONDRIA IN DIFFERENT GROWTH PHASES

	Supernatant specific activity	Mitochondria specific activity
Exponential	0.13	0.012
Stationary	0.05	0.041

Purification of Supernatant and Mitochondrial DNA Polymerase

The procedures used to purify supernatant and mitochondrial DNA polymerase are outlined in Tables 3 and 4. Both enzymes were relatively stable and could be stored in the frozen state

Table 3
PURIFICATION OF SUPERNATANT DNA POLYMERASE

Step	Total units	Specific activity
I Crude extract	446	0.12
II Protamine sulfate	216	0.22
III (NH ₄) ₂ SO ₄ precipitation	192	0.30
IV Alumina C _γ gel	118	1.17
V DEAE cellulose	62	4.1
VI Hydroxylapatite	20*	16.7

* A 280 mμ/A200 mμ = 1.45.

Table 4
PURIFICATION OF MITOCHONDRIAL DNA POLYMERASE

Step	Total units	Specific activity	Ratio activity Denatured template Native template
I Mitochondria	22	0.10	
II Crude extract	8.8	0.17	1.9
III (NH ₄) ₂ SO ₄ precipitation	7.7	0.23	1.6
IV DEAE cellulose	2.9	0.97	0.7
V Hydroxylapatite	1.4*	4.8	0.7

Mitochondria were assayed in the absence of added DNA. The addition of DNA did not stimulate incorporation of [³H]TMP into DNA. The addition of 30 μg deoxyribonuclease reduced incorporation by 28%.

* A 280 mμ/A260 mμ = 1.42.

until DEAE-cellulose chromatography. After this procedure, perhaps secondary to removal of nucleic acids, the enzymes were very labile and activity could be preserved only by addition of 50 per cent glycerol and storage at -20°. It was necessary to carry out DEAE-cellulose and hydroxylapatite chromatography with a minimum of delay on the same day. Final yields of enzyme were about 5 per cent. Supernatant polymerase was purified about 150-fold and mitochondrial polymerase about 50-fold.

Comparison of Properties of Supernatant and Mitochondrial DNA Polymerase

The partially purified mitochondrial and supernatant enzymes showed properties similar to other DNA polymerases. In both cases there was an almost absolute requirement for added DNA template, MgCl₂, and for addition of 4 deoxynucleoside triphosphates (Table 1).

Incorporation of [³H]thymidylic acid into DNA by both partially purified polymerases was proportional to enzyme concentration (Figure 2) and continued almost linearly with time for at least 30 to 60 min (Figure 3). The requirement for divalent cations, Mg²⁺ or Mn²⁺ is shown in Figure 4. The optimal concentration of the divalent cations was similar for the two enzyme preparations.

Figure 2. Relation between the incorporation of [³H]thymidylate into DNA and enzyme concentration. Fraction VI of supernatant enzyme and Fraction V of mitochondrial enzyme were used.

Figure 3. Time course of incorporation of [³H]thymidylate into DNA.

The pH optima for both enzymes were similar (Figure 5), being approximately 8.0-8.5 with Tris buffer and 6.5 with phosphate buffer.

The activities of the polymerases with different templates are compared in Table 5. *E. coli* DNA was a more effective template than salmon sperm DNA for both enzymes, and calf thymus DNA was least effective. There was little difference in relative activity of the two enzymes with native and denatured template; in both cases native DNA appeared to be somewhat more effective than denatured DNA. The degree of purification greatly altered the relative activity of the mitochondrial enzyme with native and denatured template (Table 3). The crude enzyme preferred denatured template while the purer enzyme was more active with native template. The differences in response are possibly secondary to different amounts of contaminating nuclease in the prepa-

Figure 4. Divalent cation requirement of DNA polymerase.

Figure 5. pH optima for supernatant and mitochondrial DNA polymerases. The final buffer concentration was 0.083 M. Tris malate was used at pH 6.5 and 7.0, and Tris HCl at higher pH. Nine μg of Fraction VI of supernatant enzyme, and 6 μg of Fraction V of mitochondrial enzyme were used in the standard assay system.

rations. Another factor that altered the relative activity of the enzyme with native and denatured template was ionic strength. At high concentrations of KCl or NH_4Cl incorporation of [^3H]thymidylate was greatly reduced when native DNA was template, but only slightly with denatured DNA (Table 6). The ratio of activity with denatured template to activity with native primer was therefore increased 3 to 4-fold at high ionic strength.

Table 5

ACTIVITY OF DNA POLYMERASE WITH DIFFERENT TEMPLATES

Primer	p moles [^3H]TMP incorporation in 15 min	
	Mitochondrial	Supernatant
Salmon sperm DNA, native	25.2	152
Salmon sperm DNA, denatured	18.9	86
Calf thymus DNA, native	5.4	26.3
Calf thymus DNA, denatured	5.8	14.6
<i>E. coli</i> DNA, native	61.2	190
<i>E. coli</i> DNA, denatured	31.2	138

Four and five-tenths μg of Fraction V mitochondrial DNA polymerase and 9.0 μg of Fraction VI of supernatant DNA polymerase were used in the assays.

Table 6

EFFECT OF SALT CONCENTRATION ON ACTIVITY OF PARTIALLY PURIFIED MITOCHONDRIAL DNA POLYMERASE WITH NATIVE AND DENATURED TEMPLATE

Addition	Denatured template p moles	Native template p moles	Denatured template
			Native template
None	99	81	1.2
+0.08 M KCl	74	22	3.4
+0.15 M KCl	33	10	3.3
+0.08 M NH_4Cl	76	17	4.4
+0.15 M NH_4Cl	33	10.5	3.1

Eighty-five μg of Fraction IV enzyme was used in the experiment. The assays also contained 5% glycerol and 0.0125 M K phosphate (pH 7.4).

Density Gradient Centrifugation of the Enzyme

In order to compare the molecular sizes of the enzymes, purified fractions of mitochondrial supernatant DNA polymerase were centrifuged in a glycerol density gradient. Figure 6 shows the patterns obtained with the individual enzymes and with a mixture of the two enzymes. Two components appear to be present in the supernatant DNA polymerase preparation but only one can be seen in the mitochondrial DNA polymerase. The mitochondrial DNA polymerase sediments with the slower component present in small amount in the supernatant DNA polymerase.

DISCUSSION

DNA polymerase activity in preparations of isolated yeast mitochondria appears to represent the activity present in these organelles rather than contamination by enzyme derived from the nucleus for the following reasons: (1) activity does not require the addition of DNA template; (2) the DNA synthesized by isolated mitochondria has the buoyant density of mitochondrial DNA;

Figure 6. Glycerol density gradient centrifugation of mitochondrial and supernatant DNA polymerase. Fifteen-hundredths ml of the most purified fractions of polymerase were layered on a linear 6 to 25% glycerol gradient containing 0.1 M phosphate buffer (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M EDTA and centrifuged for 13 hr at 35,000 rev/min in the SW 39 rotor of a Spinco L265 centrifuge. Twelve drop fractions were collected and each fraction was assayed for DNA polymerase activity in the usual manner except that incubations were for 30 min. A: ○—○ supernatant DNA polymerase; △---△ mitochondrial DNA polymerase. B: mixture of the two DNA polymerases.

and (3) deoxyribonuclease only slightly inhibits the reaction. The incorporation requires 4 deoxynucleoside triphosphates and therefore does not represent terminal addition or homopolymer formation. These results are similar to those previously found in rat liver by Helge and Neubert⁷ and Parson and Simpson⁸ and in yeast by Wintersberger.⁶ It is also of note that mitochondrial DNA polymerase is most active during maximal mitochondrial development in the stationary growth phase, whereas nuclear DNA polymerase is most active during the exponential phase of growth.

The DNA polymerase has been solubilized by extraction with solutions of high ionic strength, and partial purification of the enzyme has been achieved. At this level of purification, very little difference was observed in the enzymatic properties of the two enzymes. An apparent preference of crude mitochondrial DNA polymerase for denatured DNA disappeared when the enzyme was further purified. One must use great caution in interpreting preference for native and denatured template in a partially purified DNA polymerase since the difference may be related to contamination with nucleases specific for native or denatured DNA.

The lability of the enzyme prevented purification to a degree that would allow the unequivocal demonstration that the mitochondrial and supernatant enzyme were different molecular species. However, glycerol density gradient showed that the supernatant preparation contained a heavy component that was not present in the mitochondrial enzyme. The heavy component may represent enzyme derived from the nucleus whereas the lighter component may represent mitochondrial enzyme. The possibility of dimer formation,²⁰ however, has not been eliminated. The re-

cent work of Kalf and Ch'ih⁹ and Meyer and Simpson¹⁰ makes it highly probable that the mitochondrial and nuclear enzymes are different.

The activities of both supernatant and mitochondrial DNA polymerases were inhibited by high salt concentrations (0.08-0.15 M KCl). The inhibition was greater when the enzymes were assayed with native template than with denatured template. Yeast mitochondrial DNA polymerase therefore differs from the enzyme isolated from rat liver¹⁰ which is markedly stimulated high salt concentration. Rat liver nuclear DNA polymerase is not significantly stimulated by alteration of ionic strength. The difference in response to high ionic strength is therefore a means of distinguishing between liver mitochondrial and nuclear DNA polymerases.

The presence of a unique DNA polymerase localized in yeast mitochondria is in accordance with the previous finding of Mounolou, et al²¹ and of Rabinowitz et al^{17,22} that labeled precursors are preferably incorporated into mitochondrial DNA by anaerobic yeast adapting to oxygen in a non-growth sustaining medium. Specific activities of mitochondrial DNA were 15-30 times greater than that of nuclear DNA during this period of rapid development of mitochondrial respiratory function.

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TURNOVER OF CYTOCHROMES LABELED WITH δ -AMINOLEVULINIC ACID- ^3H IN RAT LIVER*

By

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Measurement of turnover rates for discrete components of the mitochondrion has served as one experimental approach to study of the mechanism of mitochondrial biogenesis. Fletcher and Sanadi¹ observed that in vivo decay of isotopically labeled mitochondrial proteins followed first order kinetics and yielded a half life of 9-10 days. Subsequent studies with mitochondrial protein fractions,^{2,3} DNA, cardiolipin, and other phospholipids⁴ generally yielded similar half lives if comparisons were restricted to mitochondria isolated from the same organ of the same species. However, the problem of reutilization of labeled precursors has complicated the interpretation of these experiments. Basing their arguments upon such data, Fletcher and Sanadi¹ have advanced the hypothesis that mitochondrial turnover occurs as a synchronous process.

Recently it has become possible to assign membrane loci to specific mitochondrial proteins⁵ and the turnover of mitochondrial proteins can now be examined in these terms. If synthesis and degradation of the mitochondrion occurs via a synchronous process, then turnover rates of inner and outer membrane proteins, as well as of matrix proteins, should be similar.

It is clear that several mitochondrial components turn over at rates inconsistent with synchronous synthesis and degradation of the entire mitochondrion. Inducible enzymes, such as alanine aminotransferase, ornithine aminotransferase,⁶ and δ -aminolevulinic acid synthetase⁷ turn over with a half life of less than one day. Proteins of the outer mitochondrial membrane have recently been shown to turn over more rapidly than proteins of the inner membrane.^{8,9}

In this study, we report turnover rates for hemeproteins after in vivo isotopic labeling with 3,5- δ -aminolevulinic acid- ^3H (ALA- ^3H).[‡] ALA is a specific heme precursor that is not reutilized. Half lives are reported for cytochrome c and cytochrome b located on the inner mitochondrial membrane, for cytochrome b_5 located on the outer mitochondrial membrane,⁵ and for microsomal cytochrome b_5 .

A preliminary account of this work has appeared.¹⁰

MATERIALS AND METHODS

ALA- ^3H (specific radioactivity = 292 mCi/mM) was purchased from Tracerlab. L-arginine- ^{14}C (guanido labeled, specific activity = 33 mCi/mM) was from Amersham-Searle. Isotopes were diluted with 0.15 M NaCl and were injected into the peritoneal cavity of adult, male Sprague-Dawley rats (220-250 g body weight). Each rat received 50 μCi of ALA- ^3H or 25 μCi of arginine-

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[‡] ALA, δ -aminolevulinic acid; ALA- ^3H , 3,5- δ -aminolevulinic acid- ^3H .

¹⁴C. After fasting overnight, rats were sacrificed at intervals by stunning and exsanguination. Livers were excised, homogenized and pooled. After ALA-³H incorporation, six livers were combined and processed; in experiments with arginine-¹⁴C, three livers were used.

Concentrations of cytochromes and heme were measured from absorbance. Total protein and apoprotein concentrations were measured by the biuret reaction¹¹ using crystalline bovine serum albumin (Pentex) as a standard. Unless otherwise noted, all preparative steps were carried out at 4°.

Preparation of mitochondria and microsomes. Mitochondria were isolated by the method of Parsons.¹² The supernatant obtained after centrifugation at 14,000 x g was recentrifuged at 14,000 x g for 20 minutes and the pellet was discarded. Microsomes were then prepared by centrifuging the supernatant solution at 100,000 x g for 60 minutes.

Mitochondria were sequentially treated to extract cytochrome b₅, cytochrome c, and heme b. The overall preparation scheme is outlined in Figure 1.

Figure 1. Preparation scheme that yields mitochondria, microsomes, cytochromes, heme, and protein from a single liver homogenate.

Mitochondrial cytochrome b₅. Mitochondria that contained 400-500 mg protein were suspended in 20 ml of 0.01 M Tris-chloride (pH 7.4) containing 10 per cent ethanol (v/v).¹³ The suspension was rapidly frozen in a dry ice-acetone slurry. The suspension was thawed, heated to 40° for 10 minutes, then refrozen and rethawed. The pellet obtained by centrifugation for 10 minutes at 48,000 x g was saved for further extraction of cytochrome c and heme b. The supernatant solution, which contained cytochrome b₅, was dialyzed against 0.01 M sodium phosphate (pH 6.6) to remove ethanol. Crystalline ammonium sulfate was added to 70 per cent saturation. After standing overnight, the precipitate was collected by centrifugation at 35,000 x g for 10 minutes. The precipitate was dissolved in 3-4 ml of the same buffer and was again dialyzed. Cytochrome b₅ concentration was calculated from absorption spectroscopy at two wavelengths reading protein reduced with dithionite against samples oxidized with potassium ferricyanide. The following relationship was used: $(A_{557\text{m}\mu}^{\text{mM}}) - (A_{575\text{m}\mu}^{\text{mM}}) = 20$.¹⁴

Cytochrome c. The pellet collected after cytochrome b_5 extraction was suspended in 20 ml of 0.15 M KCl and stirred for 10 minutes at 30°. After centrifugation at 35,000 x g for 15 minutes, the pellet was reextracted in the same way with 0.15 M KCl. One drop of 10^{-3} M $K_3Fe(CN)_6$ was added to the combined supernatant solution, which was applied to a 0.2 x 0.5 cm column containing XR-64 resin.¹⁵ The column was washed with 3 ml of water, and cytochrome c was eluted with 1 M NH_4OH directly into a dialysis sac. The cytochrome c solution was dialyzed against 0.01 M sodium phosphate (pH 6.6). The concentration of cytochrome c reduced with dithionite was determined by absorption spectroscopy: $(A_{550m\mu}^{mM}) - (A_{535m\mu}^{mM}) = 20.4$.¹⁶

Heme b. Mitochondria from which cytochromes b_5 and c had been extracted were treated as described by Connelly et al,¹⁷ except that silicic acid chromatography was omitted. Solvent extraction was used to separate heme b from heme a,¹⁸ and heme b was measured as the pyridine hemochromogen.¹⁹ Although heme a was detected, its spectral purity was variable, and its half-life was not estimated.

Microsomal cytochrome b_5 . Microsomes were suspended in 0.1 M Tris-chloride, 1×10^{-3} M EDTA (pH 8.0), and the suspension was diluted to a protein concentration of 15 mg per ml. Crystalline trypsin (Worthington) was added to a concentration of 200 μ g per ml and the mixture was incubated for 48-60 hours at 4°. ²⁰ Crystalline ammonium sulfate was slowly added to 60 per cent saturation, and after standing for 20 minutes, the precipitate was removed by centrifugation for 15 minutes at 48,000 x g. Ammonium sulfate was added to the supernatant solution to reach 85 per cent saturation, and after standing for 30 minutes, the precipitate was removed by centrifugation. 1 N HCl was added to the supernatant to adjust the pH to 5.2, and after standing for 30 minutes, the precipitate was removed. The pH of the supernatant solution was adjusted to 4.2, and after standing for 30 minutes, the precipitate containing cytochrome b_5 was collected by centrifugation.

In some experiments, cytochrome b_5 precipitated at pH 4.2 was further purified.²¹ The protein was dissolved in 1 ml of 0.01 M sodium phosphate (pH 7.5) and applied to a 1 x 40 cm column of G-75 Sephadex equilibrated with the same buffer. Fractions that contained cytochrome b_5 were pooled and applied to a 0.2 x 1.0 cm column of DEAE cellulose (DE-23, Whatman) equilibrated with 0.01 M sodium phosphate (pH 7.5). The column was washed with 10 ml of the same buffer containing 0.1 M NaCl. Cytochrome b_5 was eluted with 0.3 M NaCl in buffer.

Molar absorbancy indices* were determined for these two preparations after reduction with dithionite: for protein precipitated at pH 4.2, $A_{556m\mu}^{mM} = 19.0$; for protein prepared with gel filtration and DEAE cellulose, $A_{556m\mu}^{mM} = 29.0$. These absorbancy indices were used to calculate the concentration of microsomal cytochrome b_5 .

Absolute and difference spectra were recorded for microsomal and mitochondrial cytochrome b_5 , cytochrome c, and heme b with a Cary 14 spectrophotometer. Our values corresponded to published data.^{16,19,22}

Radioactivity. ³H was counted in a Packard liquid scintillation counter. 50-200 μ l of protein solution or alkaline pyridine hemochromogen was added to 15 ml of Bray's solution.²³ Quench correction was made by dual channel counting, and results are expressed as DPM. ¹⁴C was counted with a low background instrument (Beckman Low Beta II). Less than 5 mg of protein, dis-

* A molecular weight of 14,400 was used in these calculations.²² Protein concentration was measured by the biuret reaction.

solved in formic acid, was dried on each planchet. At this protein concentration, no self-absorption occurs.

RESULTS

The specificity of mitochondrial cytochrome b_5 extraction was examined by applying the ethanol-freezing procedure to a suspension of microsomes. No detectable cytochrome b_5 was solubilized from microsomes by this procedure. In addition, our preparation of mitochondrial cytochrome b_5 was not reduced by adding cysteine. It has been previously reported that microsomal cytochrome b_5 is reduced by cysteine, but mitochondrial cytochrome b_5 is not.²⁴ Our preparation of mitochondrial cytochrome b_5 was also reduced upon adding NADH^+ (Sigma).

The degree to which $\text{ALA-}^3\text{H}$ is specific as a label for heme and heme proteins can be estimated by comparing the specific radioactivities of labeled heme and heme protein with those of corresponding heme-free apoprotein. These results are summarized in Table 1. Acetone-HCl

Table 1
SPECIFIC RADIOACTIVITIES OF CYTOCHROMES, HEMES AND
APOCYTOCHROMES LABELED WITH $\text{ALA-}^3\text{H}$

Sample	(DPM/ μ mole) $\times 10^{-3}$
<u>Microsomes:</u>	
Total heme	1800
Total protein, acetone-HCl extracted*	10
Cytochrome b_5	2500
Apocytochrome b_5	0.01
<u>Mitochondria:</u>	
Cytochrome c	1200
Heme b	1500
Total protein, acetone-HCl extracted*	10

Samples were isolated from rat liver 24 hours after injection of isotope. Concentrations of cytochromes b_5 and c, and of heme b were measured by absorbance. Total protein was determined by biuret reaction.

* For these calculations, an average protein molecular weight of 50,000 was assumed.

(1 per cent HCl, v/v) was used to split heme from heme protein.* A comparison of the specific radioactivities of mitochondrial or microsomal proteins with their corresponding apo-proteins shows that less than one half per cent of the specific radioactivity is retained by apo-protein after extraction of heme. For microsomal cytochrome b_5 , 0.001 per cent of the specific radioactivity remains after treatment with acetone-HCl.

In vivo labeling after $\text{ALA-}^3\text{H}$ injection yields heme proteins with high specific radioactivity.

* Acetone-HCl does not split heme from cytochrome c. Hence, total apo-protein from mitochondria neglects the counts from cytochrome c present if extraction is incomplete. For microsomal apo-proteins, cytochrome c was not extracted, although small amounts are found here.²⁵

The specific radioactivity of microsomal cytochrome b_5 labeled with ALA- ^3H was 200-fold greater than that of microsomal cytochrome b_5 labeled with arginine- ^{14}C .*

Half lives were measured for the following cytochromes: cytochrome c, mitochondrial cytochrome b_5 , mitochondrial cytochrome b, and microsomal cytochrome b_5 . The biologic decay rate was calculated from determinations made beginning 24 to 48 hours after isotope injection.

As shown in Figure 2, the half life of cytochrome c was 6.1 days, measured over a 21 day interval. For heme b, which is used as an index to cytochrome b turnover, the half life was 5.5 days (Figure 3). The half life of mitochondrial cytochrome b_5 was 4.4 days (Figure 4).

Figure 2. Cytochrome c: in vivo decay of specific radioactivity after labeling with ALA- ^3H .

Figure 3. Heme b (cytochrome b): in vivo decay of specific radioactivity after ALA- ^3H labeling.

When cytochrome purification was restricted to ammonium sulfate fractionation and pH precipitation (Figure 5), the half life for microsomal cytochrome b_5 was 2.3 days. In another experiment, in which cytochrome b_5 was further purified with G-75 Sephadex and DEAE cellulose, there was a 50 per cent increase in purity. The millimolar absorbancy index was increased from 19 to 29. This preparation of microsomal cytochrome b_5 migrated as a single band during electrophoresis on 7 per cent polyacrylamide gel. The half life of this preparation of cytochrome b_5 was 2.4 days, shown in Figure 5.

Guanido labeled arginine- ^{14}C is not significantly reutilized in tissues that contain arginase activity.²⁶ The half life of microsomal cytochrome b_5 was measured after injection of this isotopic precursor, and cytochrome b_5 was purified through the Sephadex and DEAE cellulose steps. As shown in Figure 6, the half life of arginine- ^{14}C labeled microsomal cytochrome b_5 was 2.5 days.

* For this comparison, counting data are corrected for the amount of precursor injected (ALA- ^3H = 50 μCi ; arginine- ^{14}C = 25 μCi) as well as for ^{14}C counting efficiency (35 per cent).

Figure 4. Mitochondrial cytochrome b_5 : in vivo decay of specific radioactivity after ALA- ^3H labeling.

Figure 5. Microsomal cytochrome b_5 : Closed circles, decay of specific radioactivity of protein prepared by precipitation. Open circles, decay of specific radioactivity of protein further purified by gel filtration, DEAE cellulose chromatography. Proteins were labeled with ALA- ^3H .

DISCUSSION

Turnover rates measured after isotopic amino acid injection may be significantly prolonged by isotopic reutilization.²⁷ Leucine- ^{14}C or methionine- ^{35}S have been used as precursor isotopic label in most reported studies of mitochondrial protein turnover. Except in the case of cytochrome c, half lives were measured for protein fractions that were operationally defined according to their solubility properties.¹ Thus, an isotopic label that is subject to reutilization was incorporated into protein fractions of varying purity to measure protein turnover rates in vivo. The half lives of rat liver mitochondrial proteins are reported as 9-10 days after labeling with leucine- ^{14}C or methionine- ^{35}S , as shown in Table 2. Swick et al⁶ used a steady state model in which ^{14}C incorporation into arginine was measured, minimizing reutilization problems. They found a half life of 5-6 days for several rat liver mitochondrial proteins. These lower values are similar to data reported in this paper for cytochromes associated with the inner membranes of mitochondria.

With ALA- ^3H as a precursor label, isotopic incorporation is specifically into heme; incorporation of radioactivity into non-heme proteins is insignificant (Table 1). During degradation of heme proteins, heme is split and catabolized to bilirubin without reutilization of the porphyrin ring.²⁸ Since isotopic labeling after ALA- ^3H injection is restricted to heme, removal of non-heme proteins is not of crucial importance in turnover experiments. For example, increasing

Figure 6. Microsomal cytochrome b_5 ; in vivo decay of protein labeled with l-arginine- ^{14}C , purified by gel filtration and DEAE cellulose chromatography.

the purity of microsomal cytochrome b_5 by 50 per cent (based on absorbance: protein ratio) did not increase the specific radioactivity of this protein when cytochrome concentration was measured by optical absorbance at 556 $m\mu$. Both radioactivity and absorbance are based on measurements of the heme moiety. In all experiments reported in this paper, there was no evidence of cross contamination between hemoproteins or hemes in fractions that were measured for radioactivity.

Cytochromes b and c are associated with the inner mitochondrial membrane, and their turnover rates are similar ($0.4 < p < 0.5$).^{*} We have used cytochrome b_5 as a marker protein for the outer membrane, and its turnover rate differs significantly from that of cytochrome c ($p < 0.05$) but is equivocal when compared with cytochrome b ($p = 0.1$). These data support the hypothesis that the inner mitochondrial membrane, or a fraction thereof, turns over synchronously.⁴ Recently begun experiments yield preliminary data that further support this hypothesis; the half life of heme a appears similar to values found for cytochromes b and c.[†]

Total proteins isolated from the outer mitochondrial membrane are renewed more rapidly than proteins from the inner membrane. Isotope ratios, measured on isolated membrane fractions after sequential injection of ^3H and ^{14}C -leucine³⁰ showed that turnover of inner membrane protein was more rapid.⁸ After incorporation of methionine- ^{35}S , a 4.2 day half life has been re-

^{*} p values were obtained as described by Goldstein.²⁹

[†] Ashenbrunner, V., unpublished observations.

Table 2
HALF LIVES OF MITOCHONDRIAL PROTEINS

Protein	Label	Half life (days)	Reference
Water soluble	^{35}S -met	10.8	1
	^{35}S -met	9.0*	2
	^{14}C -leu	8.56	3
	$\text{Ca}^{14}\text{CO}_3^\dagger$	5.1	6
Cytochrome c	^{35}S -met	9.7	1
	^{14}C -leu	8.56	2
	$\text{Ca}^{14}\text{CO}_3^\dagger$	5.0	6
Insoluble	^{35}S -met	10.1	1
	^{35}S -met	9.5	2
Structural	^{14}C -leu	8.77	3
	$\text{Ca}^{14}\text{CO}_3^\dagger$	5.5	6
Alanine aminotransferase	Activity ‡	0.73	6
Ornithine aminotransferase	Activity ‡	0.78	6
δ -aminolevulinic acid synthetase	Activity ‡	0.05	7

* Half life based on measurements made between 14 and 28 days; decay over 35 day interval was non-linear.

† Steady state model. Values obtained over a 12 day interval.

‡ Induced enzyme activity; half life calculated from decay of enzyme activity.

ported for outer membrane proteins,⁹ a value in close agreement with our data for mitochondrial cytochrome b_5 turnover. ^{35}S labeled protein from the inner membrane was found to have a 12.6 day half life.⁹ Hence, our data, in conjunction with recent reports^{8,9} indicate that mitochondrial protein turnover occurs at several rates, and that protein from the outer membrane of the mitochondrion undergoes more rapid renewal than that from the inner membrane.

Whether or not mitochondrial moieties, such as the inner membranes, are synthesized and degraded as a unit cannot now be decisively answered. Our data, based upon turnover rates for cytochromes b and c, are consistent with this hypothesis.⁴

Table 2 summarizes the broad range of half lives reported for rat liver mitochondrial proteins. Turnover rates up to 100-fold greater than those of cytochromes have been reported for inducible enzymes. δ -aminolevulinic acid synthetase has a 10 minute half life⁷ and is located in the matrix fraction of the mitochondrion.³¹ Loci for aminotransferases have not yet been reported. The half lives for mitochondrial and microsomal cytochromes b_5 are significantly different ($p < 0.01$). This observation provides further support for the view that mitochondrial cytochrome b_5 is not a contaminant derived from microsomal cytochrome b_5 .

Our measurement of the half life of microsomal cytochrome b_5 is significantly different from that given in a previous report, in which a 5 day half life was calculated after ^{14}C -leucine

incorporation.²¹ Two alternative explanations for this disparity merit consideration: leucine-¹⁴C may be reutilized, leading to an apparent prolongation in the rate of biologic decay; or the turnover of ALA-³H labeled heme may be independent of the turnover of the holoprotein. The observation that hemes exchange in hemoglobin solution³² suggests the possibility that hemes could exchange between microsomal cytochrome b₅ and cytochrome P-450, and this might result in a shorter apparent half life for cytochrome b₅. To evaluate this possibility we measured the turnover rate of cytochrome b₅ after incorporation of arginine-¹⁴C into the protein moiety. The half lives obtained after labeling heme (ALA-³H) or protein (arginine-¹⁴C) are identical. For cytochrome c, heme exchange appears very unlikely, as heme is bound covalently.³³ Heme exchange among other membrane bound cytochromes has not yet been studied.

The validity of our measurement of cytochrome b turnover depends upon the prior removal of other potential sources for heme b, since heme b rather than cytochrome b was isolated. Hemoglobin is one potential contaminant; ALA-³H, however, is poorly incorporated by the erythron³⁴ and the half life of rat erythrocytes is 30 days.³⁵ Mitochondrial cytochrome b₅ could yield heme b, but this cytochrome has been extracted. Peroxisomes contaminate mitochondrial preparations,³⁶ but three aqueous extractions (Tris-ethanol, 0.15 M KCl, twice) followed by three non-aqueous extractions (acetone, chloroform-methanol, acetone) precede splitting of heme from protein by acetone-HCl, and probably remove catalase.

During the preparation of this paper, the turnover rate of catalase was reported after incorporation of several different isotopic precursors.³⁷ With ALA-¹⁴C, the half life of catalase was 1.8 days; with arginine-¹⁴C (guanido) it was 2.5 days; and for leucine-¹⁴C it was 3.7 days. Hence, the half life after leucine-¹⁴C labeling appears to be prolonged by reutilization of the isotope, as suggested by our data on cytochromes. Poole et al³⁷ attribute the disparity between the ALA-¹⁴C and arginine-¹⁴C values to scatter of the experimental points obtained after arginine-¹⁴C labeling. Prior studies, based on the effect of aminotriazole on catalase, support a half life of 1.4 days for catalase.³⁸

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AN ADDITIONAL SYSTEM FOR BONE DENSITOMETRY STUDIES*

By

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In vivo methods of evaluating variations in the density of human bone mineral content have been developed by Gershon-Cohen,¹ by Cameron and Sorenson,² and, more recently, by Johnston and co-workers.³ These methods involve transmission of a photon beam through a section of bone, using a radioactive source in combination with counting techniques. We have utilized variations of the technique to measure the bone mineral content of several hundred patients at the University of Chicago Hospitals and Clinics, our objectives being a study of bone demineralization diseases and the development of methods of treating them.⁴⁻⁸ Currently we are evaluating patients both with our original ¹²⁵I equipment, and with a newer instrument manufactured by Packard Instrument Company, the Osteodensitometer, utilizing primarily ²⁴¹Am.

¹²⁵I, with a dominant photon energy of 27 keV, is an ideal source for transmission measurements through small bones with little overlying tissue, because it discriminates effectively between bone and soft tissue. Its relatively low energy prevents it from being used efficiently with bones and tissues much larger than those comprising an adult human wrist. Furthermore, its half-life is only 60 days which means that the source must be replaced relatively often. ²⁴¹Am, with a principal photon energy of 60 keV, can be used as a source for transmission measurements through large bones (e.g., the calcaneus), but discrimination between bone and soft tissue is not as good as it is with ¹²⁵I. The half-life of ²⁴¹Am is 458 yr which makes installation of a permanent source possible.

The phalanx is an example of accessible cortical bone. Changes in density, however, show up first in trabecular bone. We have therefore included evaluations of the density of trabecular bone as exemplified by the calcaneus in our present work. The irregular shape of the calcaneus, however, makes it important to use as nearly as possible the same counting position on each patient, whether the measurements are part of a series of patients or are simply for self-comparison over an extended period of treatment. The ¹²⁵I finger counting device consists of a well collimated photon beam directed into a well collimated detector. Transmission of the photon beam is measured directly by means of a scintillation counter. The development and use of this unit at Argonne Cancer Research Hospital have been reported in detail previously.⁴

The Packard Osteodensitometer is designed to fill the needs of physicians studying bone density changes. The maximum versatility of this instrument ensures that the investigator may proceed along many avenues while retaining the option of changing direction as more data become available, either from his own work or from the work of others.

The late Dr. Nels M. Strandjord of the University of Chicago was interested in the develop-

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ment of a bone densitometry unit that would adequately fill the needs of investigators of bone density. He persuaded the Packard Instrument Company to develop such a unit and provided the background and early consultation necessary for its design. The resulting instrument has the following features.

- A. A variety of positioning fixtures allows transmission measurements to be made through the calcaneus, radius, ulna, humerus, fibula and phalanges. Figure 1 shows the electronics and data readout unit next to the source-detector unit which is set up for a calcaneus measurement. The source and detector assembly, which weighs 35 pounds, can be placed on the floor, on a stool or table, standing upright or lying on its side depending on the requirements of a specific measurement.

Figure 1. Source-detector unit of Packard Osteodensitometer with the electronic package and data printout assembly.

- B. ^{241}Am , ^{125}I or other isotope sources can be used in the unit.
- C. The standard scanning speeds are 0.5 cm and 1.0 cm/min; other speeds can be provided easily.
- D. Scanning can be done on both X or Y axis over any portion of an 8 cm x 8 cm area.
- E. A rapid traverse speed of 20 cm/min allows fast positioning on an X-Y coordinate for either starting a scan or taking a stationary count. The same rapid traverse is used for rapid automatic return to the starting position after completion of a scan. A STOP button halts the stationary count or scan as desirable.
- F. There is a position indicator light on a translucent grid on the source and detector assembly. Front panel pushbuttons adjacent to this grid enable the operator to set up the scan limits necessary for individual bone measurements. The position of the collimated photon

beam is indicated continually by the light beam on the grid.

- G. Data are printed out on a Monroe lister. A 6-sec or 30-sec printout interval is chosen by means of a selector switch. A buffer storage system avoids any count loss during data read-out. The actual count loss interval is only 15 μ sec. Figure 2 shows a schematic representation of the counting system.

Figure 2. Schematic of Packard Osteodensitometer counting system.

- H. The source collimation can be changed readily. Standard collimators are 2.0-, 1.5- and 1.0-mm dia x 24-mm long.
- I. Detector collimation is easily varied. Standard collimators range from 1.0 through 10.0 mm dia in increments of 1 mm, x 19 mm in length.
- J. The sodium iodide crystal specially designed and built for this unit at Packard Company is coupled to the photomultiplier with a light pipe. The crystal's small size, 16 x 16 x 6 mm, is important in keeping the instrument background count down to between 20-30 cpm. The photomultiplier tube is a 1-1/8" dia II stage EMI #9524 (Electric and Musical Industries).
- K. Shielding of the source keeps the level of radiation at the surface of the source and detector assembly housing at less than three times normal background with the shutter open.

Normally, the shutter is open only during the scan mode or the stationary count mode. Depressing the STOP button closes the shutter, as does tripping the end of scan limit switch.

If the source shutter does not close completely due to a solenoid malfunction or for any other reason, and the electronic logic determines that the shutter should be closed, a warning is provided by a flashing light in the STOP pushbutton and also by a lighted COUNT button.

The source-detector assembly is linked to the electronics printout assembly by means of two flexible cables. The weight of the electronics readout assembly is 93 pounds, and this unit houses most of the electronic components in order to keep the source-detector assembly as

light as possible for ease in positioning and handling.

The distance between the source and the detector is 20.64 cm. The collimated photon beam from the source strikes the sodium iodide crystal and generates light pulses which pass through a light pipe to a photomultiplier tube (see Figure 2). An initial preamplification stage is used within the photomultiplier tube assembly, and this signal is fed to the amplifier and pulse height analyzer section located within the electronics chassis. In this section, the amplifier gain is set and the discriminators are adjusted to provide a window appropriate for the source being used. For ^{241}Am at 60 keV, a typical window setting might be 50 to 70 keV. From the pulse height analyzer, the signal is fed to a scaler that accumulates counts for either 6 or 30 sec, transfers the counts to a buffer storage section within 15 μsec , and immediately starts to accumulate the next count. The count from the buffer storage is fed through a multiplexer section to a Monroe lister where it is printed (the lister is a Monroe calculator actuated by solenoids to list and print the data). Virtually no counts are lost during data readout. The resolving time for this system is 6 μsec , which means that one event per second (approximately 1/4 of 1 per cent) is lost at an observed 420 cps, which is a typical count rate for the calcaneus. Ten events per second (less than 1 per cent) are lost at an observed rate of 1,300 cps, which is a typical count rate for a phalanx. The maximum count rate observed on the Packard system using a 14 mCi ^{241}Am source with all collimation removed from both source and detector was 20,000 cps through 20.64 cm of air. With the 2.0-mm source collimator, the observed count rate was approximately 8,000 cps. Events lost at this rate would be 380 cps or approximately 4.7 per cent.

In our ^{125}I work, the first step is to take palm-down and lateral view radiographs of the middle finger of the left hand. The counting position, i.e., the distance from the end of the bony structure to our prescribed location on the second phalanx, and the thickness of the bone at that region are determined from the radiographs. The finger is placed palm down over the collimated ^{125}I source and beneath a crystal detector. (Figure 3 is a detailed view of the finger in position for a series of counts on the Packard Osteodensitometer.) The source and detector remain stationary for one set of counts (usually 0.5 min); the count is printed on a paper tape; the source and detector then move together 1 mm either to the left or right, and count at the new position. This procedure is repeated automatically across the entire width of the finger. The average of the counts through the center of the finger is used, together with counts taken with the finger removed, film measurements, and correction for soft tissue, to determine a linear absorption coefficient of the bone.

Figure 4 shows a cross-sectional diagram of a normal finger. During evaluation, the finger is compressed between two parallel plates, U and V. The path of the photon beam passing through the soft tissue of the finger is indicated by S. The transmitted intensity, I , of the beam equals the unattenuated intensity, I_0 , times the attenuation, $e^{-\mu_T t}$, of the beam due to soft tissues, where μ_T is the linear absorption coefficient per centimeter, and t is the thickness of the soft tissue in centimeters. This thickness, t , is also the distance between the two parallel plates V and U. I_0 is measured by removing the finger from its position between the two plates. From measured values of I , I_0 , t and use of the expression $I = I_0 e^{-\mu_T t}$ we obtain the linear absorption coefficient μ_T of the soft tissue. With the isotope ^{125}I we have found this coefficient to be 0.40 cm^{-1} ; with ^{241}Am , μ_T is 0.22 cm^{-1} .

When the beam is directed along path B, the expression must contain another factor due to the radiation absorbed by the bone:

Figure 3. Detail of patient's finger in source-detector unit of Packard Osteodensitometer.

Figure 4. Cross-sectional diagram of a finger. Path B indicates the direction of the photon beam passing through bone and soft tissue; path S is one possible path through soft tissue alone. The latter measurements are used to determine a correction factor for soft tissue.

$$I = I_0 e^{-\mu_T(t-b)} e^{-\mu_B b}$$

where μ_B is the linear absorption coefficient of bone per centimeter and b is the thickness of the bone in centimeters. The thickness b can be obtained by measuring a radiograph of the bone in question. In addition, the portion of bone through which counts are to be made is determined from the radiograph. The distance from the end of the finger to the point in question is indicated by a "finger locator" calibrated in mm.

Figure 5 is a sketch of an idealized finger bone cut through the section of measurement. The cortical bone is in the shape of the letter D, with the upright on the palmar side. It is evident that the part of the cortical bone to which the greatest force is applied in using the hand has a flattened surface, thus minimizing local stress within the bone. The back of the finger with a curved member forms an ideal structure for strengthening the flat portion. Lower density medullary bone is present at the center of the bone. Such a medullary center helps to reduce the overall weight of the finger.

Figure 5. Palm-up diagram of normal and osteoporotic bone in cross-section showing the "D" shape of the finger bone.

In advanced cases of osteoporosis, we, as well as others, have observed a thinning of the cortex (see right-hand section of Figure 5). We have also found that the cortical bone disappears from the inside and not from the outside of the finger bone (see also Meema and Meema⁹). In our transmission measurements we measure through the flat palmar section and thus are measuring both the cortical and medullary changes.

The finger counting method with ^{241}Am is essentially the same as with ^{125}I , the basic difference being that the ^{241}Am source and detector assembly moves constantly, printing counts every 6 sec as it scans across the finger. In the ^{241}Am set-up the collimator diameters are 1.0 mm for the source collimator and 5.0 mm for the detector collimator.

The ^{125}I method has proved valuable in studies of postmenopausal osteoporosis. Davis, Strandjord and Lanzl⁶ found that estrogen therapy after menopause significantly retarded the rate of bone demineralization in aging females. Forland, Strandjord, Paloyan and Cox⁷ have also shown that bone mineral content is decreased in a series of confirmed hyperparathyroid cases. Most of these latter patients appeared to have normal bone mineral content when examined radiographically, but their relatively low linear coefficients of absorption suggested that bone demineralization caused by elevated levels of parathyroid hormone had begun.

The ^{241}Am method includes measurement films of each bone studied. Positioning is similar to that for the ^{125}I method except that slightly different criteria are used for different bones.

We are measuring the fingers of several series of patients on both apparatuses with different sources. These series include normal and abnormal adult patients of all ages and both sexes. Figure 6 shows the average overall bone coefficient $\hat{\mu}_B$ with ^{125}I plotted versus the average over-

Figure 6. $\hat{\mu}_B$ with ^{241}Am versus $\hat{\mu}_B$ with ^{125}I —normal females.

all bone coefficient $\hat{\mu}_B$ with ^{241}Am for premenopausal females, postmenopausal females treated with estrogens and postmenopausal females not given estrogens. The second phalanx of the third digit of the left hand was measured in all cases.

In current studies of the calcaneus we are measuring the narrowest region of the "hour-glass" configuration seen in the lateral view (see Figure 7). The complete series of counts across each heel is recorded, and the lowest count is used as a measure of bone density. The lowest count, i.e., the region of greatest bone absorption, appears on the sole side in most of the patients so far examined, but it may also occur through the posterior region of the calcaneus. The position labeled "4.5 cm" indicates the section of the calcaneus used for all evaluations to date.

Figure 7. Diagrams of calcaneus from two radiographic views.

A typical count of the calcaneus is made as follows:

A. The patient's foot is strapped into the radiograph template which is located on a standard 8" x 10" x-ray film cassette. The resulting film shows the location of the calcaneus with respect to the superimposed 8 cm x 8 cm scanning area. From this radiograph, the investigator determines the desired scan level on the Y axis and where the beginning and end points on the X axis should be. A second anterior-posterior radiograph taken at 30° allows the thickness of the calcaneus to be measured at the scanning level (the film measurement is multiplied by a correction factor of 0.94 to give the true thickness of the bone). This information is required for calculation of the linear coefficient of absorption.

B. The source and detector assembly is programmed to produce the scan. Using the rapid traverse buttons adjacent to the source position indicator grid, the source is positioned at the appropriate axis setting for the scan level as determined from the radiograph. The scan limits are then set up on the X axis. When the limits have been set, evaluation can begin.

C. The foot positioning fixture is placed in the detector, polyethylene bags of water or tissue equivalent gel are loaded into the fixture, and counts are made through the water phantom thus produced.

D. Finally, the patient's foot is positioned in the fixture as in Figure 8, and the air space is filled with polyethylene bags of water or tissue equivalent gel. The total thickness of bone, tissue, and tissue equivalent is 12.35 cm. Typical collimation is 2.0 mm on the source and 10.0 mm on the crystal. Counting time is at 30-sec intervals. When the patient's foot is in position, the START scan button is depressed and data are printed every 30 sec until the assembly

reaches the end of the scan, when it rapidly returns to the starting position.

E. Currently, we are using the lowest count through each calcaneus as an indicator of the bone mineral content; subsequent re-evaluations can be made from the stored data.

Figure 8. Patient in position for calcaneus scan.

The ratio of ^{241}Am counts through water alone to the low count through the calcaneus immersed in water has been calculated for each patient on whom we have performed both calcaneus and phalanx scans. Our data on the calcaneus are as yet insufficient to provide us with significant information on variations associated with different disease states. Their value will increase as measurements from large series of patients accumulate.

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AN AUTOMATIC PATIENT-CONTOUR MEASURING APPARATUS*

By

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The determination of isodose distributions for the purposes of radiation treatment planning often requires obtaining one or more cross-sections of a given patient through the tumor volume. The required accuracy of the cross-sections depends on the location of the tumor in the patient and the proposed treatment plan. For instance, a wedge pair treatment of the maxillary sinus would require a contour of greater accuracy than a three field treatment of the bladder. Any method used to obtain these contours should be capable of the highest required accuracy.

At present the most popular method of obtaining a contour is by means of an adjustable curved rule. The contour obtained generally shows loss of fine detail and its accuracy is dependent on how meticulously the procedure is carried out. A more accurate method of obtaining a contour is by means of a device consisting of a series of movable rods in individual clamps around the periphery of a circular or elliptical support. This device is unwieldy and its resolution is limited by the number of rods. Contours are also constructed by a variety of devices based on the pantograph principle. It has been our experience that all three methods are awkward, time consuming, and generally incapable of the required accuracy.

AUTOMATIC CONTOURING SYSTEM

To improve upon the situation, we have developed and placed into clinical operation at the Argonne Cancer Research Hospital (ACRH) an automatic contouring system based on an optical-electronic range finder coupled to the polar plotter. The optical portion of the system has been incorporated into the rotating structure of a moving field cobalt-60 teletherapy unit.¹ The patient's contour is recorded on a polar plotter as the range finder revolves about the patient who remains in a recumbent position. Scanlon² described a manual system that has some of these features, but it is inconvenient and slow.

In our system the measurement of distance is based on the use of two slightly-convergent light beams which are directed onto the patient and thereby form two light spots on the skin. The separation of the spots is proportional to the distance between the patient's skin surface and the position at which the convergent light beams cross, i.e., the crossover point.

The images of the spots are focused onto a slit wheel which rotates at constant speed in front of a photomultiplier producing pulses whose temporal separation depends on the spatial separation of the spots. Thus, the distance between the patient and the crossover point is converted to a time interval between electronic pulses.

These pulses are then used to generate a square wave which has a width proportional to the time interval between pulses. The area under the square wave is integrated by a capacitor, pro-

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ducing a D.C. voltage. This voltage controls the pen position on the radial arm of the plotter with a zero offset corresponding to the position of the crossover point. Thus the pen position corresponds to the distance between the skin surface and the center of rotation of the teletherapy unit. The angular position of the plotter, on the other hand, is synchronized to the angular position of the therapy unit by means of selsyn motors. Thus the contour is recorded on the plotter as the unit revolves about the patient.

OPTICAL AND MECHANICAL OPERATION OF THE SYSTEM

The layout of the optical-mechanical part of the system is shown in Figure 1. A light source and series of prisms and lenses project two slightly convergent light beams toward the patient, producing two light spots. These light beams are alternately interrupted by a rotating

Figure 1. Optical-mechanical portion of the automatic contour system.

shutter wheel. (The phasing of the shutter is detected by a second light source and photodiode.) The images of the light spots are focused by a lens through a pentaprism onto the plane of a slit wheel. Because of the relative orientation of the two wheels, which are mounted on the same shaft, even numbered slits scan the image of the left beam and odd numbered slits scan the image of the right beam. A photomultiplier tube located behind the slit wheel generates a pulse when a slit encounters an image of a light spot.

When the skin surface is at the crossover point, the two images appear at the same position on the slit wheel and the pulses are uniformly spaced. As the skin moves away from the crossover point the images separate and the pulses become nonuniform.

Under certain conditions an approximately linear relationship exists between the distance from the crossover point and the time interval between pulses. Figure 2 is an unfolded sketch of the system to demonstrate this relationship.

In this figure

$$\Delta' = \Delta \left(\frac{l}{d + x} \right) \quad (1)$$

Figure 2. Unfolded view showing light beam geometry. The crossover point, where the light beams converge, is positioned to fall on the axis of the focusing lens.

where Δ is the separation of the two light spots on the patient's skin,

Δ' is the separation of the images of these spots on the rotating slit wheel,

d is the distance from the lens to the crossover point,

x is the distance from the crossover point to the patient's skin, and

l is the distance from the lens to the rotating slit wheel.

The rotating slit wheel converts the separation Δ' into a time interval t determined by (see Figure 3):

$$t = \frac{\Phi}{\omega} + \frac{\theta}{\omega} = \frac{2}{\omega} \sin^{-1} \frac{\Delta'}{2r} + \frac{\theta}{\omega}$$

$$t \cong (1/\omega) (\Delta'/r) + (\theta/\omega) \quad \text{for small } \frac{\Delta'}{r} \quad (2)$$

where ω is the constant angular velocity of the slit wheel,

Φ is the angular separation between images,

r is the radial distance from the axis of the slit wheel to the images, and

θ is the angle between slits, $\pi/4$.

By substitution of equation (1) into (2):

$$t \cong \frac{l\Delta}{\omega(d+x)r} + \frac{\theta}{\omega} \quad (3)$$

* NOTE: Point P is the position of light spot images when the patient's skin surface is at crossover distance.

Figure 3. Front view of slit wheel showing the locus of light spot images and the range of detection.

From geometry: (see Figure 4)

$$r = r_o - a\left(\frac{\ell}{d+x}\right),$$

but

$$a = s\frac{x}{d},$$

therefore,

$$r = r_o - \left(\frac{x}{d}\right)\left(\frac{s\ell}{d+x}\right) \quad (4)$$

where r_o is the distance between the axis of the slit wheel and the axis of the focusing lens, and s is the distance between the center of the projector lenses and the center of the focusing lens in the "side view" plane (see Figure 2).

Also:

$$\Delta = bx/d \quad (5)$$

where b is the separation of the centers of the projector lenses in the "top view" plane (see Figure 2).

Then substituting equations (4) and (5) into equation (3) gives;

$$t \cong \frac{b\ell x}{\omega d(r_o d + x(r_o - s\ell/d))} + \theta/\omega \quad (6)$$

In equation (6), x is the only variable dependent on the position of the skin surface. Thus t is approximately equal to a constant plus a term directly proportional to x if

$$r_o = s\ell/d. \quad (7)$$

Fortunately, this condition can easily be met in the design of the equipment.

There are, however, still some inherent errors in the system as described. Returning to equation (2), the factor Δ'/r is the first term in the series expansion of the arc sine, while the second term gives an approximation to the error.

$$\text{Error} \cong \frac{1}{3} \left(\frac{\Delta'}{2r} \right)^3 \quad (8)$$

This error increases with displacement from crossover.

Another inherent error is due to the variation in the diameter of the light spot. The projected light source is in focus at crossover so the light spot diameter increases with displacement from crossover. Finally, there is an error that is a function of the solid angle of the light spot as seen from the focusing lens.

The net effect of these systematic errors was measured and used to make a template for remachining the slit wheel to correct for these errors.

ELECTRONIC OPERATION OF THE SYSTEM

The light signals from the optical-mechanical system described above are detected and analyzed by the electronic system (Figure 4). The light passing through the slit wheel is converted by the photomultiplier into pulses with varied spacing (Figure 4, curve a). The light from the

Figure 4. Block diagram of the electronics of the system. All components to the left of the slip rings are mounted on the rotating structure of the cobalt-60 teletherapy unit.

second source which is interrupted by the shutter wheel is converted by the photodiode into a square wave indicating which light beam is projected (Figure 4, curve b). This square wave is amplified and used to trigger a bi-stable flip-flop which thereby synchronizes two electronic gates with the alternating light beams.

The photomultiplier pulses are amplified and, in turn, drive a second flip-flop through the gate circuits. Thus the pulse due to the right light beam always turns the second flip-flop on while the pulse due to the left beam always turns it off. In this way, the system distinguishes be-

tween the two light beams even though they cross over.

The output of the second flip-flop is a square wave with an on-time that is proportional to the distance between the skin and the crossover-point (Figure 4, curve c). The on-time is equal to the off-time when the skin surface is at the crossover point. This output is transmitted through the slip rings of the teletherapy unit to the recording station, capacitively coupled, and clipped. Thus, any pick up or slip ring noise is eliminated and the square wave now has equal positive and negative voltages with respect to ground. Integration of this signal results in a voltage proportional to the displacement from the crossover point.

SERVO-RECORDER SYSTEM

The voltage output from the integrator is used to drive a servo-controlled pen of a polar coordinate recorder. The recording system (Figure 5) uses a Moseley* model 2FRAM two channel amplifier and two servo-arm assemblies. One channel is used for recording the patient contour and the other for either the output of a transmission ionization chamber or an output representing the "equivalent" unit density contour. The angle between the two arms of the recorder is

Figure 5. Polar recorder with contour plotting arm mounted at the angle corresponding to the location of the optical system in the teletherapy unit. The other arm is used to plot transmission ionization chamber data.

* F. L. Moseley Company, 409 N. Fair Oaks Avenue, Pasadena, California.

equal to the angle between the range finder and the cobalt source (see Figure 6). Thus, the real and "equivalent" contours will always be in phase. The graph paper, on which the recording is made, is held down electrostatically by four Moseley "Autogrip" platens mounted on a 32 inch diameter magnesium table. This table is synchronized to the rotation of the cobalt-60 teletherapy unit by means of a pair of selsyn motors.

Figure 6. Cobalt-60 teletherapy unit with the optical system mounted on the rotating structure (upper left hand side). The cobalt source and shield are overhead.

An offset bias is used to zero the recording pen at a distance from the table's center equal to the distance from the center of rotation of the teletherapy unit to the crossover point, i.e., about 14 cm. This distance was chosen to correspond roughly to an average patient skin position. A patient contour is obtained by rotating the cobalt-60 unit through 360° while plotting the output of the optical electronic system. The rate of rotation of the cobalt-60 unit is such that a complete contour can be completed in 30 seconds.

Figure 7. Comparison of contours of an obese patient recorded by the optical electronic system (A), and using the adjustable curved rule (B).

RESULTS AND DISCUSSION

The relationship between the range finder, cobalt-60 unit and patient is shown in Figure 6. The mechanical-optical portion of the range finder is mounted behind the holes in the upper left-hand corner of the figure. The light beams are projected through the two upper holes while the light spots are focused by the lens behind the lower hole. When the optical system is beneath the treatment cot, the bottom of the cot will be plotted on the recording. By subtracting the thickness of the cot, the position of the portion of the patient resting on the cot can be determined.

The output of the automatic contouring system differs from the actual contour by less than three millimeters maximum with an average difference of only one millimeter.

Two patient contours are shown in Figure 7; one (A) recorded directly from the optical-

electronic system, and the other (B) traced by the adjustable curved rule method. The patient contoured in this case was obese and had many skin folds. It is very difficult to make an accurate contour of such a patient by the adjustable curved rule method because of the elasticity of soft tissues. The advantages of a range finder system for such extreme cases is quite evident.

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STUDIES ON THE MECHANISMS OF ESTRADIOL INDUCED RADIOPROTECTION*

By

J. S. Thompson,[†] E. L. Simmons, M. K. Crawford, and C. D. Severson

With one exception¹ increased mortality has generally been observed when estradiol was administered at the time of total body x-irradiation of mice.²⁻⁴ We have reported⁵⁻⁷ that the injection of pharmacological dosages of estradiol, or estriol, for a two week period immediately after lethal or sub-lethal irradiation, impaired the recovery of lymphatic tissues and resulted in markedly increased mortality. Total leukocyte and neutrophil recovery were also impaired by these hormones, but not to the same degree as the blood lymphocytes, thymus, spleen, and lymph nodes. The immunologic ability to reject foreign skin grafts and elaborate serum antibody was also impaired.⁹ In contrast, there was no evidence that estradiol impaired the recovery of erythroid tissues after irradiation.

These facts, plus the observation that estradiol caused a severe wasting disorder with immunologic impairment in neonatal mice,⁸ favor the hypothesis that the adverse effects of post-irradiation administration of estrogen are secondary to a primary effect on the thymus. This theory is supported by the fact that with the exception of thymic involution neither estradiol nor estriol appears to have a significant immunologic or lympholytic effect in adult intact mice.^{6,7}

In contrast, it is well established that significant radioprotection results when estrogenic hormones are administered ten days prior to the x-rays.^{2-4,9-11} This report examines the hematopoietic and immunologic factors that may be responsible for the phenomenon of radioprotection by estradiol.

MATERIALS AND METHODS

Adult female, 12 to 20 week old CF No. 1 mice from Carworth Farms, were employed throughout. They were housed four or five per cage and maintained on Rockland Mouse Diet complete and water ad libitum.

Hormone therapy. Two forms of estradiol were used in the majority of these experiments: (1) an intramuscular injection of 0.05 ml sesame oil containing 0.166 mg estradiol benzoate, Progyon (Schering Corporation, Bloomfield, New Jersey); and (2) a subcutaneous injection of 1.0 mg estradiol (NF-X, J. H. DeLamar and Son, Inc., Chicago, Illinois) dissolved in 0.1 ml of 10 per cent NNDMA (NN-dimethylacetamide, Eastman Organic Chemical, Eastman Kodak Corporation, Rochester, New York) in propylene glycol.

In addition, estrone and estriol dissolved in 10 per cent NNDMA propylene glycol, estradiol valerate in 20 per cent benzyl benzoate (E. R. Squibb and Son, New York), and estradiol cypionate in cottonseed oil (Upjohn Company, Kalamazoo, Michigan) were utilized for comparison.

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Several dosages were investigated and will be listed for each experiment. The hormone or control vehicle solutions were injected either ten days prior to (pretreatment) or on the day of (concurrent treatment) x-irradiation.

Hematologic studies. The method of Till and McCulloch¹² was used to estimate hematopoietic recovery following lethal dosages of x-irradiation. Mice were weighed and sacrificed at intervals from 4 to 12 days after exposure; the spleens were removed, weighed, and fixed in Bouin's solution; and the grossly visible yellow nodules were counted and recorded. In some experiments, exogenous bone marrow from CF No. 1 mice was administered after irradiation. These animals received 0.5 ml of Tyrode's solution containing 10^5 nucleated isologous bone marrow cells intravenously within 4 hours of x-irradiation. The ability of the host's own bone marrow to recolonize the spleen was investigated by lead shielding either 1.0 cm of the tail or a femur during 800 R x-irradiation.

Immunologic studies. Jerne's method of assaying antibody plaque-forming cells (PFC)¹³ was used to study the immunologic responses in normal and irradiated mice treated with estradiol before and after irradiation. At the varying times indicated in subsequent individual experiments, the mice were injected intravenously with 0.2 ml of Hanks' solution containing 10^8 washed sheep red cells. Four days later the animals were weighed and sacrificed, the spleen weight was recorded, and the spleen cells were dispersed through a stainless steel mesh. Aliquots of 0.1 ml of the spleen cell suspension were mixed with 2.0 ml of agarose containing 0.1 ml of 10 per cent washed sheep red cells in Eisen's solution at 45° C, and immediately poured into petri dishes containing a base layer of agarose in Eisen's solution. After incubation for 60 minutes at 37° C, the petri dishes were over-layered with 2.0 ml of reconstituted lyophilized guinea pig complement (Markham, Chicago, Illinois) diluted 1:10 in saline. After further incubation for 60 minutes at 37° C, the dishes were washed with cold saline and the plaques were counted. The number of nucleated cells per spleen was determined by means of a Coulter Electronic Counter, and the number of plaques per spleen are recorded.

X-irradiation. X-rays were delivered by a G. E. Maxitron generator operated at 250 kvp and 30 ma, with 0.25 mm Cu and 1.0 mm Al filtration. The radiation dose was 800 R in the experiments testing hematopoietic response, but was lowered to 750 R and 550 R in experiments measuring immunologic response to reduce the increased mortality related to the injection of sheep red cells. Animal deaths were recorded and expressed as percentage survival on any given day postirradiation.

Morphological studies. Groups of 5 normal mice were injected with 0.166 mg estradiol benzoate or sesame oil and sacrificed at 4, 10, and 14 days after treatment. The thymus, spleen, and mesenteric lymph node were excised and weighed. Sections were prepared and stained with hematoxylin-eosin, and methyl green-pyronin.

Uterine weight assay. After gonadectomy, mice were injected with 0.166 mg estradiol benzoate, estradiol cypionate or estriol and sacrificed in groups of 5 at intervals up to 8 weeks after injection. The uterine horns were excised and weighed.

Statistical evaluation. The significance of the survival results in Table 1 was evaluated by the χ^2 (CHI) method, and all other parameters in the other tables by the t - test method of statistical analysis.

Table 1
EFFECT OF PRETREATMENT WITH LONG- AND SHORT-ACTING
ESTROGENIC COMPOUNDS ON THE 30 DAY SURVIVAL OF
MICE IRRADIATED WITH 800 R

Treatment	Dosage (mg)	Number of mice	Survival (%)
Estradiol benzoate	0.166	16	68.7
Estradiol benzoate	0.333	8	12.5
Estradiol in P.G. *	1.0	24	75.0
Estradiol in P.G. *	2.0	11	18.2
Estriol in P.G. *	1.0	26	15.4
Estriol in P.G. *	2.0	22	45.5
Estrone in P.G. *	1.0	15	40.0
Estrone in P.G. *	2.0	12	25.0
Controls [†]	†	37	0
Estradiol cyprionate	0.01	8	0
Estradiol cyprionate	0.1	16	0
Estradiol cyprionate	1.0	24	0
Estradiol valerate	0.2	8	0
Estradiol valerate	1.0	8	0
Estradiol valerate	2.0	8	0

* P.G.: 10% NNDMA propylene glycol.

[†] Controls are mice treated either with sesame oil or with 10% NNDMA propylene glycol alone.

RESULTS

Comparative effect of estrogenic compounds on survival. Figure 1 demonstrates the protective effect of two forms of estradiol on the survival of x-irradiated mice. It was found that the maximum beneficial effect could be obtained by the injection of 0.166 mg estradiol benzoate or 1.0 mg estradiol in propylene glycol ten days before exposure to x-rays. Neither agent, at this or any other dose level, was beneficial if given at the time of irradiation. In most cases concurrent treatment impaired the subsequent survival. Table 1 compares the effect of ten day pre-administration of other estrogenic compounds. The long acting agents, estradiol cyprionate and estradiol valerate, were never beneficial and the other relatively short acting agents, estriol and estrone, were beneficial to a lesser degree than estradiol itself. It is of interest that the higher dosages of estradiol benzoate and estradiol in propylene glycol were significantly ($P < 0.05$) less beneficial than the optimal dose. Since it was important for this study that the duration of action of these agents should be established, mice were castrated and injected with varying amounts of estradiol benzoate, estradiol cyprionate, and estriol (Figure 2). The figure shows that the uterine weight increase was maximal on the fifth day after 1.0 mg estriol in propylene glycol, on the eighth day after 0.166 mg estradiol benzoate, and on the tenth day following 0.1 mg of estradiol cyprionate. The uterine response to estriol was approximately one-

Figure 1. Effect of 0.166 mg estradiol benzoate in sesame oil and 1.0 mg estradiol in propylene glycol on the 30 day survival of x-irradiated mice. Estrogens were injected 10 days prior to x-ray.

Figure 2. Effect of 3 estrogenic compounds on uterine weight in castrated female CF No. 1 mice.

half that to the other two compounds, and abated quickly. The single injection of estradiol cypionate sustained the uterine weight for eight weeks.

Hematopoietic response. Sequential sacrifice of groups of mice after 800 R total body x-irradiation demonstrated that mice injected with estradiol 10 days before x-ray (pretreatment) had a significantly greater number of hematopoietic colonies on days 8 and 10 (Table 2) than

Table 2

EFFECT OF ESTRADIOL PRETREATMENT ON SPLENIC WEIGHT AND COLONY FORMATION IN MICE IRRADIATED WITH 800 R

Day after Irradiation	Number of Surviving Mice†	Treatment	Spleen		Colonies per spleen
			Weight (mg)	Index*	
4	5	Control	25.6	11.2	5.6
	5	Estradiol	25.6	10.4	0
6	5	Control	28.6	12.8	0
	5	Estradiol	27.3	11.5	2.2
8	5	Control	36.7	15.8	0.2
	5	Estradiol	25.1	10.9	12
10	5	Control	33.7	15.2	0
	5	Estradiol	48.9	20.6	16
12	5	Control	38.3	18.9	2.2
	5	Estradiol	94.5	37.8	F‡

* Spleen index: Spleen weight (g)/body weight (g) x 10⁴.

† Original number of mice per group: 5.

‡ F: Fusion, colonies either too large or too many to be distinguished.

those injected on the day of x-ray (concurrent treatment) (Table 3) or their appropriately injected controls. The increase in number of colonies in the estradiol pretreated mice was not apparent until 6 days postirradiation but continued thereafter. Colonies in control injected mice did not significantly increase in either the concurrent or pretreated experiments. There was no significant difference in the splenic weight or index between the estrogen pretreated, estrogen concurrently treated, or control animals through the first 10 days. Splenic weight, however, was significantly greater ($P < 0.001$) in the estrogen pretreated mice as compared with their controls 12 days postirradiation. No concurrently treated animals were available for comparison on this day.

To test the effects of estradiol pretreatment on internal migration and colonization by stem cells, the femur or 1.0 cm of the tail was shielded during 800 R total body x-irradiation (Table 4). A different pattern emerges under these circumstances. It may be seen that from the fourth through eighth days postirradiation, colonies were significantly greater in the control than in the experimental animals with the exception of the femur shielded group on day 6. Thereafter, colonies in the estrogen-treated mice increased to control levels. Splenic weights seemed to reflect the same general phenomenon, but significant differences were noted only on day 8 in

Table 3

**EFFECT OF CONCURRENT ESTRADIOL TREATMENT ON SPLENIC WEIGHT AND
COLONY FORMATION IN MICE IRRADIATED WITH 800 R**

Day after Irradiation	Number of Surviving Mice†	Treatment	Spleen		Colonies per spleen
			Weight (mg)	Index*	
4	10	Control	26.1	11.8	0
	10	Estradiol	21.6	10.6	0
6	10	Control	20.8	10.0	0
	6	Estradiol	23.7	11.1	0
8	10	Control	24.9	11.3	0
	10	Estradiol	31.1	16.2	0.6
10	7	Control	24.6	11.9	0.5
	6	Estradiol	35.7	18.5	3

* Spleen index: Spleen weight (g)/body weight (g) $\times 10^4$.

† Original number of mice per group: 10.

the tail shielded group ($P < 0.01$) and on days 8 and 10 in the femur-shielded animals ($P < 0.05$).

Estrogen also impaired the capacity of donor exogenous bone marrow cells to migrate and establish splenic colonies in untreated 800 R irradiated hosts ($P < 0.01$) (Table 5). The two experiments cannot be compared quantitatively since it is impossible to know the number of cells migrating from the shielded tail or femur, but qualitatively the same estrogen effect is apparent.

Immunologic response. Table 6 demonstrates the effect of a single injection of estradiol benzoate on the weight, total nucleated count, and number of plaque-forming cells (PFC) in the spleen in normal mice 4 days after the administration of sheep cells. The spleen size and index increased slightly compared to the control animals. The estrogen effect quickly abated and was no longer detectable on the fourteenth day. There was no significant change in the number of PFC in relation to the increased spleen size on day 10 or at any other time in these experiments. Although spleen size ($P < 0.01$), index ($P < 0.01$), and cell count ($P < 0.001$) were greater in the 550 R irradiated mice concurrently treated with estradiol (Table 7), the antibody response was not augmented. The apparent increase in control PFC on the 12th day is not statistically significant.

In this study neither control nor experimental mice survived to be assayed 12 days after 750 R, but comparison with Table 8 indicates that estradiol pretreatment did improve survival at this dose level. Although the difference in splenic weight, index, and cell count on day 16 in the estrogen pretreated and control animals irradiated with 550 R were not statistically significant because of great variability within the groups, the 750 R estrogen pretreated and 550 R control values did differ significantly ($P < 0.02$).

To investigate the reasons for the importance of the ten day pretreatment, normal CF No. 1 mice were injected intramuscularly with 0.166 mg estradiol. The mice were sacrificed 4, 10, 14 days later after peripheral counts and body weights had been obtained. The thymus, spleen,

Table 4

**EFFECT OF ESTRADIOL PRETREATMENT ON SPLENIC WEIGHT AND COLONY
FORMATION IN 800 R IRRADIATED MICE PROTECTED WITH FEMUR
OR TAIL SHIELDING**

Day after Irradiation	Number of Surviving Mice†	Area shielded	Treatment	Spleen		Colonies per spleen
				Weight (mg)	Index*	
4	5	Tail	Control	30.1	13.1	10.2
	5	Tail	Estradiol	24.1	10.1	0
6	5	Tail	Control	37.1	16.7	18 to F‡
	5	Tail	Estradiol	32.2	13.5	4.6
8	5	Tail	Control	61.2	25.2	23 to F‡
	5	Tail	Estradiol	29.3	11.7	10.1
10	5	Tail	Control	124.2	48.6	F‡
	5	Tail	Estradiol	88.6	34.7	15 to F‡
12	5	Tail	Control	182.1	66.1	F‡
	5	Tail	Estradiol	124.6	47.6	F‡
4	5	Femur	Control	29.2	11.0	1.2
	5	Femur	Estradiol	24.7	9.6	0
6	5	Femur	Control	29.7	12.3	2.2
	5	Femur	Estradiol	28.5	11.5	8.0
8	5	Femur	Control	47.5	18.6	26.2
	5	Femur	Estradiol	31.8	12.4	12 to F‡
10	5	Femur	Control	130.8	48.8	F‡
	5	Femur	Estradiol	67.4	27.0	F‡
12	5	Femur	Control	147.4	54.2	F‡
	5	Femur	Estradiol	151.4	50.1	F‡

* Spleen index: splenic weight (g)/body weight (g) x 10⁴.

† Original number of mice per group: 5.

‡ F: Fusion, colonies either too large or too many to be distinguished; in those spleens not fused within a group, the number of colonies has been averaged.

and mesenteric lymph nodes were excised, weighed, and prepared for histologic examination. Thymic involution and splenomegaly were noted in the estradiol recipients. Microscopic examination demonstrated an increase in pyroninophilic-staining erythroid and myeloid cells in the spleen as early as the fourth day (Figure 3). These regressed by the fourteenth day after treatment. The white pulp was not altered.

DISCUSSION

The protective effect of intramuscular injection of 0.166 mg estradiol benzoate 10 days

Table 5

SPLEEN WEIGHT AND COLONY FORMATION IN 800 R IRRADIATED MICE: EFFECT OF NORMAL AND ESTRADIOL PRETREATED BONE MARROW CELLS COMPARED TO ESTRADIOL PRETREATMENT IN NON-TRANSPLANTED ANIMALS*

Bone marrow	Treatment of donor	Treatment of host	Number of surviving mice [‡]	Spleen		Colonies per spleen
				Weight (mg)	Index [†]	
NONE	-	Control	5	26.1	11.7	0
NONE	-	Estradiol	5	25.1	10.9	12
100,000	Control	NONE	5	44.2	16.6	21
100,000	Estradiol [§]	NONE	5	28.1	11.8	5.8

* Assays were performed 8 days postirradiation.

[†] Spleen index: splenic weight (mg)/body weight (g) x 10⁴.

[‡] Original number of mice per group: 5.

[§] Bone marrow removed 10 days after 0.166 mg estradiol benzoate 1 M.

Table 6

EFFECT OF ESTRADIOL ON SPLEEN WEIGHT AND HEMOLYTIC PLAQUE-FORMING CELLS (PFC) IN NORMAL MICE

Day of assay*	Treatment	Number of Surviving Mice [†]	Spleen		Cell count PFC per	
			Weight (mg)	Index [‡]	x 10 ⁶	Spleen
4	Control	4	111.0	47.1	120.2	280,000
	Estradiol	4	137.3	60.3	160.2	220,000
10	Control	5	123.4	53.1	165.5	198,800
	Estradiol	5	197.0	85.4	227.8	176,000
14	Control	5	133.4	56.2	128.8	224,000
	Estradiol	5	128.0	51.0	126.5	345,800
18	Control	4	107.7	43.0	117.5	300,500
	Estradiol	5	125.4	50.3	144.3	448,800

* Days after estradiol injection, 10⁸ sheep red cells injected i.v. 4 days before assay in each case.

[†] Original number of mice per group: 5.

[‡] Index, spleen weight (g)/body weight (g) x 10⁴.

prior to total body irradiation has been redemonstrated in mice. In addition, it has been shown that 1.0 mg of estradiol in propylene glycol was equally as effective but that two other naturally occurring estrogens, estrone and estriol, were less so. Estrogenic compounds with sustained action, as indicated by their effect on uterine weight, however, were never beneficial. These

Figure 3. Histology of the mouse spleen 4 days after the intramuscular injection of 0.166 mg estradiol benzoate in sesame oil (A and B) or 4 days after sesame oil alone (C and D). Note the thickness of the red pulp and the increased numbers of pyroninophilic erythroid and myeloid cells in the estrogen-treated animal, spleen weight, 126 mg (A and B) as compared to the control, spleen weight 98 mg (C and D). Hematoxylin - eosin (A and C) and methyl green-pyronin (B and D), 103.2X.

◀ Figure 3 (continued)

Table 7

EFFECT OF CONCURRENT ESTRADIOL TREATMENT ON SPLEEN WEIGHT AND
HEMOLYTIC PLAQUE-FORMING CELLS (PFC) IN MICE
IRRADIATED WITH 800 R

Day after irradiation*	X-ray dosage	Treatment	Number of surviving mice†	Spleen		Cell count PFC per	
				Weight (mg)	Index‡	x 10 ⁶	Spleen
4	550 R	Control	5	34.4	15.4	4.80	58
	550 R	Estradiol	5	27.6	14.6	1.61	42
4	750 R	Control	5	21.2	11.7	1.25	14
	750 R	Estradiol	5	37.8	16.2	3.86	32
8	550 R	Control	5	32.0	15.6	9.50	28
	550 R	Estradiol	5	75.6	32.1	57.3	48
8	750 R	Control	4	33.8	15.6	17.3	15
	750 R	Estradiol	4	42.3	20.5	50.8	30
12	550 R	Control	5	46.6	20.5	32.3	528
	550 R	Estradiol	3	77.8	34.4	43.1	43

*10⁸ sheep red cells injected I.V. 4 days before assay in each case.

†Original number of mice per group: 5.

‡Index, spleen weight (g)/body weight (g) x 10⁴.

results also obtain in other strains of mice, such as the DBA/2 (C3H x 101)F₁, AKR, C3H, and 129/Jax (E. L. Simmons, unpublished data).

Why does estradiol provide protection if administered 10 days prior to irradiation but reduce survival if administered concurrently? Several theories have been proposed. Graham et al.⁴ demonstrated that an indirect effect of estradiol on the adrenal cortex was probably not responsible, and concluded that estrogen protection was a nonspecific reaction akin to that associated with foreign protein administration. A more specific effect, however, is suggested by Rugh and Wolff¹⁰ and Rugh et al.¹⁵ who observed that male mice are more radiosensitive than females and that castration improves survival in the male. These authors noted that castration was followed by an increase in circulating lymphocytes but felt that the rise was too long delayed to account for radioprotection.

The present studies indicate that radioprotection conferred by pretreatment with estradiol was associated with stimulation of erythroid and possibly myeloid elements in the spleen and had no relation to immunological competence. Although direct comparison cannot be made because of the use of different forms of estradiol in the two experiments, aqueous estradiol stimulated ribonucleic acid synthesis within 24 hours and deoxyribonucleic acid synthesis within 48 hours in the spleen of normal AKR mice.¹⁴ Comparative histologic and auto-radiographic examination revealed that this was almost exclusively confined to the splenic red pulp.

The biochemical studies also demonstrated that estradiol caused marked changes in the metabolism of both nucleic acids in the thymus, but that it had no effect on the mesenteric lymph

Table 8

**EFFECT OF ESTRADIOL PRETREATMENT ON SPLEEN WEIGHT AND HEMOLYTIC
PLAQUE-FORMING CELLS (PFC) IN MICE IRRADIATED WITH 800 R**

Day after irradiation*	X-ray dosage	Treatment	Number of Surviving Mice†	Spleen		Cell count PFC per	
				Weight (mg)	Index‡	x 10 ⁶	Spleen
4	550 R	Control	5	40.0	17.4	5.6	20
	550 R	Estradiol	5	34.8	15.4	3.7	27
4	750 R	Control	5	30.6	13.2	2.7	32
	750 R	Estradiol	5	30.0	13.2	3.0	23
8	550 R	Control	5	38.0	17.4	3.9	40
	550 R	Estradiol	5	44.2	19.2	5.9	62
8	750 R	Control	5	40.6	18.0	3.0	70
	750 R	Estradiol	5	46.0	24.8	3.9	70
12	550 R	Control	5	50.6	20.6	17.8	40
	550 R	Estradiol	5	52.8	23.0	28.9	20
12	750 R	Control	2	24.5	14.2	5.0	5
	750 R	Estradiol	4	54.5	23.9	19.6	8
16	550 R	Control	5	97.6	39.7	53.7	104
	550 R	Estradiol	5	142.0	59.4	102.1	58
16	750 R	Control	0	-	-	-	-
	750 R	Estradiol	4	279.0	112.1	162.4	45

* 10⁸ sheep red cells injected I.V. 4 days before assay in each case.

† Original number of mice per group: 5.

‡ Index, spleen weight (g)/body weight (g) x 10⁴.

node under these circumstances.¹⁴ The primary effect on the thymus, however, has particular relevance because we have shown that it secondarily impaired the recovery of other lymphatic tissues and immune reactions after irradiation.^{6,7} When estradiol was administered concurrently with x-irradiation in the present investigation it did facilitate hematopoietic repair to a certain degree, but this potentially beneficial effect was apparently not sufficient to offset the associated immunologic depression. These observations may explain why long acting estrogenic compounds, i.e., estradiol cyprionate or estradiol valerate, were not protective. Although pretreatment probably stimulated hematopoiesis, the sustained action prevented thymic function and lymphatic repair postirradiation.

Finally, since estradiol appeared to reduce the number of stem cells capable of migrating to the spleen and forming new hematopoietic colonies, the beneficial effect must reside in daughter cells located in the spleen, and presumably the bone marrow, which have arisen during the 10 days prior to irradiation. Estradiol appears to act as a stimulus that induces a pulse of hematopoiesis which reaches a peak near the tenth day. It is possible that the population of cells at this

time is qualitatively more radioresistant as well as quantitatively more numerous. Patt et al³ suggested this alternative in 1949. Since then Sinclair and Morton,¹⁶ Sinclair,¹⁷ and Mauro and Elkind¹⁸ and others have demonstrated that radiosensitivity is related to the stage of cell cycle. We have initiated studies to explore these questions.

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THE X-RAY RESISTANT CELL REQUIRED FOR THE INDUCTION OF IN VITRO ANTIBODY FORMATION*

By

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The remarkable sensitivity of the immune response to x-ray¹⁻³ has been attributed to injury to macrophages, specifically to antigen "processing" by such cells.^{4,5} The present experiments, using the in vitro response of mouse spleen cells to sheep erythrocytes,^{6,7} fail to support this suggestion.

Spleen cells were separated into two different populations on the basis of their adherence to plastic.⁸ The adherent population which comprises about 10 per cent of the total cells consists primarily of macrophages; the nonadherent population is composed primarily of small lymphoid cells. The recombination of both the adherent and nonadherent populations is required for the in vitro antibody response to sheep erythrocytes. Equally high in vitro responses are obtained whether sheep erythrocytes are added to the recombined populations and remain present during the 4 days of the response or are added only briefly to the adherent population. In this latter procedure, the adherent population is washed free of all detectable extracellular antigen before being combined with the nonadherent population; thus the first cell-antigen interaction is limited to the adherent population.

The following experiment demonstrated that the kinetics of suppression by x-ray was similar for the in vivo and in vitro responses. One group of mice was x-irradiated with doses of from 25 to 250 R and then immunized with sheep erythrocytes; a second group of similarly x-irradiated animals was sacrificed and their spleen cells were immunized in vitro. After four days the number of spleen cells releasing hemolytic antibody⁹ was measured in the living animals and also in the cultures, Table 1. When the radiosensitivity of the in vitro response was established, the two populations of cells separable in vitro were examined to determine the cell type impaired by x-ray. Two reciprocal experiments were performed: (1) adherent cells from x-irradiated mice were combined with nonadherent cells from normal mice, (2) adherent cells of normal mice were combined with nonadherent cells from x-irradiated mice. These experiments demonstrated that the immunologic function of the nonadherent population alone was sensitive to x-irradiation.

In one such experiment, spleen cells were pooled from 6 female DBA/2J mice that had received 500 R total body x-irradiation one hour previously.[†] The spleens were teased apart in Hank's balanced salt solution. Large cell aggregates, removed by 3 minute gravity sedimenta-

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‡ All x-irradiation done with Maxitron Gen. Elect. 250 (250 KVP, 30 ma). The mice, placed in lusteroid tubes on a lucite turntable (3-1/2 turns/minute), were exposed to total body x-irradiation at a target distance of 78 cm (Ave. output 58.5R/min) with 0.25 mm Cu and 1 mm Al filter (H.V.L. = 1.05 mm Cu).

Table 1
X-RAY SUPPRESSION OF THE IN VIVO AND IN VITRO
PLAQUE-FORMING CELL RESPONSE*

X-ray dose	Plaque-forming cells	
	In vivo [†]	In vitro [‡]
None	155	145
25R	99	58
50R	46	42
100R	36	12
175R	8	3
250R	2	0

* Groups of mice were irradiated one hour before either being injected i.v. with 2×10^8 sheep erythrocytes or killed and their spleen cell suspensions immunized in vitro with the same antigen. The in vitro and in vivo responses were measured 4 days later.

[†] The number recorded $\times 400$ = the number of plaque-forming cells per spleen. The number is the average count of six slides; the slides were duplicates from 3 mice.

[‡] The number recorded = plaque-forming cells for 10^6 spleen cells initially cultured per dish. The number is the average count of 8 slides; the slides were duplicates from each of quadruplicate cultures.

tion, were discarded. The suspension of remaining single cells was washed once, resuspended to a concentration of 1×10^7 cells/ml in modified Eagle's Minimal Essential Medium with 10 per cent fetal calf serum (MEM) and dispersed in 1.0 ml aliquots in 35 x 10 mm Falcon plastic petri dishes. The dishes were then incubated for 30 minutes. * The nonadherent cells were removed from these dishes by aspiration, pooled and re-incubated in new petri dishes for 30 min to remove any remaining adherent cells. The nonadherent cells were again pooled, sedimented by centrifugation and resuspended to a concentration of 1×10^7 cells/ml. The original dishes containing adherent cells were incubated with 1.0 ml of MEM containing 1×10^7 sheep erythrocytes. After 30 minutes the medium and suspended SRBC were removed by aspiration. The dishes containing the adherent cells with phagocytized and/or adherent SRBC were then washed twice by adding 1.0 ml of MEM to dishes and aspirating the fluid. These procedures were carried out simultaneously with spleen cells from normal non-x-irradiated mice.

The nonadherent cells from x-irradiated mice were added in 1 ml aliquots to the dishes of adherent cells from normal mice and the nonadherent cells from normal mice were added in similar volumes to adherent cells from x-irradiated mice. These cultures and appropriate control cultures were then incubated. Each day 0.05 ml of a "nutritional cocktail" and 0.05 ml fetal calf serum were added to the dishes. At the end of four days the cells were scraped from the dish, agitated to disperse clumps, and the number of direct plaque-producing cells was assayed by the slide modification of the Jerne technique. The results (Table 2) show that x-ray impaired

* All incubations involved maintaining the cultures at 37° C on a rocker table at 13 oscillations per minute in an atmosphere of 7 per cent O₂, 10 per cent CO₂ and the balance N₂.

Table 2

**PLAQUE-FORMING CELL RESPONSE OF VARIOUS COMBINATIONS OF CELL
POPULATIONS OBTAINED FROM NORMAL AND X-IRRADIATED MICE***

Combination	Plaque-forming cells
Normal adherent cells + normal nonadherent cells	197
Normal adherent cells + <u>x-irradiated</u> nonadherent cells	18
<u>X-irradiated</u> adherent cells + normal nonadherent cells	156
<u>X-irradiated</u> adherent cells + <u>x-irradiated</u> nonadherent cells	0
Normal adherent cells only	7
Normal nonadherent cells only	7
<u>X-irradiated</u> adherent cells only	0
<u>X-irradiated</u> nonadherent cells only	0
Normal spleen cells unseparated	170
<u>X-irradiated</u> spleen cells unseparated	0
Normal spleen cells unseparated (no antigen)	0
<u>X-irradiated</u> spleen cells unseparated (no antigen)	0

* Adherent and nonadherent cell populations were obtained from mice 1 hour after irradiation with 500R, or from normal mice. The plaque-forming cell response was measured at 4 days. Each number is the average of counts on 6 slides; the slides were duplicates from each of triplicate cultures. In vivo controls included 3 mice receiving the same dose of x-ray and 3 normal mice; the 6 mice were injected i.v. with 2×10^8 SRBC. Duplicate slide counts were made on each spleen 4 days after immunization. Spleens of x-irradiated mice contained 60 ± 49 plaque-forming cells; spleens of the control mice contained $56,400 \pm 12,600$ plaque-forming cells.

the immune function of the nonadherent population but had no measurable effect on the adherent population.

The same experimental design was used to examine the effects of dose of x-irradiation and of the interval between x-irradiation and sacrifice. In some experiments spleen cells were prepared from mice killed 1 hour after x-irradiation with doses of 250, 750 and 1000 R. In other experiments spleen cells were prepared from mice killed 6, 24 or 48 hours after receiving 500 R. In every experiment adherent cells from x-irradiated mice functioned as well as adherent cells from normal mice. In contrast, the function of nonadherent cells from x-irradiated mice, whether combined with adherent cells from normal or x-irradiated mice, was markedly impaired. The results were consistent for the range of x-ray doses and time intervals tested and have been combined for presentation in Table 3.

It is conceivable that x-ray injury to adherent cells might become manifest if the interval between sacrifice and antigenic stimulation were prolonged. To examine this possibility, adherent cells were prepared from mice 2 days after x-irradiation with 550 R. The adherent cells were cultured for 2 days and then exposed briefly to antigen before normal nonadherent cells were added to them. Thus, the total elapsed time was 4 days between x-irradiation and antigenic stimulation of cultures. The usual high plaque-forming cell response was observed 4 days after in vitro immunization, the response being equivalent to that for in vitro antigen stimulated control cultures of fresh adherent and nonadherent cells from normal mice.

Table 3
SUMMARY OF RESULTS FOR CELL COMBINATIONS OBTAINED
FROM X-IRRADIATED AND NORMAL MICE

Adherent population	Nonadherent population	Plaque-forming cells
Normal	Normal	167 ± 18*
Normal	X-ray	3 ± 1.5
X-ray	Normal	151 ± 19
X-ray	X-ray	0 ± 0

* The plaque-forming cell response is the mean ± standard error of the mean of in vitro responses from 11 experiments and represents the results of counts on 66 or more slides. Spleen cells were obtained from mice receiving 250R to 1000R, 1 to 48 hours after x-irradiation. Responses were measured 4 days after immunization.

These experiments show that the immune function of nonadherent cells is sensitive to x-irradiation, and conversely, that the immune function of adherent cells is highly resistant to x-ray injury. Other deductions can be made concerning nonadherent and adherent cells. First, nonadherent cells contain 2 subpopulations which are required for the in vitro response to sheep erythrocytes.¹⁰ Although the present experiments do not resolve whether one and/or the other of these subpopulations is x-ray sensitive, the following 2 observations are relevant to this question. On the one hand it is possible that neither subpopulation is directly injured by x-ray, but that the immune function is suppressed by some indirect mechanism such as release of a toxic product from injured cells. This seems unlikely since the plaque-forming cell response of 1×10^7 cells from x-irradiated mice was fully restored by addition of 8×10^6 nonadherent cells from normal mice. In other experiments from this laboratory, it has been found that thymocytes restore the in vitro antibody response of spleen cells from mice that have been thymectomized, heavily x-irradiated and then given syngeneic bone-marrow cells.¹¹ However, the addition in vitro of from 10^6 to 10^7 normal mouse thymus cells to cultures of unseparated spleen cells, or to cultures of separated and recombined spleen cells from x-irradiated mice, failed to restore the response. This result does not indicate whether or not the nonadherent population contains highly x-ray sensitive thymus-derived cells, but it does suggest a requirement for at least one x-ray sensitive subpopulation which cannot be substituted for by thymus cells. The other subpopulation in nonadherent cells probably consists of bone marrow-derived cells, cells which synthesize antibody and are identifiable as plaque-forming cells.¹⁰

Second, adherent cells from the mouse spleen contain a subpopulation of 1 to 10 per 10,000 cells which are essential for the in vitro response to sheep erythrocytes.^{10*} The morphology and function of the cells in this subpopulation is undefined at present, but it is clear that their immunologic function is relatively resistant to x-irradiation. It seems unlikely, therefore, that these cells divide rapidly and repeatedly to give rise to antibody synthesizing (plaque-forming) cells. Adherent cells obtained from normal or x-irradiated mice (800 R, 24 to 72 hours previously) contain "rosette-forming cells." Rosettes are formed by the rapid and dense clustering

* Attempts to substitute other phagocytic cells (e.g., mouse Kupffer cells or rat spleen adherent cells) for mouse spleen adherent cells have been unsuccessful.

of heterologous erythrocytes on the surface of about 1 per 10,000 adherent cells. Rosette-forming cells are different from background plaque-forming cells and are apparently specific for different non cross-reacting heterologous erythrocytes. Thus, it is possible to demonstrate in adherent cells a subpopulation which interacts with erythrocyte antigens, but it has yet to be determined conclusively that this interaction is required for the in vivo or in vitro antibody response.¹³

Most of the adherent cells are morphologically and functionally macrophages. It is not known whether they play any part in the immune response to sheep erythrocytes, but if they are functional, then that function is also resistant to x-irradiation.

Numerous investigators have suggested that ingestion and "processing" of antigen by macrophages is required for antibody synthesis by lymphoid cells.¹⁴ It has been further proposed that this phase of the immune response may be highly sensitive to x-irradiation. In the present experiments, the first antigen-cell interaction occurs with adherent cells only. Excess antigen is then removed so that the stimulus provided by antigen (directly or indirectly) to nonadherent cell populations is presumably only by interaction between adherent and nonadherent cells. If it is assumed that the adherent cells necessary for the immune response are phagocytic and that these cells, by interacting with antigen, process antigen in some manner, then clearly x-irradiation does not injure primarily macrophages or any processing mechanism of these cells for sheep erythrocyte antigens.

Many elegant experiments using cells to reconstitute heavily x-irradiated animals have identified radiosensitive populations required for the immune response, but it is obviously impossible to evaluate any requirement for radioresistant populations with such a model. This first demonstration, that a step or cell required for the antibody response is relatively x-ray resistant, may permit further dissection of the complex cell interactions required for the immune response.

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