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**Semiannual Report to
THE ATOMIC ENERGY COMMISSION**

SEPTEMBER 1969

ALEXANDER GOTTSCHALK, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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^{68Ga}, ^{99mTc}, ^{113mIn}
COMPARISON OF GALLIUM-68, TECHNETIUM-99m AND INDIUM-113m USED WITH
THE GAMMA CAMERA AND THE 3-INCH AND 5-INCH SCANNERS FOR
VISUALIZATION OF LESIONS IN THE BRAIN, KIDNEY,
LIVER, AND LUNG*

By

K. A. Lathrop, T. D. Cohen, R. N. Beck, and P. V. Harper

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When organs are visualized by means of internal radionuclides, maximum information is sought for the least radiation dose to the patient and the shortest examination time. Recognition of this achievement has been made more difficult by recent developments in nuclear medicine. One of these developments is the easy availability of many radionuclides all readily modified for selected organ localization and having gamma energies suitable for external detection, while depositing a small radiation dose in tissue either because of a short physical half-life, little nonpenetrating radiation, a short biological half-time, or a combination of these properties. A second development is the commercial production of a variety of imaging systems including single and multiple head scanners, the gamma camera, spark chamber, and image intensifiers. It is difficult to determine the combination of radionuclidic preparation and imaging system with the best detection capabilities for a designated lesion in a particular location of a specified organ for a given radiation dose. Parallel studies in the same patient are limited in applicability. Phantom studies, although more widely applicable, as generally used to simulate the clinical situation are still based on subjective interpretation of images. An objective criterion, the Figure of Merit of Dewey and Sinclair,¹ Beck,^{2,3} and others, has been used for comparison of the physical parameters of scanning systems. This concept may be extended to include the biological distribution and dosimetry^{4,5} of radiopharmaceutical preparations. In the present study such considerations have been used in a variety of simulated clinical scanning situations to compute Figures of Merit normalized to one rad absorbed radiation dose to the critical organ. The method itself is general in its application to any radionuclide used with any imaging system. For illustration, preparations of gallium-68, technetium-99m and indium-113m were used with the Nuclear-Chicago gamma camera and the 3-inch and 5-inch Picker Magnascanners for the detection of lesions in the brain, kidney, liver, and lung.

Physical properties of these three radionuclides are summarized in Table 1. ^{99m}Tc has some obvious advantages over ^{113m}In and ⁶⁸Ga, e.g., radiation shielding of multi-millicurie quantities may be accomplished with lighter, less bulky devices; contamination by the parent radionuclide is less serious due to its shorter physical half-life; multiple doses for use throughout the day may be prepared at one time with the relatively longer-lived ^{99m}Tc. The shorter physical half-lives of ⁶⁸Ga and ^{113m}In do not lower the radiation dosage to the patient since the decay of each of these produces substantial amounts of locally absorbed radiation. However, deep lesions might be detected more efficiently by the more penetrating gamma radiations of

* This report is taken from a paper that was presented at the Symposium on Radioisotope in Pharmakokinetik und Klinischer Biochemie, Wiesbaden, Sept. 26-28, 1968, sechsten Jahrestagung Gesellschaft für Nuclearmedizin, and is published in the Proceedings of these meetings.

Table 1

PHYSICAL PROPERTIES OF THREE SHORT-LIVED GENERATOR ISOTOPES

Property	Gallium-68	Indium-113m	Technetium-99m
Physical half-life	68 minutes	1.7 hour	6 hours
Parent, half-life	Ge-68, 260 days	Sn-113, 118 days	Mo-99, 67 hours
Principal gamma energy	511 KeV, .879 x 2 γ /d	393 KeV, .67 γ /d	140 KeV, .92 γ /d
Specific gamma constant (Γ)	5.04	1.45	0.56
$\bar{E}$ (MeV) (nonpenetrating radiation)	.53 (β^+ , ϵ)	.126 (ϵ)	.014 (ϵ)
Half-value layer			
Water	7.3 cm	6.8 cm	4.3 cm
Lead	.4 cm	.3 cm	.02 cm
Sodium Iodide	2.0 cm	2.1 cm	.24 cm

^{68}Ga and $^{113\text{m}}\text{In}$. ^{68}Ga might be used for determining the depth location of a lesion by taking advantage of the focusing capability of the positron attachment for the gamma camera. In addition, it might be possible in some instances to achieve a more favorable biological distribution by using indium or gallium rather than technetium.

The Figure of Merit embodies reciprocal relationships between radiation dosage to the patient, scanning time, resolution, and statistical reliability for detection of a lesion. Although statistical detection of a lesion and its perception by the physician examining a scan are not identical, it is evident that they are related, and mathematical models of perception similar to that used for detection have been proposed.^{6,7} Clearly, anything perceptible is detectable, but the reverse may not be true since a statistically detectable object may not be perceived by the eye-brain system. A high value for the Figure of Merit indicates a high rate of build-up for the signal to noise ratio in the region of interest and consequently a more rapid achievement of perceptibility than does a low value.

The Figure of Merit, as used in the present study, includes consideration of the biological distribution and radiation characteristics of the radiopharmaceutical preparation, the detection capabilities of the imaging system, and the size and depth of the lesion. Once determined, the biological measurements for a radiopharmaceutical may be used to calculate Figures of Merit for other imaging systems, and similarly, physical measurements for one radionuclide may be applied to other biological systems. Biological distribution data in humans are usually very difficult or impossible to obtain, necessitating reliance on data from laboratory animals for estimation of the human situation. Since it is also difficult to measure organ to tumor ratios of radionuclide concentration, the use of values obtained with realistic phantoms presents a reasonable solution for determining the object contrast for purposes of comparing radionuclides. The emitted gamma flux over an organ, however, is easily determined in a human subject with a detector of known sensitivity; and for comparative studies, relative organ count rates per injected millicurie suffice. Measurements involving the detection capabilities of the imaging system are the modulation transfer function and sensitivity, which, once available, may be applied with the

biological data outlined above for any pharmaceutical preparation of the designated radionuclide. For example, the absorbed radiation dose for ^{99m}Tc -S colloid calculated from biological distribution data, and the effective object modulation and emitted gamma flux for the specified lesion apply for any imaging system. Similarly, the MTF and sensitivity data for ^{99m}Tc obtained with, for example, the gamma camera, may be used for any ^{99m}Tc pharmaceutical.

METHODS

Calculation of the Figure of Merit (Q). This formulation takes into account the complete system involved in the scanning situation³ which consists of the patient whose physiological processes have distributed the radionuclide, and the collimator and detector with its associated electronics which collimate and produce pulses from the photons emanating from the patient. The situation in the patient is thought of as a lesion of specified size embedded in normal organ tissue. If C_t and C_o are the true mean count rates over the lesion and normal tissue respectively, the difference $(C_t - C_o)$ is regarded as the signal to be detected. The noise accompanying this signal is simply the random fluctuations in the signal itself, which can be measured by the standard deviation of the signal;

$$\text{S.D. } (C_t - C_o) = \sqrt{\frac{C_t + C_o}{r}},$$

where r is the observation time for C_t and for C_o . The criterion for detection is that the signal-to-noise ratio be equal to or greater than some assigned value;

$$\text{SNR} = \frac{\text{SIGNAL}}{\text{NOISE}} = \frac{(C_t - C_o)}{\sqrt{\frac{C_t + C_o}{r}}} = 2z,^3$$

where z = the number of standard deviations, which is multiplied by 2 since both false positive and false negative values must be considered. The observation time required to accumulate enough counts to resolve C_t and C_o in this situation is

$$r \geq \frac{4z^2 (C_t + C_o)}{(C_t - C_o)^2} = \frac{4z^2}{Q}$$

This equation defines Q as

$$Q = \frac{(C_t - C_o)^2}{(C_t + C_o)} = \frac{(\text{SNR})^2}{r} = \frac{4z^2}{r}$$

which can be interpreted as the rate at which the (signal-to-noise ratio)² is built up by the imaging system. If C_t and C_o are the maximum and minimum count rates in response to a sinusoidal plane wave of radioactivity with frequency ν , then $Q(\nu)$ can be interpreted as a measure of the detectability of such a sinusoid. In that case, $Q(\nu)$ can be expressed in terms of the aver-

age count rate $\bar{C}(\text{cpm}) = (C_t + C_o)/2$ and the image modulation $m_I(\nu) = \frac{(C_t - C_o)}{(C_t + C_o)}$,

$$Q(\nu) = 2\bar{C}m_I^2(\nu).$$

Since the image modulation is given by the product of the effective object modulation $m_o/(1 + S)$ and the effective modulation transfer function $MTF_G/(1 + P)$, we can also write

$$Q(\nu) = 2\bar{C} \left[\frac{m_o(\nu)}{1+S} \times \frac{MTF_G(\nu)}{1+P} \right]^2$$

where m_o = the object modulation,

S = scatter fraction,

MTF_G = modulation transfer function when septal penetration is negligible,

P = the penetration fraction.³

If the radiation dose to the region of greatest exposure is d (rads/mCi injected) and the average count rate is $\bar{C}$ (cpm/mCi injected), the Figure of Merit per rad of exposure is

$$\frac{Q(\nu)}{d} \left[\frac{\text{cpm}}{\text{rad}} \right] = 2\bar{C} \left[\frac{m_o(\nu)}{1+S} \times \frac{MTF_G(\nu)}{1+P} \right]^2$$

Quantities that must be measured are the line spread function, effective object modulation, biological distribution with time, and organ counts per minute per cm^2 per injected mCi. Each of these measurements is sensitive to the pulse height analyzer window, which was set at twice the full width at half-maximum except for the gamma camera, where a 20 per cent window with ratio circuitry was used.

Line spread functions were measured according to the method specified by the IAEA⁸ at appropriate distances from the collimator face. In addition, the ^{68}Ga source was enclosed by steel tubing 2.5 mm in diameter with one-mm walls to convert the positrons to the annihilation radiation. The modulation transfer functions (Fourier transform of the line spread function) were calculated using the computer program written by Sylvia Fedoruk.⁹ From these curves, MTF values for calculating Figures of Merit were chosen by assuming that the effective lesion size was approximated by a plane wave whose wavelength is twice the lesion diameter ("half-wavelength approximation"). For 1-inch lesions the frequency is thus 0.2 cycles per cm, and for 2-inch lesions is 0.1 cycles per cm. Since the line spread measurements were made for each nuclide with the collimator selected for the various situations, the effect of septal penetration is included. The determined value is actually the effective MTF, equal to $MTF_G/(1 + P)$. The focal planes of the collimators are assumed to be two inches below the body surface, the collimator faces of the camera in contact with the body surface, and the focal plane of the coincidence detector at the level of the lesion.

Effective object modulation is computed from the target to nontarget count ratio, where the target may be either a hot or a cold lesion surrounded by radioactivity. In all cases the lesion was large enough to cover completely the field of view of the collimator, a very fine resolution single hole with an MTF_G nearly equal to one and with no septum penetration. Values for the effective object modulation were determined by use of realistic phantoms as specified for each organ in Table 2.

The phantom measurements take account of the effects of isotope distribution, and absorption and scatter in the surrounding tissue, but not the effect of lesion diameter which is determined by the collimator MTF at the appropriate distance from the collimator face. All lesions are assumed to be cylindrical, one inch in diameter by one inch in thickness, except for the lung where they are two inches by two inches.

Table 2

PHANTOM DIMENSIONS FOR MEASUREMENT OF EFFECTIVE OBJECT (LESION) MODULATION: $m_o/(1 + S)$

Organ			Phantom		Lesion		Body/organ/lesion activity ratio	Remarks
	Thickness	Depth	Diameter	Thickness	Depth in organ	Thickness		
Brain	8"	-	8"	8"	3-1/2"	1"	1:1:5	
Kidney	1"	3"	8"	7-1/2"	-	1"	0:1:0	Correction made for observed body background.
Liver	4"	1"	8"	8"	0",1",3"	1"	0:1:0	Lesion depth measured from surface of organ to surface of lesion for liver and lung.
Lung	4"	1"	8"	6"	0",2"	2"	0:1:0	Lesion and organ simulated by air-filled gelatine capsules surrounded by activity in solution.

Using these systems, measurements include radiation scattered by the surrounding medium so that the calculated modulation contains the scatter fraction. By definition, when the MTF = 1, the effective object modulation, $m_o/(1 + S)$, is equal to the image modulation; i.e., the difference of the count rate over a lesion and the count rate over an equal area of normal tissue divided by the sum of these two. The modulation value for the annihilation radiation coincidence system of the gamma camera was computed from the attenuation coefficients for the 511 keV gamma in tissue, since the effects of scatter and attenuation in this situation are slight. For these measurements, the detector system used with ^{99m}Tc and ^{113m}In consisted of a collimator made by drilling a one-quarter inch hole through a 2-inch lead brick, which was backed by a 5-inch NaI crystal. For ^{68}Ga measurements, the collimator was a one-half inch hole drilled through 4 inches of lead, with additional lead surrounding the crystal to eliminate penetration of the 511 keV gamma.

Biological distributions of radiopharmaceuticals were determined in mice at intervals found appropriate for the biological half-time of the preparation and the physical half-life of the radionuclide. Biological half-times in humans were compared with those in mice by measuring radioactivity in the excreta. The radiopharmaceuticals used are summarized in Table 3. Many of these are well established clinical agents whose preparation and distributions may be found in the literature. Others were developed in our laboratories and are not to be considered as the optimum preparation that might ultimately be produced. A primary purpose of this entire investi-

Table 3

Organ	Radiopharmaceutical			Preparation
	^{68}Ga	^{99m}Tc	^{113m}In	
Brain	Ga-EDTA ¹⁰	Na Tc O ₄ ¹¹	In-EDTA	Equal volumes eluate and 0.005 M Na ₃ EDTA mixed and neutralized to pH 6-7 with NaOH. Sterilized by filtration through 0.22 millimicron membrane filter.
Kidney	Ga-Fe complex	Tc-Fe complex ¹¹	In-Fe complex	2 ml of 2.5 N HCl solution containing ^{68}Ga or ^{113m}In mixed with 0.1 ml 5% ascorbic acid and 0.1 ml of 1% FeCl ₃ · 6H ₂ O in 1% HCl neutralized to pH 7.8 with NaOH and sterilized by filtering through 0.22 millimicron membrane.
Liver	Ga-Zr colloid	Tc-S colloid ¹¹	In-Zr colloid	Dry, extracted ^{68}Ga dissolved in 0.05 N HCl containing 4 microgram/ml zirconium, neutralized to pH 5.5, using sterile reagents and containers. Sterile ^{113m}In eluate neutralized to pH 5.5 with sterile NaOH.
Lung	Ga-Fe aggregates	Tc-albumin macro-aggregates ¹¹	In-Zr aggregates	Dry, extracted ^{68}Ga dissolved in 0.05 N HCl containing 500 micrograms Fe ⁺⁺⁺ , neutralized to pH 7.5, using sterile reagent and containers. ^{113m}In eluate made basic with NaOH to about pH 11, neutralized to pH 8 with HCl and sterilized by autoclaving.

gation was to determine which radionuclide might most advantageously be developed for a given scanning situation. Indium-113m could be modified directly in the eluate (0.05 N HCl, pH 1.3). The ^{113m}In used in this investigation was eluted from a New England Nuclear generator in which ^{113}Sn is held on an ion-exchange bed of hydrous zirconium oxide. The nature of some of the preparations described probably depends on small amounts of the column material contained in the eluate, ^{113m}In eluates obtained from generators prepared with other ion-exchangers might behave differently. It was necessary to process the gallium-68 eluate (0.005 M $\text{Na}_3\text{-EDTA}$) by extracting it as gallium chloride into ethyl ether, evaporating off the ether and dissolving the ^{68}Ga into the desired reagent. Using the biological distribution parameters established for each preparation, the absorbed dose in rads to the regions of principal concentration was calculated by conventional dosimetry using the assumptions shown in Table 4. Doses computed by the newer method of absorbed fractions¹⁰ would not differ significantly from these for gammas in this energy range. The doses most pertinent to this investigation have been summarized in Table 5.

Organ counts per minute per cm^2 per injected millicurie over the region of interest were measured in human subjects by using the gamma camera together with a 1600 channel analyzer after injection of a carefully calibrated radionuclide preparation. Measurements over the kidney were corrected for body background by subtraction of counts collected over a similar area of the abdomen without kidney. Such a correction was necessary only for the kidney where the circulating radioactivity remained high during the first 12 hours after injection. These corrections were applied to the object contrast determined in the phantom. The collected counts were integrated over a uniform area within the borders of the region scanned. The number of channels per cm was determined by measuring a line source in two positions 10 cm apart. This measurement determines, for the camera configuration, the counts per minute per cm^2 per mCi or, with the dosimetric computation of rads to the critical organ per mCi, the counts per minute per cm^2 per rad. Using the relative sensitivity measurements between the scanner and camera this result can be transformed to the scanner, or, if desired, the reverse procedure may be carried out. In the case of coincidence measurements of positron annihilation using the camera, the maximum total count rate capability of about 10,000 counts per minute imposed substantial additional limitations in some cases. The relative sensitivities were determined using an infinite (12-inch diameter) plane source of the radionuclide. Since the camera has a useful area of detection of 507 cm^2 (10-inch diameter), it was assumed for purposes of comparison that the scanner views an equal area. The areas over which counts were integrated were in all cases the actual area of the lesion, and an equal area of non-target region.

Characteristics of the collimators used in making these measurements are shown in Table 6. All of these collimators, both for the camera and scanners, are more finely focused than is required for optimal detection of the fundamental object wavelength. This is particularly true of the collimators used with ^{99m}Tc with the scanners that sacrifice sensitivity in favor of resolution to a marked extent. Substantial septum penetration occurs with both ^{68}Ga and ^{113m}In for the collimators available with the 3-inch scanner. Results from measurements with this equipment thus represent practical observations rather than optimal comparisons. Effects of using optimal collimation will be considered in the discussion.

RESULTS AND DISCUSSION

The value determined for the Figure of Merit in each of the specified situations is shown

Table 4
DOSIMETRY PARAMETERS AND ASSUMPTIONS (1 mCi)

Organ	Shape	Size	$\bar{g}$	Mass (g)	$\mu\text{Ci/g}$ C_o	T_{eff}	
Kidney	Spheres	R = 3.3 cm	35	150 - Cortex 300 - Total	$\frac{50}{300} (^{68}\text{Ga})$ $\frac{150}{300} (^{99\text{m}}\text{Tc})$ $\frac{200}{300} (^{113\text{m}}\text{In})$	T_p	Kidney agents fixed in cortex (β dose) γ dose to whole organ (EDTA)
Liver	Sphere	R = 7.2 cm	59	1500	$\frac{800}{1500}$	T_p	
Lungs	Cylinder	H = 20 cm R = 10 cm	67	1000	$\frac{800}{1000}$	T_p $\frac{T_p}{2} (^{99\text{m}}\text{Tc})$	Lungs together as a unit
(Brain) Stomach and contents	Sphere	R = 4 cm	$g_p = 25$	300	$\frac{150}{300} (^{68}\text{Ga})$ + $(^{113\text{m}}\text{In})$ $\frac{300}{300} (^{99\text{m}}\text{Tc})$	T_p (Ga) $\frac{T_p}{2}$ (Tc) 36 min (In)	β dose = C_o equal in stomach and contents γ dose = mucosa at surface of sphere

Table 5
RADS PER INJECTED MILLICURIE TO ORGAN RECEIVING
HIGHEST DOSE

Organ visualized	Critical organ	^{68}Ga	$^{113\text{m}}\text{In}$	$^{99\text{m}}\text{Tc}$
Brain	^{68}Ga , Kidney $^{113\text{m}}\text{In}$, Kidney $^{99\text{m}}\text{Tc}$, Stomach	1.05	0.14	0.19
Kidney	Kidney	0.67	0.96	0.34
Liver	Liver	1.23	0.46	0.29
Lung	Lung	1.56	0.53	0.25

in Table 7. In all cases except one the Figure of Merit for the gamma camera is higher than for either scanner. The exception, ^{68}Ga in the lung with the coincidence detector system, is explained by the count rate limitation of the annihilation radiation detector, which is unable to make full use of the emitted gamma photon flux. This same consideration also lowers the value of Q/rad for ^{68}Ga in the liver using the coincidence detector, although to a much less extent, because of the greater tissue absorption. This problem of count rate limitation is not present with small areas of radioactivity, as shown by the high Q/rad values of ^{68}Ga in the brain and kidney, the latter being the only instance where $^{99\text{m}}\text{Tc}$ does not have the highest number. Positron annihilation radiation seems usable in this situation particularly where the location of the lesion can be reasonably well estimated, as in the kidney, so that its tomographic capability can be fully utilized. In the brain, this type of detection could also be exploited for the tomographic capabilities shown by the equally good values for surface and deep lesions. The thin NaI crystal (1/2 inch) of the gamma camera is highly efficient for the 140 keV gamma from $^{99\text{m}}\text{Tc}$, but not for the 393 keV energy from $^{113\text{m}}\text{In}$ which results in a lower Q/rad value.

The very high Figures of Merit for the lung represent exceedingly short detection times, a few milliseconds for 99.9 per cent confidence with $^{99\text{m}}\text{Tc}$, and suggest the superiority of this radionuclide for dynamic perfusion studies. Although values for $^{99\text{m}}\text{Tc}$ are higher than those for $^{113\text{m}}\text{In}$ in the lung, it is much easier to make a preparation of $^{113\text{m}}\text{In}$ carried on an inert hydroxide than macroaggregates of high specific activity Tc-labeled albumin. For this reason, many laboratories prefer to use the former. Perhaps the preferable alternative is perfection of a more easily prepared $^{99\text{m}}\text{Tc}$ lung-scanning pharmaceutical.

As anticipated, the 5-inch scanner with its NaI crystal of greater diameter than that of the 3-inch scanner (and consequently a longer collimator), is more efficient for $^{113\text{m}}\text{In}$, and higher Q/rad values are obtained with this instrument than with the 3-inch scanner. With ^{68}Ga , septal penetration is still quite high with the collimator for the 5-inch crystal, and the same increase in Q/rad is not realized. $^{99\text{m}}\text{Tc}$ and $^{113\text{m}}\text{In}$ have comparable values when the 5-inch scanner is used. With both rectilinear scanners, Figures of Merit are lowered for ^{68}Ga and $^{113\text{m}}\text{In}$ because the high septal penetration degrades the modulation transfer function even when using the best collimators available for this study. Line spread functions determined for ^{68}Ga were mea-

Table 6
CHARACTERISTICS OF COLLIMATORS

Radio-nuclide	Gamma camera	3-inch Magnascanner					5-inch Magnascanner				
		Colli-mator No.	Number of holes	Focus	Focal depth (inches)	50% Iso-response (inches)	Colli-mator No.	Number of holes	Focus	Focal depth (inches)	50% Iso-response (inches)
^{68}Ga	Positron head	2107	19	Medium	3	1/2	2111	85	Coarse	5	1/2
$^{99\text{m}}\text{Tc}$	1-3/4" 4000 hole	2113	73 (low energy)	Medium	3	1/4	2114	265 (low energy)	Fine	3	3/16
$^{113\text{m}}\text{In}$	3" 1000 hole	2107	19	Medium	3	1/2	2111	85	Coarse	5	1/2

Table 7

FIGURES OF MERIT (Q/rad) FOR DETECTING VARIOUSLY LOCATED LESIONS

Radionuclide	Instrument	Brain	Kidney	Liver			Lung	
				Lesion depth [†]			Lesion depth [†]	
				Surface	1 inch	3 inches	Surface	2 inches
⁶⁸ Ga	Gamma camera	8.2	230.0	5.3	5.3	5.3	62.0	62.0
	3" Magnascanner	.12	2.9	2.5	0.58	0.13	190.0	92.0
	5" Magnascanner	.14	3.5	2.3	0.47	.20	130.0	85.0
^{99m} Tc	Gamma camera	20.0	88.0	540.0	172.0	42.0	9500.0	4800.0
	3" Magnascanner	1.7	7.0	39.0	14.5	2.9	690.0	325.0
	5" Magnascanner	1.8	7.5	27.0	16.0	2.2	550.0	310.0
^{113m} In	Gamma camera	4.8	15.0	71.0	17.0	8.3	1400.0	600.0
	3" Magnascanner	1.3	3.9	17.0	3.6	1.6	200.0	86.0
	5" Magnascanner	3.8	11.0	25.0	9.6	5.5	550.0	320.0

[†] Measured from organ surface to top of lesion.

sured with the source encased in a steel tube to convert the positrons at their source. In tissue, the positrons travel farther before being annihilated, which would give greater degradation of the resolution than is evident from the experimental studies, and consequently Q/rad for this radionuclide would be lowered in actual practice.

It is interesting to consider the effect that optimal collimation would have on the results presented here. It has been shown³ that optimal geometrical collimation for a given space frequency involves a diameter of view approximately equal to the wavelength, or a full-width-at-half-maximum equal to the lesion diameter (half-wavelength approximation). Even the coarse focus collimators used here have a resolution twice as fine as this optimal value. This very coarse focus optimal collimation is of course optimal only with respect to detection, and depicts lesions very poorly. Nevertheless, it is instructive to look at the effect such an "optimal" collimator would have on the Figures of Merit. The ^{99m}Tc collimators used are comparatively fine focus, since the 50 per cent isoresponse region is one-fourth inch for the 3-inch scanner, and three-sixteenths inch for the 5-inch scanner, as compared to a 50 per cent isoresponse region of one-half inch for the collimators used with ⁶⁸Ga and ^{113m}In. They are thus considerably less optimal than the ^{113m}In and ⁶⁸Ga collimators, so that optimal geometrical collimation would increase the ^{99m}Tc Figures of Merit substantially relative to those for ⁶⁸Ga and ^{113m}In, especially for the 5-inch scanner. The magnitude of this effect has been estimated³ for cases lying at the focal distance. The Figure of Merit increase for the high energy collimators is by a factor of 4, while for the low energy collimators the increase is by a factor of 7.5 for the 3-inch scanner and 10 for the 5-inch scanner. The effect of using optimal geometric collimation is thus a relative improvement in the ^{99m}Tc Figures of Merit by a factor of 2 to 2-1/2 for the scanners. For the camera at 3 inches from the collimator face, optimization of the collimators improves the Figures of Merit by approximately a factor of 4 for both ^{113m}In and ^{99m}Tc. The question of collimator optimization does not apply to the coincidence system, which does not achieve improved resolution by excluding emitted photons. Thickening of the septa of the high energy collimators for the scanners used with ⁶⁸Ga and ^{113m}In would reduce septal penetration and hence increase the image contrast, but the sensitivity would be reduced. The overall gain in Q/rad achievable by this process is probably less than a factor of two.

When Q/rad for a radiopharmaceutical is known for one imaging system and object (A), the corresponding value for some other system (B) can be obtained readily after determining MTF_(B) and Sensitivity_(B)

$$Q/rad_{(A)} \times \left[\frac{MTF_{(B)}}{MTF_{(A)}} \right]^2 \times \frac{SENSITIVITY_{(B)}}{SENSITIVITY_{(A)}} = Q/rad_{(B)}$$

Similarly, when Q/rad for any imaging system is known for one radiopharmaceutical (D), the Q/rad for another pharmaceutical (E) of the same radionuclide used with the same imaging system for the same object may be obtained after determination of the dose to the region of greatest exposure and the cpm/mCi from the region of interest by,

$$Q/rad_{(D)} \times \frac{\frac{\bar{C}}{d} (E)}{\frac{\bar{C}}{d} (D)} = Q/rad_{(E)}$$

Inherent in this application of the Figures of Merit is the amount of radioactivity which may be injected to deliver one rad to the critical organ. For ^{113m}In and ^{99m}Tc these quantities are very comparable, the greatest variation being less than a factor of three greater for ^{113m}In in the kidney. Costs per patient dose are comparable for each of the preparations, but can be determined accurately only in terms of conditions existing within the particular clinical situation. It is our belief that when a choice exists, evaluation of a radionuclide preparation must be made by each nuclear medicine installation in terms of available imaging equipment, patient dosage, clinical volume, radiation exposure to personnel, and available technical capabilities. As far as the actual procedure of comparison is concerned, the method used here appears to encompass most of the accessible parameters.

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RADIOGRAPHIC DEVELOPMENT OF SKELETAL LESIONS IN MAN MANY YEARS AFTER ACQUISITION OF RADIUM BURDEN*

By

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The present permissible occupational levels for the bone-seeking radionuclides are tied to that for ^{226}Ra , which has served for several decades as the "prime standard." To establish these permissible levels and that of ^{226}Ra at certain points, observable effects have been related to carefully determined body content of the radionuclide under consideration. This has been done with various species of experimental animals for many of the bone-seeking radionuclides, and a scale of relative radiotoxicities has been derived. The experimental approach is not possible in man, and therefore we have attempted to derive as much information as possible from a rather large group of persons in mid-western United States who acquired a body burden of ^{226}Ra approximately 40 years ago. From these data we have related ^{226}Ra body content to the incidence of skeletal and other rare tumors arising in the tissues lining the mastoids, paranasal sinuses, and oral cavity on the one hand, and to other radiographically observable destructive bone changes, on the other.

CLINICAL MATERIAL AND METHOD OF STUDY

For the past 20 years a joint study of more than 400 persons bearing a detectable body burden of ^{226}Ra has been under way at the Argonne National Laboratory and the Argonne Cancer Research Hospital. Most of these persons were radium dial painters in the Illinois river valley region during 1920-1940; others received radium chloride intravenously or orally as a medicament between 1920 and 1933. Of the latter, members of a unique group of 41 patients were treated for mental illness in a state hospital in Illinois during 1931-1933 with a series of intravenous injections of radium chloride.¹ Twenty-two of these mental patients have had skeletal radiographic studies along with determinations of radium burdens by gamma ray spectrometry, and 11 of these 22 have had these studies repeated almost every two years since the study began in 1951. In addition, 34 other patients who received radium as a medicament and 219 former radium dial painters have had partial or complete skeletal radiographic surveys and radium body determinations done on at least one occasion.

The radiographic lesions appearing in these radium-bearing patients include coarsening of the trabecular pattern, localized areas of bone resorption, patchy sclerosis, small and large bone infarcts, aseptic necrosis, and pathological fractures of bone in addition to the aforementioned malignant tumors.²⁻⁶ A method for numerically scoring the radiographic lesions according to increasing severity was devised by our group, and with some modifications was adopted by the other radium study group at the Massachusetts Institute of Technology. This numerical score was also tied to a "clinical radiographic impression" of the severity of the lesions in

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terms of clinically disabling orthopedic disease. This impression included the broad categories "negative," "minimal," "mild," "advanced," and "advanced-malignant." During the earlier years of reading and scoring the skeletal radiographs, our group was struck by its apparent ability to estimate with a high degree of confidence the radium burden of the patients on the basis of the severity of the radiographically demonstrable lesions. This was 20 to 30 years after the acquisition of the radium burden.

Several generalizations were drawn from these observations. One was that there was a general correlation between the number and severity of the radiographically demonstrable bony lesions and the body burden of ^{226}Ra at that time. The other was that significant radiographic lesions distinguishable from usual aging phenomena were not seen at body burdens of below $0.1 \mu\text{g } ^{226}\text{Ra}$. During the immediately past decade, it became apparent on restudy of many of these patients that an increase in the number and severity of the radiographically demonstrable lesions had occurred concomitantly with a decrease in body burden of radium.

FIGURES 1-6

The retention of radium in man can be described as a power function of the form " $a t^{-b}$,"^{6,7} or as a sum of 3 exponentials.⁷ The slowest (third) component of the sum of 3 exponentials gives a retention half-time, at times after 1 year's retention, of approximately 15 years. It is therefore, not unexpected that the body burdens of radium in the patients under study by us for the past 20 years will have decreased considerably in this interval. With the passage of time, the relationship of the severity of the radiographically demonstrable skeletal lesions to current body burdens of radium is becoming less and less meaningful.

In order to look at parameters other than current or terminal body burdens of ^{226}Ra , calculations have been made by use of a sum of 3 exponentials fit, or the power function for radium retention ($.30 t^{-.44}$) of dose rates, total doses, and other parameters for each former dial painter under study. These computations include

Figure 1. Patient 03-423. Skull in 1951. Body burden ^{226}Ra greater than $0.765 \mu\text{g}$.

Figure 2. Patient 03-423. Skull in 1959. Increase in number and size of areas of bone resorption in skull. Body burden ^{226}Ra = 0.590 μg .

Figure 3. Patient 03-423. Skull in 1962. Further increase in lesions of skull. Body burden ^{226}Ra = 0.552 μg .

1. Microcuries of ^{226}Ra absorbed per day during the work period, and total microcuries absorbed,
2. Peak initial burden of radium, calculated retrospectively from the known injection history or from a reconstruction from recent body burden determinations,
3. Radiation dose rates to the skeleton in rads per day at various times after cessation of radium intake, and
4. Total accumulated dose to the skeleton at various times after cessation of intake.

The following combinations of parameters were then analyzed for correlations

Figure 4. Patient 03-423 (left). Left shoulder in 1951. Body burden ^{226}Ra greater than $0.765 \mu\text{g}$.

Patient 03-423 (right). Left shoulder in 1959. Bone resorption and sclerosis in head of humerus now prominent. Body burden $^{226}\text{Ra} = 0.590 \mu\text{g}$.

Figure 5. Patient 03-423 (left). Right hip in 1957. Small area bone resorption in acetabulum. Body burden $^{226}\text{Ra} = 0.765 \mu\text{g}$.

Patient 03-423 (right). Right hip in 1962. Increase in bone resorption in acetabulum and destruction in head of femur. Body burden $^{226}\text{Ra} = 0.552 \mu\text{g}$.

1. "Radiographic numerical score" versus
 - a. radiation dose rate to the total skeleton at the first day off work or completion of injections, and six other arbitrarily selected time periods thereafter to 40 years,
 - b. total accumulated dose in rads over a somewhat similar array of time intervals to 40 years;
2. "Radiographic impression" versus the above listed groups of dose rates and total dose;
3. "Radiographic numerical score" versus the total calculated amount of radium absorbed;
4. "Radiographic impression" versus total calculated amount of radium absorbed.

Figure 6. Patient 03-449 (left). Right foot in 1958, normal. Body burden ^{226}Ra = 1.610 μg .

Patient 03-449 (right). Right foot in 1964. Aseptic necrosis of tarsal scaphoid. Body burden ^{226}Ra = 1.135 μg .

These studies are a first approximation, and the groups include the multiple determinations of body burden and radiographic scoring done on many of the individuals studied. In addition, at this time, the radiographic impression group "advanced" contains all of those dial painters with malignancies or pathological fractures, even though the degree of bone damage demonstrated radiographically might otherwise place the individual into a very much lower radiographic impression group.

RESULTS

Increasing radiographic score and severity of impression has been noted in the past decade for many of the patients studied. During the same intervals, the body burdens of ^{226}Ra predictably have continued to decline.

For the total array of 308 studies, a degree of correlation significant at the .001 level has been noted in a preliminary study of dose, and of dose rates versus radiographic score at each

of 7 different times from 1 day to 40 years. When, however, the correlations within each radiographic impression group versus total dose, or dose rate at 1 day and at 5 years after cessation of exposure are looked at, it is apparent that the correlations are not statistically significant except for the group listed as "advanced." In this group the correlations are highly significant. Correlations are also highly significant at a "p" value of .001 for the relationship of the total 308 studies versus terminal body burden of radium and versus the total amount of radium calculated to have entered the blood stream either by the power function or sum of exponential methods. Again the correlations are significant at the .001 level for score within the "advanced" lesion group and these dose parameters. Within the other impression groups, however, the correlations are not significant.

DISCUSSION

The relationship between terminal body burden and radiographic score of the total group studied continues to be valid at this time. The proposition that there is a general relationship between terminal body content and the severity of bony lesions and number of bones involved still holds. It is to be expected, however, that with the passage of time this correlation will become more tenuous as the score increases and the body burden decreases.

The skeletal lesions described are not in themselves unique for radium poisoning, and are shared by aging phenomena, corticosteroid administration, caisson disease, and several systemic disorders. The constellation of these lesions, however, especially in patients in middle age groups, should arouse the suspicion of radium poisoning. The lack of correlation between body burden, dose, and dose rates within the "impression" categories reflects the overlap with aging phenomena, especially in the categories "negative," "minimal," "mild," and "moderate." Within the "advanced" group, the score is highly correlated with body burden, dose, dose rates, and quantity of radium which entered the blood stream.

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RADIOBIOLOGICAL PARAMETERS IN HUMAN CANCERS ATTRIBUTABLE TO LONG-TERM RADIUM DEPOSITION*

By

A. J. Finkel,[†] C. E. Miller,[†] and R. J. Hasterlik

For the past 20 years investigators at Argonne National Laboratory and Argonne Cancer Research Hospital have studied the long-term effects of radium deposited in the skeletal tissues of man and experimental animals. The results of studies with mice have related the carcinogenic action of radium to that of other bone-seeking radionuclides.^{1,2} The consequences of radium deposition in man were reported by Martland in a series of papers³ and later by other investigators^{4,5} for a group of dial painters in eastern United States and by Looney et al⁶ for a series of patients in the Chicago area.

The radium patients that we have studied comprise the following population:

Occupational exposure	
Dial painting industry	208
Radium chemists	4
Iatrogenic cases	
State mental hospital	41 ^{7,8}
Private patients	40

These numbers represent cases that we have either studied in some detail or about whose radium body burden and medical fate we have some knowledge. The basic data relating to the occupational exposure, medical history, radium burden measurements, skeletal radiography, and other pertinent items for these patients recently have been published in a joint report from Argonne National Laboratory and Argonne Cancer Research Hospital.⁹

MALIGNANT TUMORS IN RADIUM DIAL PAINTERS

The malignant tumor experience of our radium patients was discussed in some detail at the University of Utah Symposium on "Delayed Effects of Bone-Seeking Radionuclides" in September, 1967.¹⁰ The data in the present report are somewhat different, having been modified by the addition of a newly developed case of osteogenic sarcoma (Case 03-429) and the deletion of a much debated case of questionable carcinoma of the maxilla (Case 03-685). The tissues of the latter patient have been examined widely and the opinion of competent oral pathologists is that the lesion is not a malignant tumor but instead may be a benign tumor of the odontogenic epithelium or may represent a stage in the healing of a tooth socket affected by periodontal disease. For this reason we have removed it from our list of established malignancies attributable to radium deposition in bone. In addition, the mathematical treatment and evaluation of the data here goes beyond our previous paper.

* This report is taken from a paper presented at an IAEA Symposium on Radiation-induced Cancer, in Athens, Greece April 28-May 2, 1969 and will be published in the Proceedings thereof.

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The malignant tumors of skeletal tissues in radium dial painters who have had radium body burden determinations are given in Table 1. Similarly, Table 2 lists carcinomas, principally of the mastoids and the jaw and a case of "splenic leukemia" (chronic myelogenous leukemia), that have developed in dial painters. The cases of malignant tumors without body burden measurements have been omitted since they cannot contribute to an analysis of the radiation and radiobiological parameters that may be involved in radiation carcinogenesis. In our experience, the development of carcinomas of the mastoids, paranasal sinuses, and other locations close to the bones of the skull is largely a phenomenon of the 1960s and has generally occurred much later than most of the osteosarcomas and the aplastic anemias and leukemias.

For the present purposes, the presence or absence of radium-induced malignancies will be discussed in terms of these radiobiological indices: (1) current or preterminal radium body burden, (2) retrospectively estimated maximum body burden (i.e., peak initial burden), (3) estimated amounts of radium absorbed daily into the bloodstream from the gastro-intestinal tract, and (4) estimated total amount of absorbed radium during the critical work period. Many other indices can and have been computed for comparable data but they will not be utilized in this paper.

PATTERNS OF RADIUM RETENTION

The computation of the values for three of the four radiobiological indices proposed above depends on our notions of the patterns of radium retention in the skeleton during the years after various routes of administration. Two principal mathematical models have been proposed to describe the course of retention of radium and other bone-seeking radionuclides. These are the power function and the sum of a series of exponentials. In a recent paper we have evaluated both approaches in an analysis of the existing data for the state mental hospital patients who were given intravenous injections of radium in 1931-33.⁸

The power function as proposed by Norris et al^{11,12} gives the value of retained radium at time t after a single intravenous injection by

$$R = a t^b \quad (1)$$

where a is the vertical intercept or amount of retained radium at unit time, and b is the slope of the straight line that this function produces on a log-log grid.

Where radium is acquired orally and where it can be assumed that the same amount was absorbed each day, the amount of radium retained at t days after cessation of unit daily absorption during F days is given by

$$R_{\text{oral}}(F,t) = \frac{a}{b+1} [(t+F)^{b+1} - t^{b+1}]. \quad (2)$$

The family of curves generated by Equation (2) for selected ingestion periods is given in Figure 1, where the power function parameters used are: $a = 0.30$, and $b = -0.44$. The latter values have been used instead of those proposed by Norris et al^{11,12} on the basis of restudy of the same patients that Norris used and a recomputation based on additional data.⁸ It can be seen in Figure 1 that the curve for one day's ingestion is very close to that expected for a single intravenous injection. As the ingestion period becomes longer, the earlier portion of the retention curve becomes flatter, but eventually the slopes of the curves bend down and approach that for a single injection.

Table 1

MALIGNANT TUMORS OF SKELETAL TISSUES ATTRIBUTABLE TO RADIUM IN DIAL PAINTERS

Case No.	Year of birth	Work period		Diagnosis and year of diagnosis	Mean current or preterminal radium burden	Date of measurement
		Dates	Days*			
03-455	1906	7/22-8/23	395	Fibrosarcoma, left radius 1935	μCi 0.81	1958
03-402 [†]	1905	7/23-7/26	914	Fibrosarcoma, left femur 1954	1.06	1962
03-649 [‡]	1906	10/24-10/50	455	Fibrosarcoma, right ischium 1954	1.30	1951
03-619	1903	8/22-4/23	242	Fibrosarcoma, left femur 1962	1.50	1962
03-429	1908	8/23-8/27	883	Osteosarcoma, right 3rd metacarpal 1967	1.70	1966
03-401	1900	6/23-4/25	669	Fibrosarcoma, left leg 1963	2.29	1960
03-671	1906	7/22-9/22	61	Fibrosarcoma, left femur 1953	3.82	1952
03-648 [§]	1903	9/21-9/22	1090	Fibrosarcomas, left femur and right humerus 1956	7.61	1955

* Work period F computed to January 1, 1926.

[†] Each of these two cases also had malignant tumor of mastoid; see Table 2.

[‡] Case 03-649 also listed as 01-023 (M.I.T. series).

[§] Case 03-648 also listed as 01-013 (M.I.T. series).

Table 2

OTHER MALIGNANT TUMORS ATTRIBUTABLE TO RADIUM IN DIAL PAINTERS

Case no.	Year of birth	Work period		Diagnosis and year of diagnosis	Mean current or preterminal radium burden	Date of measurement
		Dates	Days *			
03-417	1909	7/24-9/25	426	Carcinoma, right gingiva 1962	μCi 0.62	1964
03-402 [†]	1905	7/23-7/26	914	Carcinoma, right mastoid 1964	1.06	1965
03-407	1905	6/23-6/46	944	Carcinoma, right mastoid 1959	1.40	1957
03-648 [‡]	1903	1/22-12/24	1090	Mixed carcinomas, right mastoid 1955	7.61	1955
03-657	1906	7/22-7/36	1279	Splenic leukemia 1934	18.00 [§]	1936

* Work period F computed to January 1, 1926.

[†] Also had a malignant tumor of bone, see Table 1.

[‡] Also had a malignant tumor of bone, see Table 1. Listed as 01-013 (M.I.T. series).

[§] Measurement by R. D. Evans; also listed as 01-002 (M.I.T. series).

Figure 1. Log-log plot of radium retention after cessation of oral ingestion for ingestion periods (F) of selected lengths of time. Daily incorporation of $1.0 \mu\text{Ci}$ is arbitrarily assumed. Power function parameters: $a = 0.30$, $b = -0.44$.

Maximum retention, which occurs at the end of the feeding period, $\underline{F}$, is given by

$$R_{\text{oral}}(F, 0) = \frac{a}{b+1} F^{b+1} \quad (3)$$

since this is at the end of the ingestion period when $\underline{t} = 0$. Again, this computation is based on the assumption of absorption of a unit amount of radium during the ingestion period. For each case where radium was acquired orally, as in the case of the radium dial painters, we computed a ratio, $\underline{Q}$, in which the estimated radium burden at the end of the ingestion period was divided by the radium calculated to be present at time, $\underline{t}$, after cessation of ingestion:

$$Q = \frac{R_{\text{oral}}(F, 0)}{R_{\text{oral}}(F, \underline{t})} \quad (4)$$

This quotient represents the ratio of the maximum radium burden to any measured burden at time $\underline{t}$ thereafter, and in this way provides a factor by which the maximum burden estimated for each patient is greater than the subsequently measured amount. Values of $\underline{Q}$ are shown in Figure 2 for selected $\underline{F}$ periods and $\underline{t}$ days after cessation of ingestion. Sample retention values of retained radium and the accompanying $\underline{Q}$ ratios computed for $\underline{t}$ days after cessation of periods of ingestion $\underline{F}$ days in duration were given in a previous paper.¹⁰

Figure 2. Ratio of estimated maximum radium burden to calculated measured radium burden at time t , both computed on the assumption of unit daily absorption of radium from gut. Power function parameters: $a = 0.30$, $b = -0.44$.

For specific cases, then, the maximum radium burden at $t = 0$ can then be estimated from the magnitude of Q (which depends upon F , t , a , and b) and the whole-body radium measurement at time t :

$$\text{Estimated maximum burden} = Q \times (\text{Measured radium burden at } t). \quad (5)$$

This estimated maximum burden represents a value for peak initial burden computed separately for each whole-body radium measurement for each patient.

For the radium dial painters whose work history was known, the length of the ingestion period, F , was computed in days on the assumption that ingestion occurred throughout the period at the same rate because of consistency in personal work habits. This computation does not correct for absence from work, but is nevertheless a reasonable approximation. In the cases of those dial painters whose work history straddled January 1, 1926, we have ignored any work period subsequent to that data on the assumption that the bulk of the ingestion occurred before the practice of pointing the brushes between the lips was discontinued.

Another index that can be computed is the amount of radium absorbed daily from the gut. This value is the ratio of the measured body radium at time t to the radium computed to be retained in accordance with Equation (2). We have termed this the Ingestion Q:

$$\text{Ingestion } Q = \frac{\text{Measured Ra at } t}{\text{Computed retained Ra at } t \text{ after unit daily absorption}}. \quad (6)$$

Since the computed retained radium is estimated on the assumption of unit absorption of radium daily from the gastrointestinal tract, the Ingestion Q value is a measure of the amount actually

absorbed daily (rather than ingested in the sense of swallowed). The Ingestion Q value presumably varied from person to person depending on work practices (tipping the brushes between the lips and general personal hygiene) as well as individual physiological factors. Again, on the assumption that these physiological factors remained reasonably constant during the period of employment, with the major exception of the January, 1926 change in work procedures mentioned above, the total amount of radium absorbed into the bloodstream from the gut is given by the product of F and Q:

$$\text{Total absorbed radium} = F \times Q. \quad (7)$$

The computed values of FQ constitute the fourth of the measures used in this paper for evaluating the long-term effects of radium deposition.

Similar computations can be made on the basis of radium retention described by a sum of a number of exponential terms. Analysis of long-term retention data of the state mental hospital patients already alluded to⁸ has established that average radium retention during the period beginning with the first year after administration can be described by a single exponential term. This exponential equation has the form $R = 0.0246Ae^{-0.693t/5472}$, where t is given in days and A is the total amount administered. This expression implies that 2.46 per cent of the injected radium (and hence also of radium absorbed into the blood from the gut) is retained with a 14.98 year half-time. The early portions of the retention curve cannot be reconstructed from this single exponential term but can perhaps be approximated in the following way. Maletskos et al¹³ reported some experimental work with ²²⁴Ra that indicated a 2.4 day half-time for 85 per cent of injected radium. We have assumed a 30 day half-time for the remaining 12.5 per cent of administered radium since there is no indication of more than one remaining exponential term after one year.

Maximum radium retention, then, can be computed by the sum of three exponentials given by

$$R = 0.85Ae^{-0.693t/2.4} + 0.125Ae^{-0.693t/30} + 0.025Ae^{-0.693t/5472}.$$

Here again a Q ratio can be computed and a peak initial burden can be estimated for each whole-body measurement made years later. Similarly, values can be computed for Ingestion Q and for total radium absorbed from the gut during the critical work period.

These several measures of radium retention and radium metabolism have been computed for all our patients for whom suitable data are available. These indices have then been examined for their pertinence in characterizing the conditions in which radium-induced malignant change has occurred and may be expected to occur in the future.

RADIOLIOLOGICAL INDICES FOR MALIGNANT TUMORS IN DIAL PAINTERS

Table 3 presents the estimates of maximum body burden, the amount of radium absorbed daily, and the total amount of radium absorbed during the critical work period for those cases of malignant tumors of skeletal tissue that are listed in Table 1. Where more than one whole-body measurement was made, the value listed is in each case the mean of the computed index. Estimates have been made by both the power function, $R = 0.30t^{-0.44}$, and the sum of the three exponentials given above. Table 4 presents similar data for the carcinomas and for the case of

Table 3

ESTIMATES OF RADIOBIOLOGICAL INDICES FOR CASES OF MALIGNANT TUMOR OF SKELETAL TISSUE
ATTRIBUTABLE TO RADIUM IN DIAL PAINTERS

Case no.	Power function $0.30 t^{-0.44}$			Sum of three exponentials		
	Estimated mean maximum radium burden	Estimated mean daily absorption	Estimated mean total absorption during critical work period	Estimated mean maximum radium burden	Estimated mean daily absorption	Estimated mean total absorption during critical work period
	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>
03-455	6.72	0.441	174.16	7.96	0.461	181.93
03-402	6.99	0.287	262.09	8.87	0.314	287.14
03-649	8.89	0.539	245.31	7.83	0.421	191.55
03-619	16.14	1.393	337.17	23.02	1.658	401.12
03-429	11.21	0.469	413.83	15.10	0.547	483.05
03-401	15.38	0.751	502.65	17.62	0.761	509.02
03-671	66.49	12.418	757.49	90.56	10.674	651.14
03-648	38.63*	1.435	1564.62	42.54	1.338	1458.09

* Recomputed since previous publication.¹⁰

Table 4
ESTIMATES OF RADIOBIOLOGICAL INDICES FOR CASES OF CARCINOMA AND LEUKEMIA
ATTRIBUTABLE TO RADIUM IN DIAL PAINTERS

Case no.	Power function $0.30 t^{-0.44}$			Sum of three exponentials		
	Estimated mean maximum radium burden	Estimated mean daily absorption	Estimated mean total absorption during critical work period	Estimated mean maximum radium burden	Estimated mean daily absorption	Estimated mean total absorption
	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>	<u>μCi</u>
03-417	5.72	0.360	153.33	7.16	0.399	169.77
03-402	6.99	0.287	262.09	8.87	0.314	287.14
03-407	7.67	0.309	291.49	8.56	0.297	280.06
03-648	98.63*	1.435	1564.62	42.54	1.338	1458.09
03-657	55.64	1.891	2418.43	38.25	1.077	1377.92

* Recomputed since previous publication.¹⁰

leukemia presented in Table 2. From these tables it can be seen that the lowest mean values for any malignancy attributable to radium are:

Current or preterminal radium burden	0.62 μCi
Estimated maximum radium burden	5.72 μCi
Estimated daily absorbed radium	0.29 μCi
Estimated total absorbed radium	153.33 μCi

These minimum values are based on a specific power function and are very close to those independently estimated from the sum of three exponentials.

RADIOGIOLOGICAL INDICES AND DEGREES OF RADIOGRAPHIC CHANGE

Most of the dial painters whom we have discussed have had skeletal radiography along with whole-body measurements of radium by gamma-ray spectrometry. The radiographic changes typical of radium deposition were found to be, in increasing order of severity: coarsening of trabeculation, localized areas of bone resorption in the cortices of long bones and skull, spotty areas of sclerosis, aseptic necrosis, pathological fracture, and development of malignant tumors of bone and adjacent tissues. A quantitative scoring system was utilized to grade the severity of the radiographic changes and for each set of roentgenograms an overall impression was recorded that characterized the general degree of skeletal damage attributable to radium. These impressions were recorded in the following categories: negative, minimal, mild, moderate, advanced, and malignant changes due to radium deposition. These broad categories were closely related to the quantitative score for the entire skeleton and in general they expressed not only the extent of radiographically detectable damage but also the degree of orthopedic disability.

In a number of cases, periodic radiography at intervals of three or four years demonstrated a progression of changes in the direction of increasing severity. For the purpose of the present analysis, all the cases were reclassified, where necessary, so as to reflect the most severe category of change that has developed to date (January, 1969). In this way, a body burden determination that was associated, for example, with only mild changes in 1957 was regrouped with the cases of malignant disease when such developed in 1967. By this procedure, the various parameters or radiobiological indices were applied to the most severe degree of pathological change that has manifested itself at the present time.

For 207 dial painters there were 313 independent determinations of radium body burden. These measurements have been sorted by the latest diagnosis and the ranges and median values for each category are presented in Table 5. In many ways, the measured radium burden is among the least satisfactory of the indices for characterizing radium damage because its magnitude depends largely upon whether it is determined soon after the burden is acquired or at long time intervals thereafter. Furthermore, the rate of change of this value also depends on the route and time scale of administration, i.e., whether the radium was received as a single intravenous injection or over a protracted period of time by ingestion or repeated injections.

It would seem then that the radium burden measurements made 35 to 50 years after exposure or administration have only a limited applicability to the delineation of conditions of radium-induced carcinogenesis. Yet, despite these considerations, there is a persistent association between the level of current or preterminal body burdens of radium and the severity of skeletal damage set forth in Table 5 principally because most of the measurements were made in a rela-

Table 5

MEASURED RADIUM BODY BURDEN IN DIAL PAINTERS
SORTED BY LATEST DIAGNOSIS

Latest x-ray or clinical diagnosis	No. of patients	No. of measurements	Measured body radium μCi	
			Range	Median
Negative	146	168	0.001 - 0.500	0.018
Minimal	21	33	0.001 - 0.761	0.150
Mild	10	29	0.041 - 1.743	0.523
Moderate	9	29	0.115 - 1.622	0.900
Advanced	10	29	0.574 - 2.200	1.170
Malignant	11	25	0.597 - 18.000	1.420

tively narrow time band many years after exposure. Nevertheless, such values as are presented in Table 5 cannot be used for evaluating the hazard of a freshly acquired radium burden. For such purposes the estimated peak initial burden, or maximum burden, may be much more valuable. This view is fortified by the finding of M. Finkel et al that the dose rate during the first days after injection of ^{90}Sr into mice provided the amount of radiation necessary to induce malignant bone tumors in these animals.¹⁴

Table 6 presents retrospectively estimated values for the maximum radium burdens in dial painters. These values have been computed for each whole-body measurement, and have been calculated by various methods, as indicated, and have been sorted by latest diagnosis. Three sets of computations, each by a different power function, are listed. In addition to the power function $R = 0.30t^{-0.44}$ we have also used $R = 0.20t^{-0.35}$ which may also be a valid average function as shown by recent studies.⁸ For purposes of comparison we have also included values computed by the power function proposed by Norris et al, $R = 0.54t^{-0.52}$. Calculations were also made with a sum of three exponentials whose values have already been presented.

The significant point of Table 6 is the indication that malignancies have not developed in those cases where the computed maximum radium burden was less than $5.0 \mu\text{Ci}$ when $R = 0.30t^{-0.44}$ was used for the calculations. Use of the function $R = 0.54t^{-0.52}$ would place this lower limit at about $7.7 \mu\text{Ci}$, while the other computations vary with the parameters and mathematical functions used. The former power function leads to an estimate of $3.0 \mu\text{Ci}$ for peak initial burden as the level below which serious orthopedic difficulties are not likely to ensue, even after 40 years. These values should be evaluated against the maximum permissible level of $0.1 \mu\text{Ci}$ for radium body burden that has been the standard for many years.¹⁵

The ranges of values presented in Table 6 also suggest that there is some probability that some of the persons who are now free from serious deleterious effects might still develop such difficulties, particularly if their estimated maximum burdens exceed $5.0 \mu\text{Ci}$.

Table 7 similarly presents computations of the estimated daily amount absorbed by the dial painters, again computed for each body burden measurement for each patient by several methods and sorted by latest diagnosis. Here, although there is an increase in the amounts absorbed as one proceeds from the negative radiography group to those with advanced changes and with ma-

Table 6

ESTIMATED MAXIMUM RADIUM BURDEN IN DIAL PAINTERS, COMPUTED BY VARIOUS METHODS,
AND SORTED BY LATEST DIAGNOSIS

Latest x-ray or clinical diagnosis	Power function $0.30 t^{-0.44}$		Three exponentials		Power function $0.20 t^{-0.35}$		Power function $0.54 t^{-0.52}$	
	Range	Median	Range	Median	Range	Median	Range	Median
	μCi	μCi	μCi	μCi	μCi	μCi	μCi	μCi
Negative	0.00 - 4.51	0.20	0.002 - 5.69	0.18	0.00 - 2.66	0.11	0.00 - 7.37	0.35
Minimal	0.00 - 6.20	1.23	0.006 - 7.25	1.91	0.00 - 3.91	0.83	0.00 - 9.53	1.81
Mild	0.35 - 9.47	3.48	0.448 - 13.61	4.65	0.22 - 6.49	2.29	0.55 - 13.52	5.20
Moderate	1.83 - 12.12	7.08	2.40 - 17.66	9.35	1.00 - 7.62	4.58	2.77 - 18.73	10.64
Advanced	3.09 - 15.29	7.18	5.31 - 21.93	9.91	2.12 - 9.78	4.90	4.41 - 23.24	10.34
Malignant	4.99 - 66.49	8.89	6.64 - 90.56	10.77	3.13 - 42.85	5.82	7.71 - 117.36	13.24

Table 7

ESTIMATED DAILY AMOUNT ABSORBED BY DIAL PAINTERS, COMPUTED BY VARIOUS METHODS,
AND SORTED BY LATEST DIAGNOSIS

Latest x-ray or clinical diagnosis	Power function $0.30 t^{-0.44}$		Three exponentials		Power function $0.20 t^{-0.35}$		Power function $0.54 t^{-0.52}$	
	Range	Median	Range	Median	Range	Median	Range	Median
	μCi	μCi	μCi	μCi	μCi	μCi	μCi	μCi
Negative	0.000 - 0.849	0.018	0.000 - 0.858	0.019	0.000 - 0.544	0.011	0.000 - 1.004	0.021
Minimal	0.000 - 0.407	0.064	0.000 - 0.420	0.073	0.000 - 0.261	0.040	0.000 - 0.481	0.077
Mild	0.024 - 0.599	0.174	0.027 - 0.596	0.206	0.016 - 0.387	0.112	0.029 - 0.704	0.219
Moderate	0.044 - 0.803	0.343	0.062 - 0.949	0.418	0.028 - 0.516	0.224	0.054 - 0.948	0.412
Advanced	0.104 - 1.388	0.279	0.147 - 1.754	0.294	0.065 - 0.876	0.177	0.125 - 1.664	0.330
Malignant	0.265 - 12.418	0.441	0.261 - 10.674	0.461	0.167 - 8.076	0.281	0.318 - 14.502	0.523

lignancy, the progression is not smooth and the separation of the groups is not as clear as it was with estimated values for maximum burden. The principal reason for this finding is the variation in the length of the work period, which is not taken into direct consideration in this computation. Despite the unevenness of the data, the development of a malignancy seems to have required a minimum absorption of 0.26 μCi daily (calculated either by the power function with $a = 0.30$ and $b = -0.44$ or by three exponentials).

When Ingestion Q is multiplied by F, the total radium absorbed during the critical work period is computed. The results are given in Table 8. Here there appear to be three clusters of values: (a) cases with negative or minimal changes, (b) those with mild or moderate changes, and (c) those with advanced orthopedic difficulties and/or malignant neoplasms. The latter appear to develop only if the total amount of absorbed radium is computed to have exceeded 130 μCi according to both the standard power function that we have been using and the sum of three exponentials. These values generally agree with the data for the four state mental hospital patients who developed malignant tumors attributable to radium. These four patients had received from 11 to 41 weekly intravenous injections of radium in amounts of 10 μCi each, so that the total amount introduced into the blood ranged from 110 to 410 μCi .¹⁰ On the other hand the computed maximum radium body burdens at the end of the injection period for these same four patients ranged from 16.9 to 26.5 μCi ,¹⁰ amounts that are generally higher than those given in Table 6 for the dial painters who developed radium-induced malignant disease.

The radiobiological indices evaluated here for 207 radium dial painters can be used to set lower limits, or threshold values, for the probable development of radium-induced malignancies. Such lower limits, based on individual measurements rather than on the mean values for the affected patients noted earlier, would have these values:

Measured radium burden after at least 35 years	0.6 μCi
Estimated maximum (i.e., peak initial) burden	5.0 μCi
Total amount of radium absorbed from the gut	130.0 μCi

These levels can roughly be used to establish the probability of developing a radium-induced malignancy in the population already studied. It would appear that none of those now negative radiographically will develop a malignancy attributable to radium, while one of the 21 persons now classified as having minimal changes may be so affected eventually. For the mild group 40 per cent of the patients now have indices that fall within the range of the malignant group, while for the moderate group this proportion ranges from 44 to 78 per cent, depending upon the index used. The group with advanced changes practically overlaps with the malignant group and might be expected to have a high likelihood of developing radium-induced neoplasms.

These admittedly crude probability estimates reinforce our conviction that the patients in the high risk groups should be maintained under reasonably close medical surveillance as long as they are alive and as long as it is feasible to do so. For these reasons, the present study cannot be considered closed, and the data and conclusions presented here are still somewhat tentative.

Finally, it should be pointed out that the radiobiological indices discussed here are only one set of the many that can be used. Evans⁵ has proposed measures such as residual average skeletal rad/day, cumulative rads, cumulative μCi -years, cumulative rad-years, and others. Evaluations in terms of delivered dose to the entire skeleton have special problems, principally those concerned with the lack of knowledge of the precise duration of the induction and latent periods

Table 8

ESTIMATED TOTAL RADIUM ABSORBED BY DIAL PAINTERS, COMPUTED BY VARIOUS METHODS,
AND SORTED BY LATEST DIAGNOSIS

Latest x-ray or clinical diagnosis	Power function $0.30 t^{-0.44}$		Three exponentials		Power function $0.20 t^{-0.35}$		Power function $0.54 t^{-0.52}$	
	Range	Median	Range	Median	Range	Median	Range	Median
	μCi	μCi	μCi	μCi	μCi	μCi	μCi	μCi
Negative	0.14 - 113.35	3.73	0.08 - 138.50	3.78	0.09 - 71.72	2.36	0.15 - 135.63	4.43
Minimal	0.20 - 160.75	32.88	1.20 - 165.77	35.67	0.13 - 103.19	21.14	0.24 - 189.88	38.77
Mild	8.96 - 393.71	111.72	9.82 - 475.54	131.91	5.71 - 249.31	71.56	10.65 - 470.77	140.14
Moderate	24.88 - 375.32	206.67	26.45 - 490.74	240.70	15.89 - 236.50	130.43	29.52 - 450.75	247.86
Advanced	135.90 - 469.23	262.15	191.83 - 614.47	308.74	85.23 - 299.96	166.28	163.90 - 563.59	313.01
Malignant	133.78 - 2418.43	312.52	157.62 - 1458.09	348.69	84.85 - 1703.33	198.95	159.73 - 2630.78	371.93

as well as of the precise volume of sensitive tissue that requires radiation for malignant transformation. Furthermore, there is considerable evidence that radium deposition in the skeleton is highly nonuniform in its distribution so that the concentration of radium in bone varies widely even in persons who acquired their burdens over a substantial ingestion period. This nonuniformity exists between major areas of the skeleton as well as on a microscopic level within each bone.¹⁶

Despite these difficulties, we are currently evaluating our data for each patient in terms of daily dose rates and cumulative doses to the skeleton at selected times during and after exposure in an effort to illumine the problem. We hope that when these dose calculations are considered together with the indices presented here, some contribution will have been made in understanding the conditions of radium-induced carcinogenesis in man.

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PURIFICATION OF MITOGENIC PROTEINS DERIVED FROM PHASEOLUS
VULGARIS: THE ISOLATION OF POTENT AND WEAK
PHYTOHEMAGGLUTININS POSSESSING
MITOGENIC ACTIVITY*

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In order to study in detail the mechanism whereby phytohemagglutinin, a protein derived from the kidney bean Phaseolus vulgaris, causes lymphocyte transformation, it was deemed necessary to obtain a highly purified transforming factor. The present report describes the isolation of such a factor from commercially available starting materials, characterizes the fractions obtained, and clarifies the relationship between the serologic and mitogenic properties of several closely related protein species encountered.

MATERIALS AND METHODS

Bacto-phytohemagglutinin P (PHAP) was obtained from Difco Laboratories (Detroit, Michigan; cat. #3110-57). Four different batches of starting material prepared by the method of Rigas and Osgood¹ were utilized and no significant differences were encountered. The contents of 1-3 vials (41 - 125 mg) of PHAP were dissolved in 5 - 10 ml of 0.15 M NaCl and dialyzed for 6 hours at 4°C against several liters of 0.01 M phosphate buffer, pH 5.5. A precipitate which constituted 5 - 10 per cent of the original material was spun down and discarded. The supernatant solution was applied to a 1 x 25 cm column of CM-Sephadex C-50 (Pharmacia Fine Chemicals Inc., capacity 4.5 ± 0.5 meq/g, particle size 40 - 120 μ) previously equilibrated with 0.01 M phosphate, pH 5.5, and eluted with the same buffer at a flow rate of 24 ml/hour in 8 ml aliquots. After elution of the initial peak, stepwise elution was carried out using 0.0167 M phosphate, pH 6.6, followed by 0.03 M phosphate, pH 8.1. Finally, a linear gradient was developed using 250 ml 0.03 M phosphate, pH 8.1, containing 0.5 M NaCl and an equivalent volume of 0.03 M phosphate, pH 8.1. In other experiments the gradient was developed using 0.2 M NaCl in 0.03 M PO_4 , pH 8.1, as the limit buffer. The resultant fractions were monitored for protein content (O.D. 280 $m\mu$), hemagglutination titer, lymphocyte transformation, pH, and conductivity. Appropriate fractions were pooled and concentrated to a volume of 5 ml or less by pressure ultrafiltration (Amicon Corporation, Lexington, Massachusetts, filter UM-10). The material was then applied to a 2.5 x 100 cm column of Sephadex G150 (Pharmacia Fine Chemicals Inc.) and eluted by upward flow chromatography utilizing 0.1 M NaCl in 0.03 M PO_4 , pH 8.1, at a flow rate of 12 - 15 ml/hour; 5 ml fractions were collected. Following appropriate assays, the fractions containing the purified transforming factors were pooled, concentrated by ultrafiltration, and stored at -20°. All chromatographic procedures were performed at 4°C.

Polyacrylamide disc gel electrophoresis at pH 4.5 was performed according to the method

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of Ornstein and Davis² utilizing Reisfeld's β -alanine-acetic acid buffer³ and an acrylamide concentration of 7 per cent. Following the run (5 ma/tube, 2 to 2-1/2 hours, 4°C) the gels were sliced at the leading edge of the tracker dye (pyronine Y), fixed and stained for 1 hour in 7 per cent acetic acid, 0.5 per cent amido black, and destained by electrophoresis in 7 per cent acetic acid. The R_F of the protein bands was determined by careful measurement and inspection utilizing the front of the tracker dye as the point of reference. In certain experiments, direct assays of hemagglutinating or transforming activity were made by slicing the unstained gels into 2 mm sections directly following electrophoretic separation, and eluting the gel slices in small volumes of tissue culture medium or phosphate buffered (pH 7.4) 0.15 M NaCl overnight at 4°C. Following this, the gel slices were removed and the assay was performed. The R_F of activity so determined was compared with the R_F of protein bands revealed by staining gels that had been prepared and run simultaneously.

Hemagglutination titers with human red blood cells were performed by the method of Salk,⁴ utilizing either glass tubes or a microtitration apparatus (Cooke Engineering Corp., Alexandria, Va.).

Leukoagglutination was performed by the method of Thompson et al.⁵ Titers were expressed as the concentration of test material necessary to completely inhibit separation of the packed leukocyte button in the capillary glass tubing. Both purified lymphocytes (vide infra) and mixed white cell populations were employed.

Heparinized blood from healthy human volunteers was used for lymphocyte transformation studies. The red cells were sedimented by gravity for 2 - 3 hours at 37°. In early studies the supernatant plasma was removed and centrifuged at 300 x g for 20'. The cell button was resuspended in 5 ml of autologous plasma and was then passed over a nylon wool column (Fenwal Co.) in a modification of the method of Hirschhorn et al.⁶ Cells prepared in this fashion contained over 90 per cent lymphocytes, but had a rbc:wbc ratio of from 3-10:1, with an abundance of platelets. In later experiments when lymphocytes free of platelets and red cells were desired, the cell button obtained after red cell sedimentation was subjected to osmotic shock lysis followed by centrifugation through a saline-plasma gradient.⁵ The leukocyte button so prepared was resuspended in 3 ml of 50 per cent plasma, 50 per cent tissue culture medium, and then passed through the nylon column. Lymphocytes prepared by the latter method were free of red blood cells and contained from 2 - 10 platelets/100 lymphocytes. Lymphocyte cultures were performed in 16 by 125 mm plastic screw-top tissue culture tubes (Falcon Plastics Cat. #3033). Each culture contained from $1.5 - 2 \times 10^6$ cells, 0.5 ml autologous cell free heparinized plasma, and 3.5 ml Eagles Minimal Essential Medium (MEM), (Spinner modified), containing penicillin and streptomycin (Baltimore Biological Corporation, Catalog #71-016R). Glutamine was added to the medium just prior to use. From 1 - 50 μ l of fractions to be tested for transforming activity were added. The cultures, with loosened screw caps, were incubated at 37° in a high humidity atmosphere of 5 per cent CO₂. At 72 hours, 0.1 μ c ¹⁴C thymidine (sp. activity 20 mC/mM) in 0.1 ml saline was added, the cells were gently suspended, and incubation was continued for another 16 hours. The cells were then spun down, and the supernatant was discarded. The cell button was transferred to 12 ml conical glass tubes, and washed twice in 3 ml phosphate buffered 0.15 M NaCl containing 5×10^{-4} M Na₂H₂EDTA, followed by precipitation with 3 ml 5 per cent TCA. After two 5 per cent TCA washes and 1 methanol wash, the acid insoluble precipitate was dissolved by heating at 80° for 15'. The material was then transferred to counting vials in

10 ml of scintillation fluid (2:1 (V/V) toluene + ethanol, 0.4 per cent PPO, 0.01 per cent (POPOP) and counted in a liquid scintillation counter. The entire harvesting procedure was performed at 4°C. When necessary, 1 unit of transforming activity was defined as the amount of material that would yield half maximum transformation in any given culture series, according to the concepts elaborated by Tormey and Mueller.⁷ Such assays were always performed with the red blood cell-platelet-free lymphocytes. All experiments were done in duplicate.

Amino acid analysis was performed according to Moore and Stein utilizing a BioCal 200 amino acid analyzer.⁸ Cystine and cysteine were determined as the carboxymethyl cysteine derivative following reduction and alkylation in 8 M urea.⁹ Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton.¹⁰ Carbohydrate analysis was performed by borate column analysis using the Technicon sugar chromatography system. Samples were hydrolyzed in 1 M HCl for 3 hours; then deacidified with Dowex 3 (carbonate form). Hexosamine was estimated by the method of Boas modified by omission of resin treatment.¹¹ Identification of the hexosamines was made during the course of amino acid analysis. Sedimentation velocity analysis was done using a Beckman Model E ultracentrifuge equipped with interference optics.¹² Diffusion coefficients were obtained by molecular sieving on a Sephadex G150 column, according to Andrews,¹³ using as markers spermwhale myoglobin, ovalbumin, bovine serum albumin, and human globulin.

RESULTS

Polyacrylamide gel electrophoresis of PHAP revealed at least 17 different discernible bands stained by amido black. A densely stained region (R_F 22.5-32.5) in which 4-5 separately discernible bands were visualized, contained all of the transforming and hemagglutinating activity, although the latter was evident only in the more cathodal segments of the gel slices bearing transforming activity (Figure 1).

The results obtained by chromatographic separation of crude PHAP on CM Sephadex are shown in Figure 2. The initial peak of material not retained by the column was devoid of both hemagglutinating and transforming activity. The second, third, and last peaks all contained potent transforming activity but only the latter 2 peaks displayed hemagglutinating activity when column fractions were analyzed directly. Close examination of the material eluted by means of the NaCl gradient revealed that within this high hemagglutinating peak there was dissociation between hemagglutinating and transforming activities, the latter eluting from the column between 5000 and 9500 umhos, while the former persisted in fractions eluted between 10,500 and 13,500 umhos. The various fractions were pooled, concentrated, and analyzed by polyacrylamide gel electrophoresis. The peak eluted at pH 6.6 contained only the most anodal of the protein bands discerned in the active region of PHAP (Figure 1). The material from the peak eluted by the NaCl gradient contained the more cathodal protein bands. These two fraction pools, which were designated low hemagglutinating PHAP (L-PHAP) and high hemagglutinating PHAP (H-PHAP) were further purified by passage over Sephadex G150 to remove trace contaminants. The intermediate transforming peak, which contained both H-PHAP bands and L-PHAP bands, was not studied extensively.

The elution of L-PHAP and H-PHAP from Sephadex G150 is shown in Figure 3. The specific activity of transformation of L-PHAP across the elution peak varied by only 10 per cent, which was within the limits of error of the assay employed. Analysis of Sephadex G150 purified H-PHAP

Figure 1. Acrylamide gel electrophoresis of PHAP (A) and L-PHAP (B). Unstained gels run in parallel with the stained gels shown, were sliced into 2 mm segments and assayed for mitogenic activity. The cross-hatched bars represent those segments in which hemagglutinin activity was detected.

and L-PHAP acrylamide gel electrophoresis revealed that transforming activity in the latter, and both hemagglutinating and transforming activity in the former, correlated well with the protein bands in each preparation (Figure 1). The properties of H-PHAP and L-PHAP are compared in Table 1, and detailed composition studies of L-PHAP are shown in Table 2.

Study of L-PHAP in the analytical ultracentrifuge revealed a single homogeneous peak with a sedimentation coefficient of $6.5S_{20,w}^0$ (Figure 4).

Figure 2. CM-Sephadex chromatography of PHAP. Twenty μ l aliquots were used in the transformation assays.

DISCUSSION

These experiments provide a relatively simple method for the isolation of a purified low hemagglutinating mitogenic glycoprotein from commercial preparations of phytohemagglutinin (PHAP). A closely related, somewhat less potent mitogenic fraction which is capable of strong hemagglutination can also be isolated from the same starting materials. The latter fraction is nonhomogeneous by both CM Sephadex chromatography and polyacrylamide gel electrophoresis, and contains at least 2, and possibly more, components. Preliminary attempts at better resolution of these H-PHAP components have met with partial success and have shown that the protein fraction which migrates most rapidly toward the cathode, and elutes from CM Sephadex at higher conductivities, is a less potent mitogen and a stronger hemagglutinin. The L-PHAP and H-PHAP proteins are closely related by immunologic criteria,¹⁴ and have similar amino acid and carbohydrate compositions, although H-PHAP is somewhat richer in carbohydrate and, as judged by molecular sieving, somewhat larger, than L-PHAP. The presence of multiple components in an otherwise homogeneous preparation of PHAP, when subjected to IRC-50 chromatography, has been described by Rigas et al¹⁵ who suggest that such components are artifacts arising from dissociation of the PHAP molecules into subunits during chromatography, with subsequent random recombination. However, the chromatographic methods were clearly not the cause in our experiments, since heterogeneity of the starting material was revealed by acrylamide gel electrophoresis, and the isolated materials conformed to the original pattern when analyzed by the same method.

Several workers have previously shown that the mitogenicity of PHAP does not depend sole-

Figure 3. 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, concentrated, and compared with the starting material by acrylamide gel electrophoresis analysis (shown on right). The Sephadex G-150 elution peaks of human gamma globulin (γ G) and bovine albumin (BSA) are also shown.

ly upon the hemagglutinating factor, and have isolated hemagglutinin-poor fractions from PHAP capable of lymphocyte transformation.¹⁵⁻¹⁷ Others have shown that absorption of the hemagglutinating principle from PHAP with red blood cells does not abolish mitogenicity of such preparations.¹⁸⁻²² The present studies suggest that the residual mitogenicity of such red cell absorbed PHAP preparations is due to L-PHAP. We have confirmed this by demonstrating that the mitogenicity of H-PHAP preparations is completely abolished by red cell absorption, while that of L-PHAP is unaffected. Absorption of H-PHAP with red blood cells also removes all material capable of reacting immunologically with anti-H-PHAP, while absorption of L-PHAP with red blood cells does not affect its immunological reactivity with anti-L-PHAP. These results suggest that the mitogenicity of H-PHAP preparations is not due to the presence of contaminating L-PHAP, but rather that hemagglutinating and mitogenic properties reside on the same molecule. In confirmation of this, preliminary studies of ¹²⁵I-labeled H-PHAP show that virtually all such material can be absorbed by red blood cells. Thus, while hemagglutination is not a necessary requisite for mitogenicity (e.g., L-PHAP), at least some H-PHAP molecules are also capable of lymphocyte transformation.

Both H-PHAP and L-PHAP possess leukoagglutinating properties, and the more potent mitogen (L-PHAP) is also the more potent leukoagglutinin. To date, our studies have not revealed any dissociation between mitogenicity and leukoagglutination, but more extensive studies will be

Table 1
COMPARISON OF CERTAIN PROPERTIES OF L-PHAP AND H-PHAP

	L-PHAP	H-PHAP
1. Carbohydrate composition per cent by weight		
a. Glucosamine	2.10	2.30
b. Mannose	1.69	4.76
c. Xylose	0.17	0.48
d. Arabinose or fucose *	0.14	0.38
e. Total	4.1	8.9
2. Hemagglutination unit [†] $\mu\text{g/ml}$	227	1.05
3. Leukoagglutination unit ($\mu\text{g/ml}$)		
a. Mixed leukocytes	5.6	69.5
b. Pure lymphocytes	11.3	304
4. Transformation unit (μg)	1.2	4.2
5. V_e/V_o (Sephadex G-150)	1.52	1.48
6. Diffusion coefficient, $D_{20,w}$ ($\times 10^{-7} \text{cm}^2/\text{sec}$)	5.1	4.76
7. $A_{280}^{1\%}$ _{mu}	13.0	12.1
8. Partial specific volume ($\bar{V}$)	.73 [‡]	N.D.

* Not further identified.

[†] Minimum concentration yielding 4+ agglutination.

[‡] Calculated from amino acid composition.

N.D. not determined.

Figure 4. Sedimentation velocity analysis of L-PHAP (2.8 mg/ml) in .03M phosphate, pH 8.1, 0.1M NaCl. Beckman model E Analytical Ultracentrifuge, 52640 RPM.

Table 2
AMINO ACID COMPOSITION OF L-PHAP

	Residues	Observed residues	Mole integer
	100 amino acid residues	L-PHAP molecule	
Lysine	4.27	41.3	41
Histidine	0.86	8.3	8
Arginine	3.85	37.2	37
Aspartic acid	14.1	136.7	137
Threonine	8.62	83.4	83
Serine	10.7	103.5	104
Glutamic acid	7.14	68.9	69
Proline	3.59	34.6	35
Glycine	8.12	78.5	79
Alanine	6.23	60.4	60
Half-cystine	0.33	3.2	3
Valine	8.80	85.1	85
Methionine *	---	---	--
Isoleucine	4.45	43.1	43
Leucine	8.66	83.5	84
Tyrosine	1.82	17.6	18
Phenylalanine	6.33	61.2	61
Tryptophan [†]	2.18	21.1	21

* Trace amounts of methionine, insufficient for accurate quantitation, were detected.

[†] Determined spectrophotometrically.

needed to clarify this point, since other workers have suggested that leukoagglutination is not a requisite for lymphocyte transformation by PHAP.²³

Early in our studies we noted that although L-PHAP was a more potent mitogen than H-PHAP at low dose levels, the plateau value of peak response was always higher with H-PHAP. A similar observation had been made by Rigas et al.¹⁵ Further studies have shown that the effectiveness of H-PHAP as a more potent mitogen at high dose levels depends upon the presence of red blood cells, which contaminated our early lymphocyte preparations. When pure lymphocytes, free of red cells and platelets, were employed, the greater effectiveness of H-PHAP at high dose levels was abolished. The role of red cells in potentiating lymphocyte transformation by H-PHAP will be reported separately.

The molecular weight of L-PHAP is similar to that reported by Rigas et al.¹⁵ for PHAP (123,000), and closely resembles that recently reported by Weber (118,000).²⁴ Rigas has presented convincing evidence that PHAP molecules are made up of protein subunits held together by noncovalent forces.¹⁵ In relating biological function to chemical structure, it remains to be seen whether the various biologic properties of PHAP molecules are carried on specific subunits of different amino acid composition, or whether other structural or conformational differences, such as specific carbohydrate groups, explain the variability encountered between L-PHAP and H-PHAP.

The mitogenic properties of PHAP have been widely exploited in the study of lymphocyte function, and in the study of human chromosomes. It is hoped that the availability of a highly purified preparation of L-PHAP will aid in our understanding of the mechanism by which this and other materials elicit, from an otherwise dormant lymphocyte, such a total expression of gene function.

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THE PATHOGENESIS OF HYPERURICEMIA IN SATURNINE GOUT*

By

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Chronic renal disease is thought to cause gout rarely. This paper documents its role in the pathogenesis of saturnine gout, a disorder of exceptional historical interest. Musgrave described saturnine gout in 1703,¹ and Garrod suspected the association between lead toxicity and gout in about one quarter of his patients, inferring the cause of the hyperuricemia to be imperfect secretion of urate.² Alcoholic beverages and paint were the supposed vehicles of the lead in these patients. More recently, Richet in France,³ and Emmerson in Australia have studied about 50 patients with saturnine gout, whose exposure to lead occurred during childhood.⁴

Moonshine whiskey is the important source of lead in the patients reported here. Eighty-nine per cent of the samples of Alabama moonshine analyzed in 1965 by the Alabama Regional Laboratory contained lead. The content of 39 per cent was greater than 1,000 mcg per liter, with values higher than over 200,000 mcg per liter.⁵ The extent of moonshine in the United States can be appreciated from the estimated production in 1966 of 55.7 million gallons of illegal whiskey.⁶ The production and habitual use of lead contaminated alcohol appear to be localized now to the "moonshine belt" comprising 12 southeastern states, but this has not been true always. Benjamin Franklin wrote in July 1786 that there were complaints from North Carolina against New England rum, which was said to poison the people. . . .⁷ The Massachusetts Bay Acts of 1724, possibly the first industrial health act in the colonies, forbade the use of leaden articles in the distillation of rum.

In 1967 there were 2734 admissions to the Medical Service of the Birmingham Veterans Administration Hospital; chronic lead intoxication was diagnosed 98 times; 43 admissions were for gout, of which 37 were related to chronic lead intoxication, viz, "saturnine gout." Patients with this syndrome are usually of low socioeconomic status, with sociopathic personalities, a history of long exposure to moonshine, no family history of gout, mild anemia (the mean hematocrit in our patients was 30), hypertension, elevated urinary lead levels, with or without stimulation by calcium disodium edetate, and evidence of slowly progressive renal disease with minimal to moderate renal failure present for years. Renal biopsy shows interstitial fibrosis with few inflammatory cells, obsolescent glomeruli, perivascular fibrosis and degenerating tubular cells containing intranuclear inclusion bodies.⁸

Emmerson suggested that a defect in the tubular secretion of uric acid might be the cause of lead related hyperuricemia although noting he could not exclude other factors, chiefly genetic,⁴ and Ludwig proposed an increased turnover of nucleoprotein as the primary mechanism of hyperuricemia in his two patients with saturnine gout.⁹ Our study was designed to determine, there-

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fore, whether in addition to decreased renal excretion of urate there are other causes of hyperuricemia in this condition.

Patient Material

Three patients with acute gouty arthritis verified by finding urate crystals in the joint fluid, and chronic lead poisoning, were studied. All patients have continued drinking moonshine from their initial hospitalization to now.

Patient I, a 36-year-old social derelict, began drinking moonshine heavily in his early teens. Gouty arthritis first occurred at age 31. Hypertension and anemia (Hct 30) were noted during an admission for gout in 1965, the BUN was 40 mg per cent, and the serum creatinine was 1.8 mg per cent. Gouty arthritis was proven in 1966 by finding negatively birefringent crystals in the joint fluid. The 24-hour urinary lead level was 3.5 mg/24 hours after intravenous stimulation with 1 gram calcium disodium edetate. Urinalyses typically show +1 protein, specific gravity 1.015, and a few WBC's and RBC's per hpf.

Patient II was 40 years old. Intractable alcoholism was well established by age 34, and between 1963-1967 there were nine admissions to the hospital for alcoholism and pancreatitis. Hypertension, anemia (Hct 29) and gout, the latter beginning at age 37, were noted in 1964. Renal failure has been of slow progression. Urinalyses show a trace of protein, an occasional RBC and 2-6 WBC's. The BUN was 36 mg per cent and the serum creatinine was 1.6 mg per cent. Urate crystals were aspirated from a gouty joint in 1965. A urinary lead level of 3.5 mg/24 hours was found after 1 gram intravenous calcium disodium edetate.

Patient III was 46. He admitted drinking a pint jar of moonshine almost daily for many years, but although he was an alcoholic, his only admissions to the hospital were for gout. Sodium urate crystals were demonstrated in joint fluid obtained during an attack of acute arthritis. The hematocrit was 34. Blood pressure averaged 180/110 mm Hg. Urinary lead level without stimulation was 0.900 mg/24 hours. (The upper limit for unstimulated urinary lead per 24 hour specimen is 0.10 mg; the normal response to calcium disodium edetate stimulation should not exceed 1 mg lead per 24 hours.)

METHODS

For four days before as well as during the studies, the patients were maintained on a diet essentially free of purines, but with protein sufficient to maintain nitrogen balance. Plasma and urinary uric acid were determined by an enzymatic spectrophotometric method.¹⁰ On the 5th day, glycine-1-¹⁴C was injected intravenously in a dose of 54.32 μ c. Uric acid was isolated from urine and purified as described elsewhere,¹¹ except that the first step in the isolation procedure involved either the addition of 200 mg of uric acid in solution as carrier to 250 ml of urine, or the adsorption of urinary uric acid to charcoal (Darco G-60) and its subsequent elution with hot 0.1N NaOH. Radioassay of ¹⁴C was done in a vibrating reed electrometer after wet oxidation of uric acid in a vacuum combustion line. Seven days later the patients were given uric acid ¹⁴C, and the miscible pool and turnover of uric acid were determined as described previously, except that radioassay of ¹⁴C was done in a liquid scintillation spectrophotometer (Packard Tricarb, model 3324) using the following technique: preweighed amounts of uric acid were dissolved in 2 cc of hyamine hydroxide, then mixed with 18 cc of a mixture of PPO (2,5-diphenyloxazole), 3 gm; POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene), 100 mg; and toluene, 1000 cc. In this system

toluene- ^{14}C counted with an efficiency of about 70 per cent. In counting uric acid a modest quenching was observed which was proportional to the quantity of uric acid counted (a sample weighing 50 mg reduced the counting efficiency to 85 per cent of the efficiency at zero weight). All samples were corrected for quenching either by including internal standards, or by applying a factor derived from a curve relating degree of quenching to sample weight.

Endogenous creatinine and inulin clearances were done with forced hydration; inulin clearance was done with catheter collection of 3 periods of 20 minutes each. Urinary lead determinations were done in the Renal Research Laboratory, Veterans Administration Hospital by the method of Bessman and Layne.¹²

RESULTS AND CONCLUSIONS

In all three patients plasma urate demonstrations fell on purine deprivation; a similar fall has been noted on exclusion of exogenous purines in those patients whose gout was not related to overproduction of urate, as well as in other patients with saturnine gout⁴ (Table 1). The endogenous creatinine and inulin clearances were compatible with renal disease of moderate severity

Table 1

PLASMA URATE, URIC ACID CLEARANCE, CREATININE AND INULIN CLEARANCE IN THREE PATIENTS WITH CHRONIC LEAD POISONING

Patient	Plasma urate, mg% regular diet	Plasma urate, mg% purine exclusion	Uric acid clearance, ml/min	Creatinine clearance, ml/min
I	9.0	6.8	1.3	30
II	6.8	5.0	2.2	49
III	7.6	6.4	1.4	Inulin clearance, ml/min 50

whereas the uric acid clearances were severely depressed (Table 1). The low mean values for 24 hour urinary uric acid in per cent of turnover indicates about three-fourths of the daily production of uric acid underwent extrarenal elimination, as compared to one-fourth to one-third in normal subjects. The recovery of uric acid ^{14}C from urine in per cent of dose, seven days, corresponded closely to the urinary uric acid in per cent of turnover, validating the calculation of the uric acid pool and turnover (Table 2).

The uric acid pool size was not excessive while the patients were on a purine-free diet, and the uric acid production did not exceed the rate expected for normal non-gouty men. The generation of uric acid was normal by pattern of incorporation of glycine ^{14}C into urinary uric acid (Figure 1) and by the cumulative seven day total incorporation of glycine ^{14}C into urinary uric acid. The cumulative glycine incorporation values were corrected for extrarenal disposal of uric acid, obviously necessary when extrarenal disposal of uric acid is so great (Table 3). In normal subjects the corrected values average 0.1-0.15 per cent of the injected dose of glycine; the corresponding value in primary metabolic gout may be from 0.4-0.5 per cent of injected dose of glycine.

Table 2

URIC ACID MISCIBLE POOL, DAILY TURNOVER AND URINARY RECOVERY

Patient	Miscible pool, mg	Turnover mg/day	Urinary uric acid mg/day	Urinary uric acid % of turnover	Recovery of uric acid- ^{14}C % of dose
I	1194	427	119	27.87	26.35
II	1530	739	168	22.79	25.67
III	1200	500	132	26.57	22.68

Figure 1.

Table 3

Patient	Glycine ^{14}C incorporated into urinary uric acid, % of dose	Glycine ^{14}C incorporated into urinary uric acid, corrected for extra renal disposal, % of dose
I	.0258	0.0993
II	.0293	0.1120
III	.0208	0.0948

The data indicate that impaired renal function is the sole cause of hyperuricemia and gout in these patients. The markedly reduced C_{ur} with only moderate reduction in the C_{in} or C_{creat} suggests a defect in tubular secretion of uric acid although this was not measured as such. The $\frac{C_{ur}}{C_{in}} \times 100$ (per cent) ratio of 2.8 is far less than any of thirty-six determinations reported by Steele and Rieselbach in a variety of patients with chronic renal disease whose C_{in} ranged from 1.3 to 94.8.¹³

Plasma urate levels in this group of patients are apt to fluctuate widely in response to their erratic eating patterns and prolonged bouts of alcoholism, and the likely complication of lactic acidemia.¹⁴ This may be of additional importance in explaining the high incidence of gout in this form of chronic renal disease since it is a clinical truism that acute gouty arthritis is most likely to occur when the plasma urate concentration changes abruptly.

Although moonshining and its sequelae are more or less regional problems, lead intoxication doubtless occurs throughout the United States. Last year there were approximately 2000 known cases of acute lead poisoning among Chicago children. Fourteen of these youngsters died and neurological abnormalities were common among the survivors. One wonders whether they will develop chronic nephropathy and saturnine gout, and inferentially whether there are not now numerous sporadic cases of saturnine gout among survivors of youthful bouts of lead poisoning.

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THE RATE OF DIVISION OF ANTIBODY-FORMING CELLS DURING THE EARLY PRIMARY IMMUNE RESPONSE*

By

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There is conflicting evidence about the relative roles of division and "recruitment" of responding cells during the early primary immune response. The cells releasing primarily "19S" hemolysin for sheep erythrocytes may be enumerated using the plaque-forming cell technique developed by Jerne and Nordin^{1,2} and independently by Ingraham.³ An exponential increase in plaque-forming cells occurs during the early primary response; doubling times vary from about 5 to 10 hours depending on the dose of antigen and other experimental variables.⁴⁻¹⁴

A method using mitotic blocking agents to estimate cell cycle times of antibody-forming cells, in vivo and in vitro is presented. Results with this method indicate that the increase in numbers of antibody-forming cells which occurs during the early primary response is due primarily to exponential division of responding cells. Increased responses produced by larger antigen doses or adjuvant appear to result from an increased rate of division of responding cells. These findings are contrary to the view that the observed increase in antibody-producing cells can be attributed to continued activation or recruitment of potential antibody-producing cells.⁹⁻¹⁴

MATERIALS AND METHODS

Young adult Sprague-Dawley rats were used. The antigen was washed whole erythrocytes prepared from sterile sheep's blood. A single sample of blood stored in Alsever's solution was used in each experiment both for immunization and for titrating immune responses. The sheep erythrocytes were washed 4 times in large volumes of freshly prepared pyrogen-free saline and used immediately. Doses of sheep erythrocytes were standardized by cell count. Bordetella pertussis vaccine was used to enhance the response to sheep erythrocytes and will be referred to as adjuvant. The vaccine, Lilly, Strain 37790, Lot T-59187, containing about 50 opacity units per ml and merthiolate 1:10,000, was kindly supplied through Dr. H. Campbell, Jr., Biological Development, Eli Lilly and Company, Indianapolis, Indiana. The adjuvant was mixed with antigen or injected separately as indicated. All injections of antigen and adjuvant were into the lateral tail vein.

Plaque-forming cells. The method of Jerne and Nordin¹ for demonstrating release of antibody by single cells in agar plates was used with minor modifications.¹⁵ Each spleen was minced and passed through a wire gauze screen using a pestle and 4.0 ml of tissue culture medium. Re-

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peated pipetting of the cell suspension caused additional dispersion of cells. The volume of the suspension was brought to 5.0 ml and tissue fragments were removed by low speed centrifugation for 30 seconds. One tenth ml of an appropriate dilution of the spleen cell suspension and 0.1 ml of a suspension containing 2×10^8 sheep erythrocytes mixed with 2.0 ml of agar-tissue culture medium were layered over a thin layer of solidified agar-tissue culture medium in plastic petri plates 9.0 cm in diameter. The plates were incubated 1.5 hours at 37°C ; 2.0 ml of normal rat serum diluted 1:3 with saline was added as complement; the plates were incubated 1.5 hours at 37°C , washed and counted immediately. Except for very early responses when spleens contained few plaque-forming cells, the total number of spleen plaque-forming cells for each animal was calculated from counts on duplicate plates using dilutions of spleen cell suspensions which gave about 100 to 250 plaques per plate.

The calculated total number of plaque-forming cells per spleen was transformed by taking the logarithm of the number to the base 10. Where means are recorded untransformed, they represent the antilog of the mean of the logarithmically transformed data. All statistical calculations were performed on the logarithmically transformed data. The reasons for preferring the logarithmic to the simple arithmetic scale arise from the fact that the scatter around the untransformed means was grossly dependent upon the mean itself, the higher mean values being attended by greater variability. Logarithmic transformation of the data fully corrected this feature, so that the variance at different response levels was independent of the mean and showed no significant trend.

Antibody titers. Titrations for hemolytic and agglutinating antibody to sheep erythrocytes used the double dilution technique with an initial serum dilution of 1:10.^{15,16} During the first 5 days of the primary response to sheep erythrocytes, the agglutinin and hemolysin titers were equal $\pm$ one serum dilution using selected end points; the relationship between spleen plaque-forming cells and antibody titers was the same whether agglutinin or hemolysin titers were used. Only hemagglutinin titers are reported. For convenience in comparing the plaque-forming cell and antibody responses, titers were also transformed by taking the logarithm of the number to the base 10. Where mean titers are recorded untransformed, they represent the antilog of the mean of the logarithmically transformed data. All statistical calculations were performed on the logarithmically transformed data. Sera were also titrated for antibody not inactivated by 2-mercaptoethanol; serum diluted 1:5 in saline was added to an equal volume of 0.1M 2-mercaptoethanol, incubated at 37°C for 30 minutes, and then titrated for hemagglutinins.

Mitotic blocking agents. Colchicine, 0.5 mg per ml, injectable, was purchased from Eli Lilly & Co., Indianapolis. Velban, vinblastine sulfate, Lot No. 9EP95, was kindly supplied by Dr. I. S. Johnson, Biology Research Division, Eli Lilly & Co., Indianapolis; fresh solutions of the powder dissolved in pyrogen free normal saline were prepared for each experiment.

In vitro cultures. Dispersed spleen cells of DBA/2 Jax mice were immunized in vitro with sheep erythrocytes by the methods of Mishell and Dutton^{17,18} or a modification of the method used in this laboratory.¹⁹ Responses were measured after 1 to 4 days of culture. Cultured cells, including cells free in the culture medium and cells adherent to plastic, were harvested by scraping dishes with a plastic policeman. A modification of the Jerne technique using microscopic slides was used for enumerating plaques.¹⁸ When responses were measured 4 days after immunization, appropriate dilutions of cultured cells were made so that about 100 to 200 plaques were present on each slide. Each plaque count recorded for the in vitro cultures is the mean (to the

nearest whole number) of counts on 2 to 4 slides prepared from a pool obtained from duplicate culture dishes. Counts are expressed as the number of plaques or plaque-forming cells per 10^6 cells initially cultured.¹⁹ Four day cultures of normal mouse spleen cells that were not immunized in vitro with sheep erythrocytes had 1 or fewer "background" plaque-forming cells per 10^6 spleen cells initially cultured.

PRELIMINARY OBSERVATIONS

Responses to antigen. The spleen plaque-forming cell and serum antibody responses were measured 1 to 5 days after immunization for doses of antigen ranging from 10^5 to 10^{10} sheep erythrocytes per rat. Spleens of normal rats contained about 80 "background" plaque-forming cells for sheep erythrocytes; sera contained no detectable antibody to the antigen. Rats immunized with 10^6 to 10^7 sheep erythrocytes invariably had slightly increased numbers of spleen plaque-forming cells by 3 to 5 days; there was considerable individual variation in response, and sera contained no or very low titers of antibody. A dose of 5×10^7 sheep erythrocytes approximated the lowest dose of antigen that regularly produced reasonably consistent plaque-forming cell and antibody responses. Maximum responses were produced by 5×10^8 to 10^9 sheep erythrocytes; increasing the dose to 10^{10} sheep erythrocytes did not appreciably affect the response during the first 5 days. A dose of 10^9 sheep erythrocytes was selected as a "saturation" dose of antigen; i.e. a dose capable of producing a maximum response.

In repeated experiments, rats were injected with 5×10^7 or 10^9 sheep erythrocytes and killed 1 to 5 days after immunization. The results have been combined and plotted on Figure 1. One day after immunization, spleen plaque-forming cells were slightly increased above background. Between days 1 and 4, the increase in plaque-forming cells was exponential for both doses of antigen, but the rate of increase was higher for the larger dose of antigen. The average doubling time of plaque-forming cells from days 1 to 4 was 8.3 hours for the lower dose and 6.9 hours for the higher dose of antigen. The number of plaque-forming cells increased slightly from the 4th to the 5th day. The rise in serum titer from days 3 to 5 correlated reasonably well with the rise in plaque-forming cells. All measurable antibody on days 3, 4 and 5 was inactivated by 2-mercaptoethanol.

Responses to antigen and adjuvant. The adjuvant effect of various doses of pertussis vaccine was measured using 10^9 sheep erythrocytes, i.e. a "saturation" dose of antigen. Doses of 0.4 ml or more of adjuvant produced death or severe weight loss, and therefore were not used. Doses of 0.2 or 0.1 ml of adjuvant produced equal enhancement. Decreasing the dose of adjuvant to 0.01 ml reduced enhancement by about one half. On the basis of these findings, a dose of 0.15 ml of adjuvant was arbitrarily selected as capable of producing maximum enhancement, and this dose was used for all subsequent experiments. Enhancement was equivalent whether this dose of adjuvant was mixed with the antigen in vitro or was injected separately at the time of immunization.

In repeated experiments rats were injected with 10^9 sheep erythrocytes and 0.15 ml of adjuvant and were killed 1 to 5 days after immunization. The results have been combined and plotted on Figure 1. One day after antigen and adjuvant, spleen plaque-forming cells were slightly increased above background but were equal in number to those detected after immunization with 5×10^7 or 10^9 sheep erythrocytes alone. Between days 1 and 4, the increase in plaque-forming cells was exponential with an average doubling time of 5.2 hours. The number of plaque-forming

Figure 1. Spleen plaque-forming cell and serum antibody responses of rats immunized intravenously with 5×10^7 , 10^9 sheep erythrocytes or 10^9 sheep erythrocytes and adjuvant. The adjuvant was 0.15 ml of B. pertussis vaccine given intravenously at the time of immunization. Each point represents the mean of the $\log_{10}$ transformed data $\pm$ the standard error of the mean plotted as a vertical bar. The point at day 0 is the mean for 35 unimmunized rats; the point at day 1 is the mean for 32 rats, 8 immunized with 5×10^7 sheep erythrocytes, 16 immunized with 10^9 sheep erythrocytes, and 8 immunized with 10^9 sheep erythrocytes and adjuvant. Each other point is the mean for 14 or more rats.

cells increased slightly from the 4th to the 5th day. The rise in serum titer from days 3 to 5 correlated well with the increase in plaque-forming cells. Antibody present before the 5th day was inactivated by 2-mercaptoethanol. On the 5th day, low titers of antibody not inactivated by 2-mercaptoethanol were present in the sera. Adjuvant enhancement of the responses to 10^9 sheep erythrocytes is summarized in Table 1. Plaque-forming cell responses were enhanced to a greater extent than antibody titers; this discrepancy between the responses will be discussed later.

Controls for experiments using adjuvant included rats injected with 0.15 ml of adjuvant but no sheep erythrocytes. The animals were killed 1 to 8 days later. Such rats had from 200 to 2,000 "background" spleen plaque-forming cells, the greatest increase occurring 3 or 4 days after animals were given adjuvant. By 8 days spleens again contained usual numbers of "background" plaque-forming cells. Sera from rats receiving only adjuvant had no measurable antibody to sheep erythrocytes. Adjuvant given alone increased the number of background plaques, but adjuvant given 1, 2, 3 or 4 days before antigen did not enhance the response. In contrast, adjuvant given after antigen caused enhancement of responses as shown in the following experiment. Each of 44 rats was injected with 10^9 sheep erythrocytes. Twenty-four hours after immunization, 8 rats were sacrificed for spleen plaque-forming cell determinations; 18 rats were injected with 0.15 ml of adjuvant, and the remaining 18 rats served as controls. Groups of 6 rats that received

Table 1
ADJUVANT ENHANCEMENT OF RESPONSES TO 10^9 SHEEP ERYTHROCYTES

	Plaque-forming cells per spleen [*] mean of $\log_{10} \pm$ S.E.			Serum antibody titers [*] mean of $\log_{10} \pm$ S.E.		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
10^9 SRBC + adjuvant	5.08 ± 0.04	6.26 ± 0.04	6.33 ± 0.04	1.94 ± 0.17	3.33 ± 0.11	3.66 ± 0.06
10^9 SRBC	4.22 ± 0.04	5.12 ± 0.05	5.19 ± 0.08	1.32 ± 0.09	2.54 ± 0.06	2.99 ± 0.06
Difference [†]	0.86	1.14	1.14	0.62	0.79	0.67
Enhancement [‡]	7X	14X	14X	4X	6X	5X

^{*} Each mean is for 14 or more rats.

[†] The difference equals the mean for spleen plaque-forming cells or antibody titers of rats immunized with antigen and adjuvant minus the mean for animals immunized with antigen alone.

[‡] Enhancement equals the antilog of the difference.

antigen alone, and groups of 6 rats that received adjuvant one day after immunization were killed 2, 3 and 4 days after immunization. The spleen plaque-forming cell responses are plotted on Figure 2. Adjuvant enhancement was measurable 2 days after immunization, i.e. one day after adjuvant was given; the response was exponential from days 1 to 4. Enhancement was almost as great as when adjuvant was given with antigen, as may be seen by comparing the responses of adjuvant treated animals in Figures 1 and 2.

Figure 2. Enhancement of the spleen plaque-forming cell response by adjuvant, 0.15 ml of *B. pertussis* vaccine, given one day after immunization with 10^9 sheep erythrocytes. Each point represents the mean of the $\log_{10}$ transformed data $\pm$ the standard error of the mean plotted as a vertical bar. The point at day 1 is the mean for 8 rats; each other point is the mean for 6 rats.

EXPERIMENTAL OBSERVATIONS

A mitotic blocking agent could be used to estimate in vivo cell cycle times of plaque-forming cells providing that the blocking agent did not inhibit release of antibody by cells during interphase, and that cells blocked in mitosis did not release sufficient antibody to be identified. Under these conditions treatment of immunized animals with a mitotic blocking agent would render plaque-forming cells undetectable only as the cells entered mitosis. The reduction in numbers of plaque-forming cells would be directly proportional to the length of drug treatment, as long as the interval was less than the mean cycle time of plaque-forming cells. Cell cycle times could be calculated using a mathematical model appropriate for exponentially increasing popula-

tions of cells, as described in the appendix. The following experiments demonstrate the use of mitotic blocking agents for estimating cell cycle times in this way.

Use of mitotic blocking agents to estimate in vivo cell cycle times of plaque-forming cells. Colchicine or Velban given to immunized rats at various times before sacrifice caused reduction in numbers of plaque-forming cells recovered from spleens. The extent of reduction was dependent on the dose of the drug, the method of immunization, and the interval between drug administration and sacrifice. These parameters of the phenomenon were examined in a large series of experiments. Doses of drugs selected for experiments reported here were: colchicine 1.3 mg/kg or Velban 3.0 mg/kg. The drugs at these doses produced equal effects and could be used interchangeably; reducing either drug dose by one third caused significantly less effect, but increasing the dose 3-fold caused no greater effect than the doses selected. The extent of suppression of spleen plaque-forming cells was dependent on the duration of treatment of animals with the blocking agent. For example, 3 days after immunization with 5×10^7 sheep erythrocytes, 5 rats received no injections and groups of 5 rats were injected with Velban, 3 mg/kg, either 4 or 8 hours before sacrifice. The mean numbers of spleen plaque-forming cells were: untreated 5,896 (3.77 ± 0.13); Velban treatment for 4 hours 4,621 (3.67 ± 0.19); and Velban treatment for 8 hours 2,706 ($3.43 \pm .11$).*

The extent of drug suppression was also dependent on the immunization procedure as well as the duration of treatment with the blocking agent. For example, 3 days after immunization with 10^9 sheep erythrocytes and adjuvant, 5 rats received no injections and groups of 5 rats were injected with Velban, 3.0 mg/kg, either 3 or 5 hours before sacrifice. The mean numbers of spleen plaque-forming cells were: untreated 240,932 (5.38 ± 0.06); Velban treatment for 3 hours 50,100 (4.70 ± 0.06); and Velban treatment for 5 hours 21,215 (4.33 ± 0.08). In another experiment, 24 rats were immunized with 10^9 sheep erythrocytes; 24 hours after immunization 12 rats were injected with adjuvant. Three days after immunization 6 rats in each group were injected with colchicine, 1.3 mg/kg, 5 hours before sacrifice. Drug treatment reduced the number of spleen plaque-forming cells from 7,406 (3.87 ± 0.09) to 3,980 (3.60 ± 0.12) in rats immunized with antigen alone, while reduction was from 22,402 (4.35 ± 0.09) to 1,456 (3.16 ± 0.12) in rats that received adjuvant 24 hours after immunization. Thus, the extent of suppression produced per hour of drug treatment was greater in animals having more rapid doubling times of spleen plaque-forming cells resulting from immunization with higher antigen doses, or adjuvant treatment.

Cycle times measured 3 days after immunization were compared with doubling times of spleen plaque-forming cells in the following experiment. Thirty rats were immunized with 5×10^7 sheep erythrocytes; 30 rats were immunized with 10^9 sheep erythrocytes and adjuvant. Five rats in each immunized group were killed 2, 3 and 4 days after immunization. On the 3rd day, additional rats in each immunized group were injected with colchicine, 1.3 mg/kg, 2.5, 5.0 or 7.0 hours before sacrifice.

The results are presented in Tables 2 and 3. The extent of drug suppression was a function of the duration of drug treatment and the immunization procedure. In similarly immunized rats, cycle times were comparable for the various intervals of drug treatment. The mean cycle time

*The numbers in parentheses are means of logarithmically transformed data $\pm$ the standard error of the mean.

Table 2

**COLCHICINE SUPPRESSION OF SPLEEN PLAQUE-FORMING CELLS ON THE
THIRD DAY OF THE PRIMARY RESPONSE**

Immunization	Hours of colchicine treatment*	Plaque-forming cells per spleen†		
		Day 2	Day 3	Day 4
5×10^7 SRBC	None	569 (2.76 $\pm$ 0.05)	5,860 (3.77 $\pm$ 0.15)	20,790 (4.32 $\pm$ 0.07)
	2.5		3,850 (3.58 $\pm$ 0.24)	
	5.0		864 (2.94 $\pm$ 0.26)	
	7.0		311 (2.49 $\pm$ 0.17)	
10^9 SRBC + Adjuvant	None	3,230 (3.51 $\pm$ 0.12)	118,150 (5.07 $\pm$ 0.10)	965,470 (5.98 $\pm$ 0.12)
	2.5		55,830 (4.75 $\pm$ 0.10)	
	5.0		11,640 (4.07 $\pm$ 0.26)	
	7.0		7,670 (3.88 $\pm$ 0.04)	

* Rats treated with colchicine on the 3rd day after immunization received the drug, 1.3 mg/kg 2.5, 5.0 or 7.0 hours before sacrifice.

† The number of plaque-forming cells per spleen is the antilog of the mean for 5 rats; the log of the mean for 5 rats $\pm$ the standard error of the mean is given in parentheses.

Table 3

COMPARISON OF CELL CYCLE AND CELL DOUBLING TIMES (See Table 2)

Immunization	Hours of colchicine treatment	Cell cycle time in hours*	Mean cell cycle time in hours†	Mean cell doubling time in hours‡
		Day 3	Day 3	Day 2 to 4
5×10^7 SRBC	2.5	9.2		
	5.0	6.2	7.7	9.2
	7.0	7.6		
10^9 SRBC + Adjuvant	2.5	5.7		
	5.0	5.8	6.4	5.8
	7.0	7.7		

* Cell cycle time is estimated from the extent of colchicine suppression of plaque-forming cells using the method outlined in appendix 1.

† Mean of the estimates for the 3 time intervals.

‡ Cell doubling time is estimated from numbers of plaque-forming cells present in the spleens of non-colchicine treated immunized rats on days 2, 3 and 4. Method of calculation is outlined in appendix.

of 7.7 hours for plaque-forming cells in animals immunized with the low antigen dose was somewhat shorter than the mean doubling time of 9.2 hours. The mean cycle time of 6.4 hours for spleen plaque-forming cells in animals immunized with the higher dose of antigen and adjuvant was somewhat longer than the mean doubling time of 5.8 hours.

Drug reduction of plaque-forming cells was compared on various days after immunization in the following experiment. Thirty rats were immunized with 5×10^7 sheep erythrocytes, and 30 with 10^9 sheep erythrocytes and adjuvant. Ten rats from each group were killed 2, 3 and 4 days after immunization. On each day, the numbers of spleen plaque-forming cells in 5 animals given colchicine 5 hours before sacrifice were compared with the numbers of spleen plaque-forming cells of 5 similarly immunized rats that received no colchicine.

The results are presented in Tables 4 and 5. For similarly immunized rats, drug reduction of numbers of spleen plaque-forming cells was remarkably constant 2, 3 and 4 days after immunization, although numbers of plaque-forming cells increased enormously during this time. Thus, the effect of the drug was independent of the numbers of plaque-forming cells in spleens but was

Table 4

COLCHICINE SUPPRESSION OF SPLEEN PLAQUE-FORMING CELLS ON THE SECOND, THIRD AND FOURTH DAYS OF THE PRIMARY RESPONSE

Immunization	Hours of colchicine treatment*	Plaque-forming cells per spleen†		
		Day 2	Day 3	Day 4
5×10^7 SRBC	None	825 (2.92 ± 0.11)	2,099 (3.22 ± 0.08)	35,800 (4.55 ± 0.06)
	5	249 (2.40 ± 0.06)	909 (2.96 ± 0.13)	9,952 (4.00 ± 0.19)
10^9 SRBC + Adjuvant	None	2,971 (3.47 ± 0.11)	113,733 (5.06 ± 0.06)	1,178,995 (6.22 ± 0.06)
	5	296 (2.47 ± 0.21)	8,414 (3.92 ± 0.08)	103,886 (5.02 ± 0.10)

* Rats treated with colchicine received 1.3 mg of drug per kg body weight 5 hours before sacrifice.

† The number of plaque-forming cells per spleen is the antilog of the mean for 5 rats; the log of the mean for 5 rats ± the standard error of the mean is given in parentheses.

Table 5

COMPARISON OF CELL CYCLE AND CELL DOUBLING TIMES (See Table 4)

Immunization	Hours of colchicine treatment	Cell cycle time in hours*			Mean cell cycle time in hours† Day 2 to 4	Mean cell doubling time in hours‡ Day 2 to 4
		Day 2	Day 3	Day 4		
5×10^7 SRBC	5	8.1	10.4	7.7	8.7	8.5
10^9 SRBC + Adjuvant	5	5.8	5.6	5.5	5.6	5.2

* Cell cycle time is estimated from the extent of colchicine suppression of plaque-forming cells using the method outlined in the appendix.

† Mean of the estimates for the 3 days.

‡ Cell doubling time is estimated from the numbers of plaque-forming cells present in the spleens of non-colchicine treated immunized rats on days 2, 3 and 4. The method of calculation is outlined in the appendix.

dependent on the method of immunization. Cell cycle times calculated from drug reduction ranged from 8.1 to 10.4 hours in rats immunized with 5×10^7 sheep erythrocytes and 5.5 to 5.8 hours in rats immunized with 10^9 sheep erythrocytes and adjuvant. The mean cycle times and mean doubling times from days 2 to 4 were comparable in similarly immunized animals.

The results for non-drug treated, similarly immunized rats, presented in Tables 2 and 4, were combined; mean cell doubling times calculated from the increase in spleen plaque-forming cells occurring between days 2 to 4 are presented in Table 6. Also, in these 2 experiments, additional rats in both immunized groups were injected with colchicine 5 hours before sacrifice on the 3d day. Mean cycle times estimated from the combined results are also presented in Table 6. The cell cycle and doubling times are again in good agreement, particularly for animals immunized with 10^9 sheep erythrocytes and adjuvant.

Table 6
COMPARISON OF CELL CYCLE AND CELL DOUBLING TIMES
(Selected data from Tables 2 and 4 combined)

Immunization	Cell cycle time in hours*	Cell doubling time in hours†
5×10^7 SRBC	7.4 (5.7-17)	8.8 (7.8-10.2)
10^9 SRBC + Adjuvant	5.6 (5.3-6.5)	5.6 (5.1-6.0)

* The cell cycle time on the 3d day estimated from the extent of suppression of plaque-forming cells produced by 5 hours of colchicine treatment using the method outlined in the appendix. The numbers in parentheses represent the approximate 95% confidence limits.

† Cell doubling times estimated from the numbers of plaque-forming cells in spleens of all similarly immunized non colchicine treated rats 2, 3 and 4 days after immunization, using standard methods outlined in the appendix. The numbers in parentheses represent the 95% confidence limits.

The following observations provide more direct support for the assumption that the drugs reduced the numbers of plaque-forming cells by blocking such cells in metaphase.

Effect of Velban on mouse spleen cells immunized in vitro with sheep erythrocytes. Dispersed normal spleen cells were cultured in vitro with sheep erythrocytes. The early kinetics and magnitude of the plaque-forming cell response closely parallels that observed in vivo.^{17,18} Similar results have been obtained in this laboratory with the additional finding that two functionally different spleen cell populations are apparently required for in vitro induction of the immune response.¹⁹ One population consists of larger phagocytic cells or macrophages that adhere to plastic; the second population consists of smaller cells that do not adhere to plastic during short culture periods and are morphologically lymphocytes. The following observations suggest that close physical interaction between macrophages and lymphocytes continues during the in vitro immune response.

Cultured mouse spleen cells were collected 4 days after in vitro immunization; the harvesting of cultural cells was done with as little mechanical agitation of the cell suspension as pos-

sible. Plates for determining numbers of plaque-forming cells and stained smears were prepared from the suspension before and after 30 seconds agitation on a vortex mixer. Mechanical agitation of the suspension increased from 4 to 38 the number of plaques produced per 10^6 cells initially cultured. Most plaques produced by non-dispersed cells contained a central cluster of cells, Figure 3; stained smears showed that most of the clusters consisted of numerous lymphocytes surrounding one or more large mononuclear cells. Each plaque produced by the mechani-

Figure 3. A hemolytic plaque produced by a cluster of cells from a culture of mouse spleen cells 4 days after in vitro immunization with sheep erythrocytes.

cally dispersed cells contained a single centrally located cell. Microscopic examination of suspensions confirmed that mechanical agitation had dispersed the cells; in addition, microscopic examination of cultures before harvesting showed clusters of cells in the culture dishes, indicating that clusters do not result from aggregation of cells during or after harvesting. In 3 other experiments of similar design, mechanical dispersion of the cultured cells increased the number of plaques by 9-fold or more, indicating that most plaque-forming cells are in clusters.

Addition of Velban to cultures blocked many cells in metaphase and reduced the number of plaque-forming cells as shown in the following experiments. On the 4th day of culture of in vitro

immunized spleen cells, Velban diluted in balanced salt solution was added to give a concentration of 10^{-6} g per ml of culture. The salt solution alone was added to control cultures. Cultures were harvested 6 hours later. Before mechanically dispersing the cells, samples were taken for smears. These samples were treated briefly with hypotonic KCL, fixed in acetic acid-ethanol, spread on slides in 55 per cent methanol/45 per cent acetic acid, air dried and stained. The remainder of the samples were mechanically dispersed and plates were prepared to determine the total numbers of plaque-forming cells in drug-treated and control cultures. In non-Velban treated cultures, about 1 per cent of cells were in metaphase; the per cent of cells in metaphase was not discernibly greater in clusters of cells. In marked contrast, about 10 per cent of all cells in cultures treated with Velban for 6 hours were in metaphase, and 20 to 80 per cent of cells in clusters were in metaphase. Figure 4 shows a small cluster of 11 cells, 4 of which are in metaphase. Velban treatment for 6 hours reduced from 61 to 13 the total number of plaque-forming cells for 10^6 spleen cells initially cultured. The cell cycle time based on Velban suppression was 8.2 hours.

In 3 other experiments, cultures were treated with Velban 4 days after in vitro immunization. The numbers of plaque-forming cells in cultures treated with the drug for 3 or 6 hours were compared with the numbers in control cultures. The reduction in plaque-forming cells was directly proportional to the duration of drug treatment, Table 7. Cell cycle times calculated from the results of Velban treatment for 3 or 6 hours differed only slightly, and the cycle times corresponded quite well with doubling times calculated from the exponential increase in plaque-forming cells, Table 7.

DISCUSSION

The use of Velban or colchicine to estimate cell cycle times in these experiments was based on the premise that cells blocked in mitosis do not release sufficient antibody to be identified, but that release of antibody by cells in interphase is not affected by the drugs. The first assumption is consistent with observations that amino acid incorporation into protein is markedly decreased in other types of cells blocked in metaphase by colchicine,²⁰ possibly as a result of disaggregation of polyribosomes.²¹ Many observers have found occasional (<1 per cent) cells in the centers of plaques in mitosis, but mitosis may have occurred in culture after release of antibody, as has been reported recently.¹³ However, we have observed no plaque-forming cells in mitosis when cells were obtained from animals treated with Velban or colchicine, though cells in mitosis were evident in stained smears of spleen cells obtained from such animals. Several observations support the second assumption, i.e. that neither drug affects release of antibody by cells in interphase. The distribution of size of plaques produced by spleen cells from immunized rats was the same whether the cells were obtained from rats treated with Velban or colchicine or were from untreated rats. Presumably, the drugs did not produce their effects by causing progressive diminution of antibody synthesis and by this mechanism cause reduction in numbers of detectable plaque-forming cells. Very large doses of Velban or colchicine given 10 to 15 minutes before sacrifice or very high concentrations of the drugs added to the culture medium did not reduce numbers of plaque-forming cells, indicating that the drugs are not directly toxic to these cells. The extent of reduction was directly proportional to the duration of drug treatment, so long as the interval between drug administration and animal sacrifice was less than the calculated cell cycle time. The drug effect was independent of the number of plaque-forming cells in

Figure 4. A small cluster of cells from a culture of mouse spleen cells 4 days after in vitro immunization with sheep erythrocytes. Velban was added to the culture 6 hours before harvesting. Four of 11 cells are in metaphase.

spleens. It seems more reasonable to assume, therefore, that these findings are the result of the well known action of colchicine or Velban to arrest cells in metaphase, than to assume that these drugs have some other time-dependent, antigen-dose dependent, toxicity for plaque-forming cells.

Velban has been used to measure cell cycle times of tumor cells in a model very similar to the one described here.²² Inhibition of plating efficiency of tumor cells was proportional to the duration of treatment with the drug; cell cycle times estimated by this method agreed well with in vivo methods. It is of interest that in this model, Velban treatment of cells for a com-

Table 7

CYCLE TIMES FOR PLAQUE-FORMING CELLS OF IN VITRO IMMUNIZED
MOUSE SPLEEN CELLS

Hours of Velban treatment*	Exp. 1		Exp. 2		Exp. 3	
	Plaque forming cells†	Cell cycle time in hours‡	Plaque forming cells†	Cell cycle time in hours‡	Plaque forming cells†	Cell cycle time in hours‡
None	65	-	41	-	132	-
3 hrs.	35	8.8	20	7.6	82	7.4
6 hrs.	16	8.0	7	7.1	15	7.1

* Cultures, 4 days after in vitro immunization, were treated with Velban 3 or 6 hours before harvesting.

† Plaque-forming cells are recorded as the number per 10^6 spleen cells initially cultured.

‡ The cell cycle time was calculated using the model presented in the appendix. The corresponding doubling times calculated from the increase in plaque-forming cells from days 2 to 4 for experiments 1 to 3 were: 7.5, 8.4 and 6.5 hours respectively.

plete estimated cell cycle time abolished the capacity of only 90 to 95 per cent of tumor cells to grow in culture. In repeated experiments, drug reduction of plaque-forming cells never exceeded 95 per cent. It is not known why Velban or colchicine treatment for an estimated cell cycle time or longer does not abolish all plaques. The calculated cycle times represent the mean of a range of cycle times; the frequency distribution of cycle times has not been determined. The 5 per cent to 10 per cent of cells surviving the effects of the drug for the length of a mean cycle time may represent the tail of distribution of cycle times, which could have a marked degree of skewness. In any event, the tumor cell model indicates that the phenomenon is not peculiar to antibody-forming cells.

Using a hot pulse labeling technique with spleen cell cultures immunized in vitro, Dutton and Mishell concluded that essentially all antibody-forming cells appearing in cultures could arise by cell division.²³ We arrive at the same conclusion using Velban in spleen cell cultures immunized in vitro. Most plaque-forming cells in culture are aggregated in clusters. In cultures treated with Velban for 6 hours, many of the cells in clusters were in metaphase; furthermore, Velban caused a marked reduction in the total number of plaque-forming cells. These observations support the premise that the drug caused reduction of plaque-forming cells by arresting these cells in metaphase.

It might be argued that plaque-forming cells arise from an exponential rate of conversion from some other population of cells which had not previously produced antibody. In such a model, it might be expected that mitotic blocking agents would interfere with the continued conversion of cells solely by preventing multiplication of the precursor cells. Plaque-forming cells already present would not be affected by the mitotic blocking agents. Even if all plaque-forming cells arose by such conversion from a cell population with a cell cycle time as short as 5 hours, the maximum possible suppression by treatment with a mitotic blocking agent for 5 hours would be 50 per cent. The data presented here are not consistent with such a model.

Recruitment or continued activation of potential antibody-producing cells was assumed to have occurred during the early primary immune response, because generation times as short as 5.4 or 6.1 hours had not been reported for immunocompetent or other mammalian cells.¹⁴ This general argument for recruitment of cells, based solely on an assumed maximum rate for division of mammalian cells, is invalidated by observations that one population of lymphocytes in calf thoracic duct lymph has a cell cycle time of 5.5 to 6.0 hours.^{24,25}

Adjuvants might cause recruitment of additional responding cells, but the present findings provide no support for this possibility. The number of spleen plaque-forming cells is equivalent 24 hours after immunization whether animals were immunized with antigen alone or antigen and adjuvant. When adjuvant was given 1 day after immunization, enhancement 2, 3 and 4 days after immunization was almost as great as when adjuvant was given at the time of immunization; thus, enhancement cannot be accounted for by assuming appreciable recruitment of additional responding cells during the first 24 hours after immunization. If appreciable recruitment of cells occurred more than 1 day after adjuvant was given, this would result in an increased slope of the $\log_{10}$ normal plot of the plaque-forming cell response beginning when additional cells entered the population of already responding cells. No such upward inflexions have been observed.

It might be argued that the plaque-forming cell technique measures, at a point in time, the number of cells capable of synthesizing and releasing sufficient antibody to produce detectable plaques of lysis. If adjuvant had the effect of increasing the amount of antibody synthesized or released by cells, then this effect would result in increased numbers of cells that could be enumerated by the plaque-forming cell technique. Adjuvant enhanced the plaque-forming cell response to a greater extent than it enhanced serum antibody titers. This discrepancy between adjuvant enhancement of the responses might be accounted for if the amount of antibody synthesized by a cell in vivo is proportional to the length of interphase. Possibly, cells dividing every 5 hours synthesize less antibody during each cell generation than cells dividing every 7 or 9 hours. In any event, the data do not support the suggestion that individual cells from adjuvant treated animals synthesize more antibody.

The increased responses produced by increased doses of antigen or adjuvant are apparently the result of increased rates of division of responding cells. This is indicated not only by steeper slopes of $\log_{10}$ normal plots of responses, but also by shorter cell cycle times obtained when mitotic blocking agents are used. Admittedly, our experiments are not sufficiently accurate to exclude recruitment of a few additional responding cells either as a component of the continued response to a single dose of antigen or as part of the enhanced response with adjuvant. Our findings, however, are definitely contrary to those of Sterzl and Tannenbergs who concluded on the basis of autoradiographic studies that spleen plaque-forming cells arose in large part from cells that had not undergone recent mitosis.^{9,11,12} We believe there are technical and conceptual difficulties with autoradiographic studies of this type; additional work will be necessary to resolve inconsistencies between these and the present studies.

Cell doubling times during the early primary response are equivalent by the two methods used here. After this phase, and during the secondary response, the kinetics are more complex; first, because of the appearance of cells releasing "7S" hemolysin, and second because the numbers of plaque-forming cells decrease. The decrease may be due to either loss of cells from the spleens by death or emigration, or by change in function of cells. Mitotic blocking agents may provide a simple means for obtaining useful information on the kinetics of cell responses

during the late primary and secondary responses; however, the model, as presented here, cannot be applied directly to such studies without considering these parameters.

The results are consistent with a cell selection theory of antibody formation.²⁶⁻²⁹ Antigenic stimulation causes cells to proliferate and to synthesize antibody; the magnitude of the response is dependent primarily on the rate of division of initial responding antibody-producing cells and their progeny. The rate of division of antibody-forming cells might be determined by the rate of interaction between these cells and macrophages that have phagocytized antigen. In the present studies, lymphoid cells, some of which are undoubtedly plaque-forming cells, were found clustered around macrophages. Anatomical connections between lymphocytes and macrophages have been described.^{30,31} Higher antigen doses or an adjuvant might enhance the rate of interaction between the two different cell types and by this effect increase the rate of division of antibody-forming cells.

APPENDIX

In the body of the paper, a distinction is made between cycle time and doubling time. Doubling time is calculated from the increase in the spleen plaque-forming cells occurring 1 to 4 days after immunization; it is the time required for the population to double. It is assumed that the number of plaques formed follows an equation of the form

$$y(t) = Ke^{bt}.$$

Taking logarithms

$$\ln y(t) = \ln K + b \ln t$$

the logarithms of the observed number of plaques are assumed to be normally distributed. Thus, the situation is that of simple regression analysis. Standard bounds can be calculated for the doubling time from the variance of the slope.³⁴

The cell cycle time is estimated from the reduction in numbers of observable spleen plaque-forming cells produced by mitotic blocking agents.

A mathematical model was constructed to estimate cell cycle times and to determine confidence limits of the estimates. The model was constructed from the following postulates:

- a) The antibody-forming cell population is increasing exponentially.
- b) These cells are completely asynchronous with respect to stage of the mitotic cycles.
- c) All cells have a fixed cycle time, $\underline{c}$, which is the interval between successive mitoses.
- d) All cells in mitosis at the moment of administration of the mitotic inhibitor become arrested in metaphase. (All cells that subsequently enter mitosis are also arrested in metaphase.)
- e) All antibody-producing cells in mitosis, whether arrested in metaphase or not, do not form observable plaques.
- f) There is a sharply defined point somewhere at the beginning of mitosis when cells no longer produce a plaque and a point near the end of mitosis when daughter cells again produce observable plaques. The time that the cells do not produce plaques corresponds roughly to the mitotic time and is called $\underline{a}$.

g) To make analysis tractable, it is assumed that a cell doubles immediately upon entry to mitosis. This assumption does not affect the analysis since cells in mitosis are unobservable.

Let $z(t)$ be the total number of cells present at time t . Assumption (a) implies that

$$(1) \quad z(t) = ke^{bt}$$

for some positive constants k and b . Assumption (c) implies that the number of cells present doubles in time c , thus $e^{bc} = 2$ or $b = \ln 2 / c$. The constant k represents the number of cells present at the time that the response to immunization begins.

By assumptions (a) and (g), the number of cells entering mitosis between times t_1 and t_2 , ($t_1 < t_2$) is the same as the number of cells added during this interval, i.e.,

$$(2) \quad m(t_1, t_2) = z(t_2) - z(t_1)$$

The number of observable plaque-forming cells at time t is the total number of cells less those in mitosis. The number of cells in mitosis will be twice the number which entered mitosis in the interval $t-a$ to t . Thus, the number of cells observable at time t will be

$$\begin{aligned} (3) \quad y(t) &= z(t) - 2m(t-a, t) \\ &= 2z(t-a) - z(t) \\ &= ke^{bt} (2e^{-ba} - 1) \end{aligned}$$

Assuming that a mitotic blocking agent is injected x hours prior to observation, the effect is to render unobservable all cells in mitosis at the time of injection (i.e. $2m(t-x-a, t-x)$ cells) and all cells entering mitosis thereafter. Thus, twice the number of cells which entered mitosis in the interval $t-x-a$ to t are unobservable. Let $w(t, x)$ be the number of observable cells at time t when mitotic inhibitor was injected x hours prior to t (where $x < c-a$ to avoid negative numbers being predicted). Then,

$$\begin{aligned} (4) \quad w(t, x) &= z(t) - 2m(t-x-a, t) \\ &= 2z(t-x-a) - z(t) \\ &= ke^{bt} (2e^{-b(x+a)} - 1) \end{aligned}$$

Inspection of the data showed that the scatter about the mean in the experimental groups was grossly dependent on the mean. Logarithmic transformation adequately corrected this and was used throughout these calculations to achieve homoscedasticity.

Having made this transformation, it is assumed that the data are drawn from Gaussian distributions and that these distributions have means given by $\ln y(t)$ or $\ln w(t, x)$ and common variance.

To estimate the parameters, b and a , likelihood methods originated largely by Fisher³² were used. These methods give the probability density of the possible outcomes of the experiment, given the parameters of the model, namely a , c , k , and the variance of the above mentioned Gaussian distributions.

The principle of maximum likelihood is to take as estimates of the parameters those values which maximize the probability density at the point representing the actual outcome of the experiment. Fortunately, it is a property of this model that for fixed values of a and c , it is easy to compute the values of the other parameters that maximize the likelihood. (Details will be made available upon request.) A tabulation can be prepared of this likelihood maximized over these parameters against a and c and the maximum obtained.

Asymptotically, twice the negative logarithm of the ratio of the likelihood at a non-optimal parameter point to the maximum likelihood is distributed as chi-square with the degrees of freedom equal to the number of estimated parameters (four in this instance). Thus, it is possible to

set approximate confidence limits on the estimates. A recent exposition of these methods is presented by Rao.³³

The likelihood function changed very little for different values of $\underline{c}$ and $\underline{a}$ if $\underline{c}-\underline{a}$ remained the same. Hence, $\underline{c}-\underline{a}$ is the quantity being estimated in these experiments, given this model. Therefore, the results for $\underline{a} = 0$ are those reported in the paper. It is possible to substitute appropriate estimates for $\underline{a}$. Reported estimate for mitotic time of lymphoid cells ranges from 15 to 45 minutes.^{25,31} Estimates and confidence limits may be increased by this amount.

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STUDIES ON IMMUNOLOGIC UNRESPONSIVENESS DURING SECONDARY DISEASE.
III. EFFECT OF DONOR STRAIN ON ACQUISITION OF MUTUAL TOLERANCE*

By

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We have previously reported that C3H/Anf mice exposed to 900 R total-body X-radiation and given transplants of 10 million AKR bone marrow cells develop a chronic, lingering, secondary disease manifested by the usual signs of wasting and nonspecific immunologic unresponsiveness to rat erythrocytes.^{1,2} The intravenous injection of 50 million normal host or normal donor-type spleen cells 21 days after irradiation and transplantation improved survival and ameliorated subsequent course of secondary disease in the majority of mice. This effect appeared to be specific since cells transferred from unrelated but H-2k similar CBA or 101 mice were never beneficial. In contrast, the recipients were harmed by cells preimmunized to donor or host antigens.

The facts suggested that added normal host or donor spleen cells failed to respond primarily to the foreign antigens, and were thus able to repopulate and save the lymphoid-depleted host.

The present studies were performed to investigate the phenomenon further, and to extend the observations to a radiation-induced chimera afflicted with acute secondary disease, i.e., the C3H/Anf mouse with transplants of C57BL/6 bone marrow.

MATERIALS AND METHODS

Fourteen-to-eighteen-week-old female C3H/Anf, AKR, and C57BL/6 mice from Cumberland View Farms, Clinton, Tennessee, were used in these experiments. They were fed Rockland Mouse Diet complete, allowed water ad lib, housed 4 to a cage, and inspected daily for signs of secondary disease and mortality.

Basic experimental system. The basic system was similar to that previously reported.^{1,2} Host C3H/Anf (hereafter referred to as C3H) mice were confined in perforated lusteroid tubes that were rotated on a lucite wheel 79 cm from the source of x-rays. They received 900 R in air, total-body X-radiation with the following factors: 250 kvp, 30 ma, and a half value layer (HVL) of 1.02 mm Cu. Within 4 hr of irradiation, the hosts were injected with 10 million bone marrow cells of either syngeneic C3H, H-2 compatible AKR, or H-2 incompatible C57BL/6 origin. Hereafter, the host and bone marrow donors are indicated by the symbols C3H-C3H, C3H-AKR, and C3H-C57BL/6.

If no bone marrow was transplanted, primary hematopoietic failure led to death within 16 days. On this basis, it was assumed that mice surviving 21 days after 900 R did so by virtue of a successful primary bone marrow transplant. This time limit was arbitrarily selected as the onset of the secondary period, and animals dying during the subsequent 90 days were considered

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to have succumbed to secondary disease. Studies demonstrating the influence of spleen cells added at the 21st day (late addition) were related to the chimeric status and immune reactions of the host at this time.

In addition, the host response to spleen cells inoculated at 48 hr (early addition) was compared to that following similar injections 21 days postirradiation. The effect of donor anti-host antisera on this reaction was investigated.

Preparation and administration of spleen cells. The methods of immunization, tests for viability, and administration have been previously described.³ For immunization, 10×10^7 dispersed spleen cells in 0.5 ml Freund's adjuvant were injected intraperitoneally and aliquots of 50×10^6 cells in 0.2 ml of Hanks' solution were given intravenously and intraperitoneally. Three weeks later, intraperitoneal and subcutaneous booster injections of 5×10^7 cells in Hanks' solution were given. One week later, the immunized animal's spleen cells were harvested and administered to mice that had previously been X-irradiated. Fifty million dispersed spleen cells in 0.5 ml Hanks' solution were injected via the lateral tail veins.

Chimeric evaluation. Chimerism was analyzed 21 days after irradiation and bone marrow transplantation by two methods. Erythrocyte analysis was performed with antisera specific for either the donor or host. These antisera were prepared by repeated intravenous inoculation of washed erythrocytes into adult mice. The presence of C3H red blood cells was tested for with AKR anti-C3H and C57BL/6 anti-C3H antisera. C57BL/6 erythrocytes were detected by a C3H anti-C57BL/6 serum, and AKR red blood cells by a C57BL/6 anti-AKR serum that had been absorbed with C3H erythrocytes at 37° for 30 min before use. A modification of the agglutinin method described by Severson and Thompson⁴ to detect mouse hemagglutinins was employed in this analysis.⁵ The discriminant spleen assay method of Simonsen and Jensen³ was also utilized to determine the presence of chimerism 21 days after irradiation. Newborn C3H, AKR, and C57BL/6 mice were injected intraperitoneally with 10×10^6 dispersed spleen cells harvested from normal adult C3H, AKR, or C57BL/6 mice, irradiated mice transplanted with C3H, AKR, or C57BL/6 bone marrow, and mice preimmunized to antigens identical to those of the newborn host. Half of each litter received spleen cells. The remainder, as a control, received Hanks' solution. The test mice were sacrificed 8 days later and the total-body and spleen weights were recorded.

Immune reactivity of the host. Two additional methods were employed to study immune responses by the host 21 days after irradiation. The presence of donor anti-host and host anti-donor hemagglutinins and leukoagglutinins was investigated in serum obtained at this time from C3H mice transplanted with AKR and C57BL/6 bone marrow. As controls, the immune response in non-irradiated C3H animals preimmunized with repeated intravenous injections of 50×10^6 dispersed AKR and C57BL/6 spleen cells was compared to that in irradiated C3H mice protected with syngeneic bone marrow. Leukoagglutinin antibody was measured by a modification⁵ of the procedure described by Amos and Peacock⁶ and hemagglutinin antibody by the method indicated above.

Adoptive immunity was utilized to test the bone marrow recipients for graft-versus-host, host-versus-graft, or tolerant responses. Spleen cell donors were hyperimmunized to rat erythrocytes by an initial intraperitoneal injection of 0.4 ml of Freund's adjuvant containing a 50 per cent suspension of washed rat red cells. One and three weeks later, 0.2 ml aliquots of 50 per cent erythrocytes in saline were injected subcutaneously and intraperitoneally. One week follow-

ing the last injection the spleen cells were harvested and 50×10^6 cells were intravenously injected into normal hosts, and hosts with bone marrow transplants. Seven days after transfer, 0.15 ml ^{51}Cr -labeled rat erythrocytes¹ were injected. The disappearance of circulating antigen was measured, and fourteen days later the hosts were exsanguinated to determine the hemagglutinin titer.

Late test of tolerance. Full thickness AKR and C3H skin grafts were placed on both flanks of C3H mice 130 to 150 days after bone marrow transplantation and spleen cell injection. The grafts were inspected each day until rejection was complete or for 60 days.

Preparation and administration of donor anti-host antisera. Donor anti-host antisera were prepared in AKR and C57BL/6 mice by the following procedure: approximately 50 million dispersed C3H spleen cells in 0.2 ml were emulsified in an equal volume of Freund's adjuvant and injected intraperitoneally into adult AKR or C57BL/6 female mice. Two weeks later these animals were grafted with C3H skin, and 7 days thereafter a booster injection of 50×10^7 C3H spleen cells in saline was administered. One week later the antisera were obtained by axillary exsanguination.

Lethally irradiated mice were passively immunized 24 hr after bone marrow transplantation with a single intravenous injection of 0.5 ml of a 1:4 dilution of the donor anti-host antiserum.

RESULTS

Effect of added normal spleen cells. Figure 1 demonstrates the survival of lethally irradiated C3H mice protected with 10×10^6 syngeneic C3H, allogeneic H-2 compatible AKR, and allogeneic H-2 incompatible C57BL/6 bone marrow cells. Virtually all mice given allogeneic marrow transplants developed the usual signs of secondary disease but these symptoms appeared earlier and were more severe in the mice with C57BL/6 bone marrow transplants.

Figure 1. Survival of C3H mice during a 90-day period after 900 R total-body irradiation and transplantation with syngeneic C3H, H-2 compatible AKR and H-2 incompatible C57BL/6 bone marrow (B.M.).

The late addition of 50×10^6 host C3H or donor AKR spleen cells strikingly improved survival (Figure 2) in C3H-AKR mice. These survivors did not show any of the signs of secondary disease and were considered to be "saved." By contrast, when irradiated C3H mice were protected with C57BL/6 bone marrow the late injection of 50 million spleen cells was never beneficial (Figure 3). Normal C3H cells had no effect, and donor C57BL/6 cells regularly hastened mortality.

Figure 2. Effect of 50×10^6 "host" C3H or "donor" AKR spleen cells (NS) on the survival of C3H mice lethally irradiated and transplanted 21 days previously with allogeneic AKR bone marrow (B.M.).

To determine the factors that led to these contrasting results, the chimeric status and some immune responses were analyzed in identically irradiated C3H mice transplanted with C3H, AKR, or C57BL/6 bone marrow.

Chimeric status. The existence of a chimeric state was determined by two methods. First, specific antisera demonstrated that the red cell type was 100 per cent donor AKR or C57BL/6 in all of the experimental mice tested 21 days after irradiation and transplantation. Second, discriminant spleen assays confirmed the presence of donor-type AKR or C57BL/6 antigens in irradiated C3H hosts, but the reactions differed with the donor cell type. Baseline responses to normal or preimmunized spleen cells are indicated in Table 1. It should be noted that normal allogeneic cells in every case caused a significant increase in the spleen index ($p < .01$ to $< .001$), and the greatest increase occurred when the donor and newborn host differed at the H-2 locus, i.e., 1.875, when C57BL/6 cells were injected into C3H newborn. In addition, early but gross signs of wasting associated with a failure to gain weight or actual weight loss developed in those newborn inoculated with H-2 incompatible cells, i.e., the experimental C3H newborn mice injected with C57BL/6 spleen cells weighed 19.5 per cent less than their control littermates ($p < .005$). Although normal H-2 compatible cells did not cause runting, the most profound weight loss occurred in newborn C3H mice injected with spleen cells harvested from AKR

Figure 3. Effect of 50×10^6 "host" C3H or "donor" C57BL/6 spleen cells (NS) on the survival of C3H mice lethally irradiated and transplanted 21 days previously with allogeneic C57BL/6 bone marrow (B.M.).

Table 1
HOST-GRAFT INTERACTIONS WITH SPLEEN CELLS FROM NORMAL
AND IMMUNIZED MICE

Newborn host	Spleen cells	No.*	Spleen index†	% control weight‡
C3H	C3H NS	9	0.922 ± 0.125	+ 0.6 ± 9.1
	AKR NS	11	1.348 ± 0.291	+ 2.4 ± 4.9
	C57BL/6 NS	8	1.875 ± 0.593	- 19.5 ± 11.8
	C57BL/6 IS	5	1.430 ± 0.399	- 29.2 ± 13.1
	AKR IS	8	0.956 ± 0.298	- 51.4 ± 11.4
AKR	AKR NS	5	0.999 ± 0.091	+ 4.3 ± 0.6
	C3H NS	7	1.231 ± 0.183	- 0.5 ± 6.1
C57BL/6	C57BL/6 NS	7	1.016 ± 0.039	- 0.3 ± 4.4
	C3H NS	9	1.826 ± 0.322	- 6.4 ± 11.5

* Number of litters, each consisting of 2-4 control and 2-4 experimental animals.

† Index numbers ± 1 standard deviation, derived from ratio of organ to whole-body weight of experimental animal compared to its litter-mate controls.

‡ Per cent control weight ± 1 standard deviation, calculated from the ratio of body weight of experimental animals compared to their litter-mate controls.

NS 10×10^6 normal spleen cells.

IS 10×10^6 anti-C3H spleen cells.

mice preimmunized with C3H tissues (-51.4 per cent with $p < .001$). The comparatively small or absent increase in the spleen cell index in the runted mice indicates relative lymphoid hypoplasia and is consistent with the wasting.

When the spleen cells were obtained from C3H mice transplanted 21 days previously with AKR bone marrow (C3H-AKR), the spleen index was increased in both host-type C3H ($p < .001$) and donor-type AKR ($p < .025$) newborn recipients (Table 2). Additional support for splenic chimerism in the C3H-AKR mice is offered by the fact that the values were quantitatively similar to those obtained when normal C3H or AKR cells were injected into their respective allogeneic newborn recipients (Table 1). It is of interest to note that C3H-AKR cells did not induce wasting in either case. This suggests that, within the limits of the test, neither accelerated graft-versus-host nor host-versus-graft reactions were operative in these animals 21 days post irradiation.

Table 2

HOST-GRAFT INTERACTIONS WITH SPLEEN CELLS FROM IRRADIATED MICE
WITH BONE MARROW TRANSPLANTS

Newborn host	Spleen cells	No. *	Spleen index [†]	Change in body weight (%) [‡]
C3H	C3H-C3H CS	7	1.020 ± 0.137	+ 2.0 ± 2.5
	C3H-AKR CS	12	1.220 ± 0.136	+ 1.0 ± 5.0
	C3H-C57BL/6 CS	9	1.549 ± 0.265	- 11.8 ± 5.7
AKR	C3H-AKR CS	9	1.232 ± 0.209	+ 0.8 ± 6.9
C57BL/6	C3H-C57BL/6 CS	12	1.171 ± 0.166	+ 3.3 ± 9.8

* Number of litters, each consisting of 2-4 control and 2-4 experimental animals.

[†] Index numbers derived from ratio of organ to whole body weight of experimental animal compared to its litter-mate controls.

[‡] Change in body weight calculated from ratio of body weight of experimental animal to its litter-mate controls.

CS - 10×10^6 chimeric spleen cells harvested from C3H mice transplanted 21 days previously with C3H (C3H-C3H), AKR (C3H-AKR), or C57BL/6 (C3H-C57BL/6) bone marrow.

By these same criteria, primarily donor-type C57BL/6 antigens were detected in C3H-C57BL/6 mice (Table 2) since their cells increased the spleen index (1.549) and induced moderate wasting (-11.8 per cent of control weight) when transferred to host-type C3H newborn, but had no significant effect in donor C57BL/6 newborn recipients.

Immune response. Serum anti-host or anti-graft leucoagglutinin or hemagglutinin antibody could not be demonstrated in either type of bone marrow chimera 21 days postirradiation. Discriminating responses, however, were demonstrable by investigating the fate of adoptive immunization in these experimental animals. The ability of preimmunized spleen anti-rat cells of C3H, AKR, or C57BL/6 origin to survive and function was investigated in normal syngeneic hosts, in normal allogeneic hosts, and in the radiation chimeras. Tables 3 and 4 summarize these experiments, and the differences that are discussed were all highly significant ($p < .001$). Adoptive immunity was characterized in normal syngeneic hosts by a reduction in circulating antigen, i.e., the period at which 50 per cent of the ^{51}Cr -labeled rat erythrocytes remained in

Table 3
ADOPTIVE IMMUNITY IN NORMAL MICE

Host	Spleen cells	No. *	Antigen elimination (T 1/2 - days)†	Agglutinins (log ₂)‡
C3H	Hanks'	9	2.2 ± 0.05	6.2 ± 0.14
AKR	Hanks'	12	2.5 ± 0.42	6.1 ± 0.23
C57BL/6	Hanks'	10	2.3 ± 0.20	6.7 ± 0.15
C3H	C3H IS	12	0.2 ± 0.02	10.0 ± 0.00
AKR	AKR IS	9	0.7 ± 0.33	10.3 ± 0.15
C57BL/6	C57BL/6 IS	9	0.2 ± 0.20	9.3 ± 0.15

* Number of animals.

† Time of elimination of 50 % of circulating radioactivity, mean ± 1 standard deviation.

‡ Log₂ ± 1 standard deviation of the number of 2-fold dilutions of an initial 1:10 concentration.

IS - 50 x 10⁶ immune spleen cells.

Table 4
ADOPTIVE IMMUNITY IN C3H RADIATION CHIMERAS

Marrow donor	Spleen cells	No. *	Antigen elimination (T 1/2 - days)†	Agglutinins (log ₂)‡
C3H	Hanks'	12	3.1 ± 0.74	0
	C3H IS	11	0.2 ± 0.00	9.0 ± 0.85
	AKR IS	10	0.2 ± 0.03	7.4 ± 1.10
	C57BL/6 IS	5	2.8 ± 0.61	0
C57BL/6	Hanks'	11	5.0 ± 1.30	0
	C3H IS	14	1.1 ± 0.59	0
	C57BL/6 IS	13	0.1 ± 0.00	No survivors
AKR	Hanks'	12	4.8 ± 1.42	0
	C3H IS	9	0.4 ± 0.33	9.4 ± 0.14
	AKR IS	14	0.3 ± 0.22	13.3 ± 0.67
	C57BL/6 IS	9	4.9 ± 1.70	0

* Number of animals.

† Time of elimination of 50% of circulating radioactivity, mean ± 1 standard deviation.

‡ Log₂ ± 1 standard deviation of the number of 2-fold dilutions of an initial 1:10 concentration.

IS - 50 x 10⁶ immune spleen cells.

the circulation, from 2.2 - 2.5 to 0.2 - 0.7 days, and a higher hemagglutinin response (Table 3). As expected, C3H spleen cells maintained this capacity, but antibody production by allogeneic AKR and particularly by C57BL/6 cells was significantly impaired upon transfer to C3H-radiation chimeras (Table 4).

Host C3H spleen cells failed to produce antibody after transfer to C3H mice transplanted 21 days before with C57BL/6 bone marrow, indicating a persistent graft-versus-host reaction. Donor C57BL/6 cells appeared to be immunologically active and always killed their hosts before antibody could be measured.

Particularly important, however, is the observation that both host C3H and donor AKR cells functioned normally, or better, in the C3H-AKR chimeras, suggesting that a mutually tolerant state had developed during the 21 days since transplantation. Although the prolonged circulation of labeled rat erythrocytes in the hosts transplanted with AKR bone marrow indicates some degree of nonspecific immunologic depression, the mutually tolerant state cannot be totally explained on this basis. It may be seen that C57BL/6 spleen cells, which were foreign to both the donor and host in this instance, were unable to convey any degree of adoptive immunity.

The persistence of tolerance was investigated 130 to 150 days after bone marrow transplantation and spleen cell injection in the same C3H mice that had previously demonstrated mutual donor-host tolerance 21 days after irradiation. All experimental mice retained syngeneic C3H grafts through the 60 days of observation. Twelve of twelve mice that had been transplanted with AKR bone marrow but injected with C3H spleen cells at 21 days rejected their AKR grafts within 12 days. Twelve of twenty C3H mice transplanted with AKR bone marrow and injected with AKR spleen cells retained the AKR graft, but rejection occurred within 25 days in the remaining 8 animals.

Influence of donor-anti-host antisera on the "killing effect" of added normal donor spleen cells. In direct opposition to their beneficial effect at 21 days, 50×10^6 normal AKR spleen cells injected 48 hr after AKR bone marrow transplantation induced a rapidly fatal graft-versus-host reaction (Figure 4). If the host was passively immunized with 0.5 ml of a 1:4 dilution of donor AKR-anti-host C3H antisera 24 hr before the spleen cell inoculation, some of the recipients survived. This effect was highly variable from experiment to experiment, but it is noteworthy that only two deaths occurred during the final 60 days of observation, suggesting that secondary disease was minimal or absent in these survivors. Normal AKR or C3H serum had no effect.

Donor anti-host antisera did not alter the capacity of C57BL/6 spleen cells to kill C3H-C57BL/6 chimeras (Figure 5).

Finally, donor anti-host antisera never had any beneficial effect in experiments employing aggressive preimmunized cells injected either 48 hr or 21 days after irradiation and bone marrow transplantation.

DISCUSSION

These studies have demonstrated once again that donor and host spleen cells added 21 days post-irradiation are beneficial to lethally irradiated C3H mice transplanted with H-2 compatible AKR bone marrow, and have elucidated several factors important to this phenomenon. First, genetic proximity seemed to be very important, since the late injection of normal host-type cells to C3H mice transplanted with H-2 incompatible C57BL/6 cells was never beneficial and donor-type spleen cells hastened mortality. Second, although both C3H-AKR and C3H-C57BL/6

Figure 4. Effect of donor-anti-host antiserum on the "killing effect" of 50×10^6 normal donor AKR spleen cells (NS) added 48 hr after lethal irradiation of C3H mice and AKR bone marrow transplantation (B.M.).

Figure 5. Effect of donor-anti-host antiserum on the "killing effect" of 50×10^6 normal donor C57BL/6 spleen cells (NS) added 48 hr after lethal irradiation of C3H mice and C57BL/6 bone marrow transplantation (B.M.).

donor-host pairs were chimeric as demonstrated by red cell analysis and discriminant spleen assay, and non-specifically hypo-responsive to heterologous erythrocytes, their responses with respect to tolerance and graft-versus-host reactions were sharply contrasted. Whereas a vigorous graft-versus-host reaction persisted in the C3H-C57BL/6 chimera, mutual tolerance between host and donor tissues was demonstrated in the C3H-AKR type. In the latter case, phenotypically unrelated C57BL/6 spleen cells were rejected, indicating that tolerance between donor-host tissues was specific^{7,8} and not an expression of non-specific unresponsiveness.

It is generally held that the nonspecific immunologic impairment that occurs in murine secondary disease^{1,9-11} is distinct from specific immunologic tolerance,⁷ an antigen overload type of paralysis,¹² or immunologic enhancement.^{13,14} Our studies suggest that nonspecific unresponsiveness and specific tolerance are independent variables that may or may not co-exist in the same animal. It may be, however, that the distinctions have been too rigidly applied, and Zeiss¹⁵ has reported that "third party" unresponsiveness may occur in rats made tolerant at birth without evidence for apparent antigen overlap or runt disease.

Nevertheless, the inability of the C3H host to react to donor AKR tissues (and vice versa) allowed the added donor and host-type spleen cells to function and presumably to contribute to improved survival of the C3H-AKR chimera. On the one hand, added C3H spleen cells appeared to cause the rejection of the donor tissues and, when tested 130 days later, tolerance to AKR skin grafts was no longer demonstrable. On the other hand, AKR spleen cells seemed to perpetuate mutual tolerance as measured by the acceptance of both C3H and AKR grafts in the majority of cases at the same time. Unfortunately, no control non-injected C3H-AKR chimeras without added spleen cells survived to this period. Davis et al¹⁶ have reported mutual tolerance as late as 12 to 15 months post-irradiation between coexistent donor and host lymphatic tissues in 3 of 11 LAF₁-C3H chimeras.

Third, in order to understand better the mechanisms leading to mutual tolerance it was necessary to establish first whether the donor-host non-reactivity was a continuum or whether it had been actively acquired during the 21 days post-irradiation. For this reason AKR spleen cells were injected 48 hours after irradiation. The observation that donor spleen cells at this time consistently killed their C3H-AKR hosts was similar to that previously reported by Vos and Weyzen¹⁷ in other strains, and demonstrates that tolerance was not operative shortly after irradiation. These data, plus the information that non-tolerant cells injected at 21 days failed to respond primarily to their respective foreign antigens, strongly suggest that the mechanisms leading to tolerance in this case were not based solely on a depression or exhaustion of reactivity by established cells, but were more likely dependent on a process that could affect new cells as well.

Although other explanations may be possible, we have proposed¹ that the host C3H and donor AKR populations were actively stimulated to elaborate serum antibody reactive with the opposite tissue type.¹⁸⁻²¹ Such a response could act to reduce anticipated reactions by impairing the primary response or by protecting the target tissues from contact with the added competent cells.^{18,19} Although direct attempts to demonstrate donor anti-host and host anti-donor antibody were negative, this failure may reflect the continued absorption of antibody on the chimeric tissues.²² For this reason an indirect test was performed which, although inconclusive, demonstrates that donor anti-host antiserum may have spared some of the C3H-AKR hosts from the early addition of donor AKR spleen cells.

Voison and Kinsky²³ and Batchelor and Howard²⁴ have reported that donor anti-host antiserum sometimes ameliorated graft-versus-host reactions in mice. Field et al²⁵ have stated that antiserum had no effect, but that parabiosis transferred refractoriness to graft-versus-host disease in F₁ hybrid rats. Recently, a schedule of pretreatments with small amounts of donor-type cells plus passive host-anti-donor antiserum has been shown to suppress delayed type hypersensitivity to sheep red blood cells,²⁶ and to prolong markedly survival of (Lewis x BN) F₁ to parent rat kidney grafts.²⁷

The facts that graft-versus-host reactivity persisted in our C3H-C57BL/6 chimeras and that passive donor-anti-host antiserum could not ameliorate the early spleen effect does not negate the possibility that a similar response occurred in both donor-host pairs, but rather re-emphasizes the suggestion that genetic proximity may be critical for the acquisition of specific tolerance. One may speculate that the presumed antibody response was sufficient when the donor-host tissues were H-2 compatible, but was not capable of counteracting the reactions in H-2 incompatible chimeras. Alternatively, the nature of the antibodies usually formed by C57BL/6 mice may be another factor. Antibody of the 7 s type has been shown to be primarily responsible for tumor enhancement¹⁹ and for feedback inhibition of the immune response.²⁸ Anderson and associates²⁹ indicate that the C57BL/6 strain persists in elaborating 19 s to the partial exclusion of 7 s antibody, and suggest that this may account for the "superior reactivity" of this strain against homografts and in graft-versus-host reactions. It is of interest to note that in parallel studies testing the effect of donor spleen cells added 21 days post-irradiation in several different H-2 incompatible donor-host combinations, only mice transplanted with C57BL/6 bone marrow were consistently harmed by the addition of normal donor spleen cells (unpublished data).

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FRACTIONATION OF HUMAN SERUM HIGH DENSITY LIPOPROTEIN IN UREA SOLUTIONS: EVIDENCE FOR POLYPEPTIDE HETEROGENEITY*

By

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Previous studies from this laboratory on the kinetics of cleavage of the protein of human serum high density lipoprotein (apo HDL) by carboxypeptidase A led us to suspect the existence of at least two chemically distinct polypeptide chains.¹ Additional experiments were therefore planned to give a firmer support to such an observation and, if necessary, to develop methods for the isolation of the heterologous components. We report here on the results obtained by gel filtration carried out in the presence of urea, an agent shown previously²⁻⁴ to promote dissociation of apo HDL in solution. Clear evidence was obtained to indicate that this protein is made up of chemically distinct components which also differ in physical and immunological properties. While work was in progress, papers from other laboratories⁵⁻⁷ on the heterogeneity of apo HDL were published and those results will be discussed in relation to our current findings.

A preliminary account of this work has appeared.⁸

MATERIALS AND METHODS

Materials. HDL₂ (d 1.063-1.125) was separated from the sera of healthy male Caucasian donors, 20-24 years of age, group A, Rh⁺, by ultracentrifugal flotation.¹ The purity of the preparations was checked by immunological means.¹ The protein moiety, apo HDL₂, was obtained by extraction with 3:2 ethanol:ether mixture at -10°³ and dissolved, unless otherwise specified, in 10⁻² M Tris (hydroxymethyl) amino methane buffer, pH 8.6, 10⁻³ EDTA. For gel filtrations, the apoprotein solutions, 7-10 mg/ml, were dialyzed against the same Tris buffer brought to 8 M with urea at 27°. After preliminary experiments (see Results) a 48-hour dialysis time was selected. Apo HDL₂ was also studied after reduction and alkylation⁹ in 8 M urea or after succinylation.¹

Gel filtration studies. After a series of pilot studies with various types of Sephadex (G-25, G-50 fine, G-100 and G-200, Pharmacia Fine Chemicals, Sweden) and column beds, the following conditions were found to be optimal for the fractionation of apo HDL₂: type of Sephadex, G-200; bed dimensions, 2.5 x 90 cm; amount of apo HDL₂ loaded, 20 mg; equilibrating and eluting buffer, 10⁻² M Tris, pH 8.6, 10⁻³ M EDTA, 8 M urea; direction of flow, ascending; flow rate,

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Abbreviations used: HDL₂, high density lipoprotein of d 1.063-1.125; apo HDL₂, protein moiety of HDL₂ obtained by extraction with ethanol ether; SDS, sodium dodecyl sulphate.

8 ml/hour; temperature, 27°. Under these experimental conditions, each fractionation procedure lasted about 60 hours during which time the column effluent was continuously monitored at 280 m μ either in a Gilford 2000 recorder (Gilford Instruments Co., Oberlin, Ohio) attached to a Beckman DU spectrophotometer or in an ISCO recorder (Instrument Specialties Co., Lincoln, Neb.). Protein recoveries were in the order of 80-90 per cent. The fractions within each peak were pooled, dialyzed against 10⁻² M Tris, pH 8.6, 10⁻³ EDTA to remove urea, and then concentrated by vacuum dialysis at 4°. In all of these steps the solutions remained clear.

When larger quantities of apo HDL₂ (40-50 mg) were fractionated, Sephadex G-200 beds, 5 x 90 cm, were employed in Pharmacia K-50 columns (5 x 100 cm), using the same Tris-urea buffer as above and an ascending flow rate of about 10 ml/hour. Under these conditions, the fractionation time was approximately 5 days.

Analyses of Sephadex fractions.

Polyacrylamide gel electrophoresis. The technique was essentially that of Davies¹⁰ with some modifications to allow for gel polymerization in the presence of urea (4, 6, and 8 M). Acrylamide (Eastman Kodak Co., Rochester, N. Y.) in monomer concentrations of 7.5 per cent was used. Analyses were made either in Tris buffer, pH 8.3¹⁰ or acetate buffer, pH 4.5¹¹ using a Buchler apparatus (Buchler Instruments Co., Fort Lee, N. J.). Electrophoresis was carried out at 4° for 50 minutes or 4 mA per tube (6 x 90 mm). The gels were fixed and stained for protein in 1 per cent Amido Schwarz in 7 per cent acetic acid,¹⁰ and for lipids by Oil Red O.³ After destaining, some of the gels were scanned in a Canalco Mod F Microdensitometer (Canal Industries, Canal, Bethesda, Md.).

Microanalytical isoelectric focusing fractionation of apo HDL₂ and derivatives was carried out in a polyacrylamide-urea bed (acrylamide: 5 per cent urea; urea: 6-8M) containing carrier ampholytes (Ampholine, pH 3-10, LKB Produkter AB, Bromma, Sweden) using an adaptation of the method of Wrigley.¹² In the presence of urea, chemical polymerization (Procedure B) was the preferred method. After electrophoretic separation, the protein zones were precipitated by 5 per cent trichloroacetic acid and stained, without removal of the ampholytes, by Coomassie Brilliant Blue (11-152B, Consolidated Laboratory, Inc., Chicago Heights, Ill.), according to Riley and Coleman.¹³

Molecular weight determinations. Three methods were used:

Gel filtration. The experiments were carried out in 2.5 x 90 cm G-200 Sephadex columns using 10⁻² M Tris, pH 8.6, 10⁻³ M EDTA, 8 M urea as the equilibrating and eluting buffer. β -mercaptoethanol was omitted after determining that it did not affect the results. These columns were calibrated with non-enzymatic proteins of known molecular weight (Kit N 8109A, Mann Research Lab., New York, N. Y.). They were dissolved and dialyzed for 24 hours against the eluting buffer and then applied to the column either alone or as a mixture. The molecular weights of the unknowns were calculated from the calibration curve obtained by plotting the log of the MW of each standard vs its respective elution column in ml.¹⁴

Polyacrylamide gel electrophoresis in the presence of SDS. Acrylamide monomer concentrations of 5 per cent and 10 per cent were prepared in 0.1 per cent according to Warner.¹⁵ The electrophoretic conditions in 0.1 M phosphate buffer, pH 7.1, were those described by Shapiro et al.¹⁶ After migration, the gels were fixed with 20 per cent sulfosalicylic acid and then stained with 0.025 per cent Coomassie Brilliant Blue. By the use of markers of known molecular weight, a calibration curve was obtained by plotting log MW vs migration of each protein.¹⁶

Sedimentation equilibrium experiments. The studies were carried out in a Spinco Mod E analytical ultracentrifuge using both interference and absorption optics. The high-speed meniscus depletion method¹⁷ was used with the interference system employing a 6-channel centerpiece of Yphantis and sapphire windows. The experimental details of this technique as applied to apo HDL have been described¹ except that all studies were carried out with proteins that had been dialyzed against 10^{-2} M Tris buffer, pH 8.6, 10^{-3} M EDTA, 8 M urea.

The studies by absorption optics were carried out with a unit equipped with an electronic speed control, photoelectric scanning, and multiplex system. Double sector 12 mm cells were used fitted in a multicell Spinco analytical rotor to allow for the simultaneous analysis of unknowns with standards of known MW (cytochrome C and chymotrypsinogen). The solvent and the solution sides of each cell contained 10 and 15 μ l of Fluorochemical FC #306394 (Beckman Instrument Co., Palo Alto, Calif.). Proteins in the concentration of 0.2 to 0.3 optical density units were studied in 10^{-2} M Tris buffer, pH 8.4, 8 M urea. Speeds from 20,000 to 40,000 RPM were used and equilibrium was assumed after successive scanings (280 m μ) taken at 3-hour intervals showed no change. All runs were carried out at 24°. The values of the apparent molecular weight (MW_{app}) were obtained from the relation:

$$MW_{app} = \frac{2RT}{(1 - \bar{v}\rho)} \cdot \frac{1}{w^2} \cdot \frac{d \ln c}{d(r^2)}$$

where the term $d \ln c/d(r^2)$ was obtained from the slope of the $\ln OD$ vs r^2 plot. All computations were carried out in a Mod 300 Wang electronic calculator (Wang Labs. Inc., Tewksburg, Mass.) using a specially designed program. Values of $\bar{v}$ were calculated from amino acid analysis.¹⁸ Correction for the effect of 8 M urea on these $\bar{v}$ values were carried out by assuming that there was some binding of urea to the proteins and subtracting for the factor 0.02.¹⁹ Such assumptions proved valid for the markers (chymotrypsinogen and cytochrome c) which were studied in 8 M urea under ultracentrifugal conditions similar to those employed for the apo HDL₂ fractions. Direct determinations of the $\bar{v}$ of the protein fractions in 8 M urea by pycnometry proved unsatisfactory.

Spectroscopic studies. Circular dichroic spectra at 27° were recorded in a Cary Mod 6001 spectropolarimeter equipped with a circular dichroism attachment. The experimental conditions described for the study of apo HDL₂²⁰ were applied to each apo HDL₂ fraction. Spectra were also recorded as a function of temperature according to the technique previously reported.^{21,22} Protein solutions (0.5-1 mg/ml) in 0.01 M phosphate buffer, pH 8.4, were studied in 0.1 mm pathlength cells (Pyrocell, Shoreham Co., Inc., Winfield, N. J.) which were placed in a hollow, aluminum block through which water of desired temperature was circulated. Spectra were recorded after temperature equilibration was reached.

Ultraviolet absorption spectra were recorded in a Cary Mod 14 spectrophotometer at 27° using 1 cm path length quartz cells. The studies were carried out in the presence and absence of 8 M urea. The values were all corrected for scattering.²³

Immunological studies. Antisera were prepared by injecting male albino rabbits (2-3 kg) in their foot pads with 1.0-1.5 mg of apo HDL₂ or fractions emulsified with equal volumes of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). After 3 weeks, the animals received a booster injection intramuscularly (2-4 mg of antigen suspended in the adjuvant) and 10

days later they were bled by heart puncture. After determination of their titers, the antisera were stored at -20° in 1:100,000 (w/v) merthiolate.

Immunoprecipitin reactions between antigens and specific antisera were conducted by the technique of double immunodiffusion in 1 per cent agarose (Fisher Scientific Co., Chicago) as previously described.³

Amino acid analysis. Protein hydrolysates (6 N HCl, 21 hours, 110°) were separated in a fully automated Biocal 200 Amino Acid Analyzer (Biocal Instrument Co., Richmond, Calif.). In the computations, corrections were made for amino acid losses during hydrolysis.³ Cystine was determined as carboxymethyl cysteine following S-carboxymethylation of apo HDL₂ or its fractions.³ Tryptophan was determined according to Goodwin and Morton.²⁴

Other analyses. Total protein content was determined by the Lowry method²⁵ or as N x 6.25 after nesslerization.²⁶ Total cholesterol and phospholipids were determined as previously described.

Reagents. Urea, reagent grade (J. T. Baker Chemical Co., Phillipsburg, N. J.) was recrystallized from ethanol. Solutions prepared with recrystallized urea gave comparable results with those filtered through a mixed bed resin (AG-501-X8, 20-50 mesh, Biorad, Richmond, Calif.).

RESULTS

Fractionation of apo HDL₂ by Sephadex G-200; analysis of the fraction by polyacrylamide gel electrophoresis and disc gel electrofocusing. Apo HDL₂ was fractionated by polyacrylamide gel electrophoresis in 8 M urea into 8 components, 2 major (third and fourth bands from cathode) and 6 minor (Figure 1). From the densitometric scanning, the area corresponding to the third band was about 20 per cent that of the second band. Analysis by disc gel electrophoresis in 6 M urea gave similar results; with 4 M urea the various bands were visible but their separation was less satisfactory.

In the absence of the dissociating agent the number of bands was increased and separation among them was less sharp than with urea.

Gel filtration of apo HDL₂ through Sephadex G-200 in 8 M urea produced a clear separation of five peaks, labeled in order of elution at I, II, III, IV, and V (Figure 2). Peak II was a minor component not constantly seen, and will not be given consideration in these studies. When, after collection and concentration, peaks III, IV, and V were re-run in the same column either individually or as a mixture they emerged in the same elution volume without further resolution. On the other hand, re-chromatography of peak I gave rise to peaks III and IV, although about 50 per cent of the material still emerged as peak I.

When apo HDL₂ was examined after succinylation, Sephadex G-200 chromatography showed a significant reduction of peak I (< 1 per cent), no peak II, and a relative distribution of peaks III, IV, and V similar to that of apo HDL₂. Succinylation also produced an increase in the rate of anodic migration of each protein component.

The S-carboxymethylated product had a chromatographic behavior essentially similar to that of apo HDL₂. By electrophoresis a more distinct separation was noted between the bands corresponding to peak III and IV due to an increased mobility of the latter.

By polyacrylamide gel electrophoresis in 8 M urea, a correlation was found between rates of elution from the Sephadex column and electrophoretic mobility. It may be noted that the broad peak V corresponds to the distinct bands by polyacrylamide gel electrophoresis. The percentage

Figure 1. Polyacrylamide gel electrophoresis in 8 M urea of apo HDL₂. The upper panel indicates a densitometric scanning. For technical details, see text.

Figure 2. Gel filtration (Sephadex G-200 in 8 M urea) of apo HDL₂. The lower panel indicates the behavior of the various fractions isolated from the column by polyacrylamide gel electrophoresis in 8 M urea.

area distribution of peaks I, II, III, IV, and V, based upon protein determinations by the Lowry method was 5, 2, 68, 20, and 5 respectively, which is in close agreement with the densitometric data of the gel stained by Amido Schwarz.

Disc gel electrofocusing indicated differences in isoelectric point among Sephadex fractions III, IV, and V. Moreover, each of them exhibited a heterologous pattern characterized by four closely migrating bands stained with unequal intensity by Coomassie Blue (Figure 3). No significant differences were noted between gels containing 6 and 8 M urea.

Figure 3. Disc gel electrofocusing in 6 M urea of apo HDL₂ Sephadex fractions III, IV, and V. For experimental details, see text.

Lipid content of Sephadex fractions. The Sephadex fractions showed no presence of either cholesterol or phospholipids, with the exception of peak I where quantitative thin layer chromatographic analysis indicated about 5 per cent lecithin by weight.

Molecular weight determination of the various Sephadex fractions. Peaks I, III, IV, and V appeared in a single band of different mobilities by polyacrylamide gel electrophoresis in 0.1 per cent SDS. From the calibration curve using known MW markers (see Methods), significant differences in the apparent molecular weights of peaks I, III, IV, and V were noted (Figure 4 and Table 1). These figures compared well with those obtained from the calibration curve obtained from Sephadex G-200 chromatography in 8 M urea (Table 1). By analytical ultracentrifugation a linear $\ln y/r^2$ plot was obtained only with peak V both by Rayleigh interference and absorption optics. With all other products (peak II was not studied) the curve departed from linearity at the cell bottom probably relating to aggregation (Figure 5). The values of MW obtained by ultracentrifugation as listed in Table 1 are those computed from the minimum slope near the meniscus. In peak III the clear evidence of a component with minimum MW of about 17,400 was noted with the most concentrated specimen (1-2 mg/ml at 40,000 RPM). Other authors^{6,7} have suggested that apo HDL or subfractions in urea exhibit preferential bound water.

Figure 4. Plot of molecular weight vs distance of markers and Sephadex fractions from apo HDL₂ separated by polyacrylamide gel electrophoresis (0.1% SDS).

Table 1
APPARENT MW VALUES OF APO HDL₂ SEPHADEX FRACTIONS OBTAINED BY THREE INDEPENDENT PHYSICAL METHODS*

Apo HDL ₂ fractions	Sephadex G-200 8 M urea	Disc gel electrophoresis 0.1% SDS	Equilibrium ultracentrifugation†
	x 10 ³		
I	52.0 - 53.5	54.0 - 55.0	-
III	25.8 - 27.0	27.5 - 28.2	16.6 - 17.0‡
IV	16.4 - 17.2	17.0 - 17.4	17.2 - 17.6
V	11.2 - 12.1	10.6 - 11.4	11.0 - 11.8

* The values from two determinations are reported.

† The values were calculated from the minimum slope near the meniscus.

‡ This represented only a small portion of the samples. The major component had an apparent MW of about 28,000.

Figure 5. Equilibrium ultracentrifugation of apo HDL₂ fractions III, IV, and V: $\ln y$ vs r^2 plot.

Should this be the case (and this, in our opinion remains to be proven) the values listed in Table 1 may have to be reduced about 15 per cent.

Immunological studies. Peaks I, III, IV, and V all reacted against apo HDL₂ antisera. Apo HDL₂ and peak I gave a reaction of identity whereas with III, IV and V, the reaction was only partial (Figure 6A). Sera against fraction III failed to react with fraction V (Figure 6B). The results obtained with peaks III and IV antisera are summarized in Table 2. Antisera against peak V were not obtained. Differences in antigenic properties were noted between peaks III, IV, and V. Particularly, fraction V did not share antigenic determinants with either III or IV.

Spectroscopy studies. The circular dichroic spectra at 27° of apo HDL and Sephadex fractions (Figure 7) indicated differences among the various products. All of these exhibited either a trough or a shoulder at 222 m μ and a band at 208 m μ with the exception of peak V where the minimum was at 205 m μ . Ellipticity values were in the following order: peak III > peak IV > peak V. Peak V also exhibited a shift of the crossover point to 198 m μ and a significant decrease of the ellipticity of the positive band with a maximum at 193 m μ . Peak I had a spectrum significantly different from the others and resembled closely that previously described for HDL₂²⁰ with a predominance of the $[\theta]_{222}$ over the $[\theta]_{208}$ band.

Figure 6. Patterns of immunoprecipitation (double diffusion technique) of apo HDL₂ fractions III, IV and V. The same antigens were used for A and B. Central well 1: anti apo HDL₂; central well 2: anti peak III.

Table 2

SUMMARY OF IMMUNOLOGICAL RESULTS OF APO HDL₂ AND ITS SEPHADEX FRACTIONS

Antigen	Antisera		
	Apo HDL ₂	III	IV
Apo HDL ₂	i*	p. i.†	p. i.
I	i	p. i.	p. i.
III	p. i.	i	p. i.
IV	p. i.	p. i.	i
V	p. i.	neg.‡	neg.

* i = total identity.

† p.i. = partial identity.

‡ neg. = no reaction.

In the temperature studies, all carried out in the absence of urea, no significant spectral changes were noted between 4° and 27°. Between 27° and 62° there was a marked thermal transition with maximal changes of the intensity of the 222 and 208 mμ bands of 60 per cent (fraction III), 50 per cent (fraction IV) and 40 per cent (fraction V). Between 62° and 72° (the highest temperature studied) there was a tendency for a plateau. The spectral changes were compatible with an α-helix → random coil transition and were completely reversible upon cooling. Peaks

Figure 7. Circular dichroic spectra of apo HDL₂ and Sephadex fractions.

III, IV, and V exhibited different titration curves (Figure 8), distinct UV absorption spectra, and such differences were markedly accentuated in highly alkaline media up to pH 13.0 (Figure 9). The UV spectrum of fraction III at pH 8.5 in 8 M urea was characterized by a maximum at 278 mμ (276-280 mμ), a shoulder at 289 mμ and a minimum at 252 mμ. At pH 13.0, two maxima were noted at 283 and 289 mμ (283 mμ < 289 mμ) with a minimum at 272 mμ. Further, the intensity of the maximum at pH 13.0 were significantly higher than at pH 8.6.

Fraction IV, at pH 8.6 in 8 M urea, had a maximum at 276 mμ with a small shoulder at 281 mμ and a minimum at 250 mμ. At pH 13.0 the peak maximum mμ (tyrosine ionization) was increased in intensity over the 276 mμ maximum seen at pH 8.6. In addition, there was no second maximum attributable to tryptophan.

Fraction V in 8 M urea exhibited at pH 13.0 one maximum at 292 mμ and a small shoulder at 298 mμ. The results of all of the above experiments were essentially the same in the absence of urea, except for some difference of the relative intensity of the 283 and 289 mμ bands.

Amino acid analysis. The results (Table 3) indicated marked differences in amino acid composition among the various fractions and between them and apo HDL₂. Peak I had a rather similar composition to apo HDL₂ although some differences were observed. Peaks III and IV differed markedly from each other; the former contained no cystine and very small amounts of isoleucine,

Figure 8. Titration curves of apo HDL₂ Sephadex fractions III, IV and V. (Protein concentration 0.425 mg/ml.)

Figure 9. Ultraviolet absorption spectra of apo HDL₂ Sephadex fractions III, IV and V at pH 8.4 and 13.0. (Protein concentration 0.425 mg/ml.)

Table 3

AMINO ACID COMPOSITION OF APO HDL₂ AND SEPHADEX FRACTIONS

Amino acid	Apo HDL ₂	I	III	IV	V
	Moles amino acid/100,000 g protein				
Aspartic	59.4 ± 0.9 [*]	64.4 ± 1.0	70.1 ± 1.20	30.0 ± 0.42	64.0 ± 1.0
Threonine	37.0 ± 0.48	36.2 ± 0.5	36.2 ± 0.50	48.0 ± 0.71	45.4 ± 0.70
Serine	48.0 ± 0.52	44.2 ± 0.56	46.0 ± 0.60	47.0 ± 0.70	68.8 ± 0.84
Glutamic	134.2 ± 1.80	130.1 ± 1.7	138.6 ± 1.8	125.2 ± 1.50	136.9 ± 1.60
Proline	36.8 ± 0.40	37.1 ± 0.40	42.0 ± 0.5	23.7 ± 0.32	22.5 ± 0.40
Glycine	33.2 ± 0.38	38.4 ± 0.40	39.2 ± 0.50	22.3 ± 0.36	34.8 ± 0.38
Alanine	55.4 ± 0.60	53.1 ± 0.62	64.2 ± 0.70	39.0 ± 0.54	58.0 ± 0.70
Cys 1/2	5.7 ± 0.06	3.8 ± 0.04	trace	16.0 ± 0.02	trace
Valine	48.2 ± 0.52	44.2 ± 0.58	49.0 ± 0.60	42.0 ± 0.62	43.5 ± 0.64
Methionine	7.8 ± 0.09	6.3 ± 0.08	7.0 ± 0.08	10.0 ± 0.02	8.0 ± 0.08
Isoleucine	5.2 ± 0.08	8.0 ± 0.09	1.8 ± 0.02	12.0 ± 0.02	6.6 ± 0.08
Leucine	105.2 ± 1.3	103.4 ± 1.40	141.2 ± 1.60	62.0 ± 0.76	70.0 ± 0.88
Tyrosine	27.6 ± 0.34	15.6 ± 0.20	28.1 ± 0.4	23.8 ± 0.32	24.3 ± 0.36
Alanine	26.0 ± 0.34	24.8 ± 0.34	26.4 ± 0.36	24.1 ± 0.36	34.5 ± 0.46
Lysine	68.8 ± 0.85	63.9 ± 0.82	63.4 ± 0.82	78.2 ± 0.89	49.2 ± 0.67
Histidine	10.9 ± 0.10	8.9 ± 0.10	18.2 ± 0.2	1.6 ± 0.02	5.8 ± 0.07
Arginine	34.2 ± 0.41	30.1 ± 0.42	55.8 ± 0.7	1.0 ± 0.01	21.1 ± 0.32
Tryptophan	15.0 ± 0.02	12.6 ± 0.14	20.0 ± 0.3	-	4.0 ± 0.06

^{*} Values represent average of 4 determinations ± S.E.

the latter had little histidine and arginine and no tryptophan. The amino acid composition of peak V was distinct from that of III and IV.

Studies on HDL₂ (Figure 9). By disc gel electrophoresis, HDL₂ was fractionated into several bands with distribution similar to that seen in apo HDL₂. This was particularly evident in the absence and in the presence of 4 and 6 M urea, with some decrease in mobility induced by the denaturing agent (Figure 9). In 8 M urea, the separation among bands was less distinct although heterogeneity was still detected (Figure 10). All of the bands were stained by both Amido Schwarz and Oil Red O.

DISCUSSION

Our experiments indicate that gel filtration in 8 M urea of the lipid-free protein of human serum HDL₂ affords the separation of 4 components (a fifth component—fraction II—was only occasionally seen) differing in molecular weight, amino acid composition, immunological, and spectral properties. The heaviest component, designated as fraction I, which contained about 5 per cent phospholipid by weight, probably represented an aggregate of the other fractions on the basis of (1) similarity of its amino acid composition with the starting product, (2) appearance of components of lower MW upon re-chromatography, (3) its marked decrease in apo HDL₂ preparations examined after succinylation. We cannot rule out the possibility, however, that the aggre-

Figure 10. Polyacrylamide gel electrophoresis of HDL₂ in the absence of urea (1), 4 M urea (2), 6 M urea (3), and 8 M urea (4).

gate may be associated with a distinct large MW species, occurring in apo HDL₂ in rather low concentration, since a small amount of fraction I was detected by gel filtration in all experimental conditions employed and was also seen by polyacrylamide gel electrophoresis in 8 M urea.

Fraction III was the most abundant component of apo HDL₂, about 70 per cent of the total. It had an apparent MW of approximately 27,000 (evidence for a lower MW component of about 17,000 was obtained by ultracentrifugation) by three independent physical methods (ultracentrifugation, gel filtration, and disc gel electrophoresis, all in 8 M urea) and had a characteristic amino acid composition showing trace or low amounts of isoleucine and no cystine. In terms of molecular weight, it is of interest to point out the similarity between the values for the largest portion of fraction III obtained in these studies and those previously reported for succinylated apo HDL₂.¹ The latter exhibited homology by equilibrium ultracentrifugation which had also been the case for apo HDL₃ studied in guanidine hydrochloride,²⁷ and for apo HDL₂ and apo HDL₃ in sodium dodecyl sulphate.^{9,28} To reconcile the previously suggested MW homology of apo HDL with the evidence for heterology obtained in the present experiments (see data on fractions IV and V) one must assume that either the lower molecular weight components were overlooked, or that under the given experimental conditions, they formed aggregates of the same size as fraction III. Concerning the 17,000 MW component noted in fraction III under appropriate ultracentrifugal conditions, the question arose as to whether it could represent a small contamination by fraction IV which had a similar MW. This was not the case, however, since identical results were obtained with fraction III filtered 2 or 3 times through Sephadex, and no evidence of peak IV was obtained in these products by either polyacrylamide gel electrophoresis or disc gel electrofocusing.

In terms of concentration, fraction IV was the second largest component of apo HDL₂. It had a MW of about 17,000, trace amounts of histidine, arginine and no tryptophan, was relatively rich in cystine and had distinct immunological properties from either fractions III or V. Although ho-

mogeneous by disc gel electrophoresis in SDS, it was heterogeneous in urea gels or by disc gel electrophoresis, suggesting the presence of subcomponents differing in charge density but with identical or very similar MW. The presence of cystine in this fraction (in contrast with fraction III) was also indicated by the disc gel electrophoresis of S-carboxymethylated apo HDL₂ which showed an increased anodic mobility of the band corresponding to Sephadex fraction IV.

Fraction V was the most difficult to work with, because of its low content in all preparations used. This fraction had the lowest MW, a different amino acid composition from fractions III and IV, and no antigenic determinant in common with the other components. More clearly than the other fractions it showed heterogeneity by disc gel electrophoresis in 8 M urea, and this was corroborated by disc gel electrofocusing, which suggested chemical heterogeneity, and a very similar or identical MW (see data on disc gel electrophoresis in SDS and equilibrium ultracentrifugation).

Differences among the various apo HDL₂ components were also shown by circular dichroism studies where a progressive increase of disorder—indicated by the decrease of the amplitude of the 222 mμ band—was noted from fractions III to V. Whether such differences were merely due to differences in amino acid composition among these components, or whether aggregations added to the effect, was not established. A similarity was noted between the spectrum of fraction I and that previously reported for whole HDL₂.²⁰ It may be pointed out that this fraction more closely resembled the starting lipoprotein in that it contained some lipid (about 5 per cent) and probably all of its protein units, as based upon amino acid analysis.

While this work was in progress, other authors obtained evidence of the chemical heterogeneity of apo HDL. Rudman et al⁵ used preparations from lyophilized human serum (d 1.063-1.21) extracted with 1:1 ethanol-acetone. This lipid-free product was resolved into three fractions on Sephadex G-200 columns equilibrated and eluted with 1 N acetic acid. The amino acid analyses of these fractions after purification were taken to be compatible with the presence of two polypeptide chains, A and B, having the tendency to form mixed molecular aggregates. A molecular weight for the two subunits of 20,000-25,200 was estimated by gel filtration in the presence of SDS.

Shore and Shore,⁷ who fractionated the protein moieties of human serum HDL of d 1.083-1.124 and 1.126-1.195 on DEAE cellulose in 8 M urea, also obtained evidence for two distinct polypeptide chains, although their properties differed from the fractions prepared by Rudman et al. Sedimentation equilibrium studies in 8 M urea and 7 M guanidine-hydrochloride of the protein moiety of HDL of d 1.126-1.195, together with quantitative C-terminal analysis, indicated that the two peptides (R-thr and R-gln) probably occur in equimolar amounts and have a MW of about 15,000.⁸

When compared with the results of Rudman et al and Shore and Shore, our data provide evidence that apo HDL has a structural complexity greater than previously suspected: such a conclusion also is applicable to HDL₃ (unpublished data). It may be pointed out that Rudman et al did not claim to have achieved complete purity of the "monomeric" unit, whereas Shore and Shore left about 30 per cent of the protein unfractionated. It is possible therefore that peptides like those in our peak V may have been overlooked, and that the relative proportions of the two polypeptide chains described by each author may have been incorrectly estimated. In fact, the amino acid data given by Rudman et al are more compatible with a 3:1 weight ratio for peptides A and B than the 1:1 ratio suggested by these authors. Inconsistencies can also be found in the data of Shore and Shore⁷ who assumed equimolar amounts of R-thr and R-gln peptides in one of their major

DEAE chromatography fractions (peak V) which, on the other hand, by polyacrylamide gel electrophoresis showed two bands with a marked difference of staining intensity (Amido Schwarz). A comparison of our amino acid data with those of Shore and Shore⁷ indicate that the R-thr and R-gln peptides of those authors closely resemble those of our fractions III and IV, especially in terms of almost total absence of cystine and isoleucine (fraction III or R-thr), or histidine, arginine and tryptophan (fraction IV or R-gln). Since in one case gel filtration, and in the other case ion exchange chromatography was used, it is evident that these peptides are different both in size and charge. According to Shore and Shore,⁷ who estimated a MW of about 15,000 for both subunits, the R-thr peptide would be present in 8 M urea as a dimer, in contrast to the R-gln peptide which would be present in a monomeric form. Our molecular weight determinations appear to support the concept that the various apo HDL₂ components exhibit a different degree of aggregation in urea solutions, and give minimum MW values (see Table 1) for fractions III and IV that are near but not identical with R-thr and R-gln respectively. Our figures would become closer to those of Shore and Shore if we should assume, like those authors, that there is a marked preferential binding of water when apo HDL is dissolved in 8 M urea. In the absence of definitive experimental data for the $\bar{v}$ of our peptides, we are unable to choose between this possibility and the alternate (used in our computations) favoring the interactions between protein and urea. Utilization of the amino acid data to validate the molecular weight figures may not be appropriate in our case, since we suspect, on the basis of the heterogeneity shown by the three Sephadex fractions by disc gel electrophoresis and also by DEAE chromatography (unpublished), that they may each contain heterologous chains. Thus, values reported in Table 2 are likely to represent only average amino acid compositions.

In view of the peptide heterogeneity demonstrated in apo HDL₂, one is led to ask whether HDL₂ represents a single lipoprotein species with distinct polypeptide chains or whether HDL₂, as we prepare it in the ultracentrifuge, is a mixture of HDL species each with distinct peptide moieties. Our inclination to favor the second hypothesis comes from the result of the analysis of HDL₂ by polyacrylamide gel electrophoresis (Figure 10) which indicates a close similarity in the patterns of this lipoprotein and its delipidated product either in the presence or absence of urea. It should be possible, therefore, to isolate these lipoprotein components on a preparative scale and to define whether their protein moieties are of the same nature as those obtained from apo HDL₂ in our current studies. Work in this direction is now in progress in this laboratory.

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ON THE MECHANISM OF ERYTHROPOIETIN-INDUCED DIFFERENTIATION
V. CHARACTERIZATION OF THE RNA FORMED AS A RESULT
OF ERYTHROPOIETIN ACTION*

By

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The induction of erythrocyte formation from primitive precursor cells of the blood-forming tissues appears to be initiated by erythropoietin.¹ Even though the purification of erythropoietin is not yet completed, its availability in a partially purified form and its ability to initiate cytodifferentiation provide an attractive system for studying the biochemical mechanisms underlying this process. Previous work from this laboratory has demonstrated that erythropoietin stimulates heme synthesis,² hemoglobin synthesis,³ glucosamine incorporation into stroma,^{4,5} and iron uptake⁶ by rat bone marrow cells in tissue culture, and that these effects are inhibited by actinomycin D. We have also shown that erythropoietin increases the rate of RNA synthesis in this system as early as 15 minutes after its addition.⁷ These findings indicate that the stimulation of RNA synthesis plays an important early role in the process of erythroid differentiation induced by erythropoietin.

Recent studies of RNA metabolism in animal cells have demonstrated two different classes of rapidly labeled, quickly sedimenting RNA.⁸⁻¹¹ One of these, sedimenting at 45 S, is the precursor of ribosomal RNA. The other component is heterodisperse, includes sizes greater than 50 S, and, though its function is still obscure, fulfills some of the requirements for messenger RNA. Lingrel has recently shown, in addition, that the rapidly labeled RNA of anemic rabbit bone marrow cells contains 45 S RNA as well as two nonribosomal components.¹²

The data in this paper show that the RNA formed as a result of a few minutes of erythropoietin action, is not restricted to the 12-24 S region of the density gradient as was indicated in an earlier paper.⁷ On re-examining the problem we find that erythropoietin stimulates the synthesis of at least six types of RNA. Three of these components are in the 4-10 S region, one is 45 S ribosomal RNA precursor and two others, which are distinct from ribosomal RNA, sediment at 55-65 S and 150 S. Some properties of the RNAs formed as a result of erythropoietin action, and their possible roles in cytodifferentiation will be discussed.

MATERIALS AND METHODS

Materials. New-born calf serum was bought from the Colorado Serum Company, NCTC-109 from Microbiological Associates, and tissue culture dishes from Falcon Plastics. (³H) uridine (5000 Ci/mole), (¹⁴C) uridine (51.5 Ci/mole), and (³H) methionine (106.5 Ci/mole) were bought from New England Nuclear; (¹⁴C) cytidine (20 Ci/mole) from Schwarz; and (³H) cytidine (1000 Ci/mole), (³H) guanosine (500 Ci/mole), and (³H) adenosine (500 Ci/mole) from Nuclear Chicago. Actinomycin D was a commercial sample. Sheep plasma erythropoietin concentrate, a

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gift from The National Blood Resources Branch of The National Heart Institute was further purified in this laboratory.¹³

Conditions of culture. Rat bone marrow cells were cultured as previously described,² except that the medium was composed of 60 per cent NCTC-109: 35 per cent new-born calf serum: 5 per cent rat serum (containing ferric nitrate at a concentration of 7.3×10^{-5} M). Cells in 1.0 ml of medium were cultured in 35 x 10 mm dishes, and cells in 3.0 ml of medium in 60 x 15 mm dishes. Pulsing conditions and radioactive precursors are indicated for each experiment. Cells were chased by adding actinomycin D at a final concentration of 8 μ g/ml, and unlabeled precursors at a final concentration of 0.005 M.

Tests of the adequacy of chase conditions (Table 1) show that addition of 8 μ g/ml of actinomycin D, plus 0.005 M-uridine and 0.005 M-cytidine, reduces control uridine incorporation into RNA by 98 per cent and abolishes the component of RNA synthesis that is stimulated by erythropoietin. Actinomycin D alone, at the same concentration, reduces control RNA synthesis by only

Table 1

TEST OF CHASE CONDITIONS FOR INHIBITION OF RNA SYNTHESIS

	Cpm	%
Control	1355	100
Erythropoietin	1829	135
Actinomycin D + nucleosides	27	2
Erythropoietin + actinomycin D + nucleosides	28	2

Twelve cultures containing 25×10^6 nucleated cells per ml in 1.0 ml total volume were incubated for 5 hours; six of these had 0.10 units of erythropoietin per ml added. After this time 8 μ g of actinomycin D and 5 μ moles each of uridine and cytidine were added to 3 control and 3 stimulated cultures. All cultures were then pulsed with 1 μ c per ml of [³H] uridine for 20 minutes and the radioactivity in the total RNA was determined by the TCA method.

68 per cent but still totally abolishes stimulated RNA synthesis. When the RNA from actinomycin treated cells was examined on a sucrose gradient, we found that the synthesis of erythropoietin-stimulated RNA was inhibited at all regions of the gradient except for a small amount of RNA in the 4-10 S region which may represent terminal labeling of t-RNA or a small component of RNA synthesis which is not DNA dependent.

Determination of total RNA synthesis. Total RNA radioactivity was determined after lysing the washed cell pellets with ice-cold 5 per cent TCA and washing the acid-insoluble precipitates three times with ice-cold 5 per cent TCA, once with 95 per cent ethanol, and once with ether. The precipitates were dissolved in 0.20 ml of formic acid, heated at 100°C for 10 min, and then rapidly cooled to denature the DNA. The samples were counted in 10 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole per liter of a 2:1 mixture of toluene: 2-ethoxyethanol, in a Packard Model 3375 liquid scintillation spectrometer.

Isolation of RNA.

Method I. This procedure is a modification of the method previously described.⁷ Three ml of culture medium, containing the cells, plus 1.5 ml of saline wash were added to 4.0 ml of homogenizing solution (2 per cent sodium dodecyl sulfate, 1 per cent naphthalene disulfonate,

0.002 per cent sodium dextran sulfate, 0.04 per cent bentonite, and 0.02 M-Tris (pH 7.6)). The contents were quickly mixed and then shaken with 4.0 ml of water-saturated phenol containing 0.1 per cent 8-hydroxyquinoline. After centrifugation at 15,000 g for 3 min, the aqueous phase was removed, deproteinized twice more with one-half volume of phenol, and the nucleic acid was precipitated with 2 volumes of 95 per cent ethanol. The precipitate was washed twice with ethanol, dissolved in 5 ml of acetate buffer (0.10 M sodium chloride, 0.01 M sodium acetate (pH 5.1)), and reprecipitated with 5 ml of 1 per cent cetyltrimethyl ammonium bromide. This precipitate was washed once with acetate buffer and then reconverted to sodium nucleate by mixing with 1 ml of acetate buffer (modified to contain 1 M sodium chloride) and 2 ml of ethanol. The precipitate was dissolved in 1.5 ml 0.01 M-Tris, 0.001 M magnesium chloride (pH 7.6), and DNA was hydrolyzed with 5 μ g/ml of DNase (freed from RNase by chromatography on DEAE cellulose) at 25°C for 5 min. Equal volumes of homogenizing solution and phenol were then added, the contents were mixed, centrifuged, and the nucleic acids in the final epiphase were precipitated with 3 volumes of absolute ethanol. All steps, except the DNase hydrolysis, were carried out at 0°-5°C. This procedure gives a yield of approximately 90 per cent of the labeled RNA precipitable with TCA (Table 2).

Table 2
YIELD OF LABELED RNA WITH DIFFERENT METHODS
OF ISOLATION

Method	Erythropoietin (0.08 units)	Cpm	% recovery
TCA	-	6970	100
TCA	+	9540	137
I	-	6270	90
II - one phenol treatment	-	7100	102
II - one phenol treatment	+	9550	137
II - two phenol treatments	-	6410	92
II	-	5360	77
II	+	7450	107

For the purpose of this table we assume that the TCA method yields all of the labeled marrow cell RNA. Cultures contained 25×10^6 nucleated cells per ml in 3.0 ml total volume, and, where indicated, 0.08 units of erythropoietin per ml. After five hours of incubation, they were pulsed for 20 minutes with 1 μ c per ml of [3 H] uridine. These results are the averages of 3 experiments.

Method II. This procedure is a modification of the method of Scherrer and Darnell.¹⁴ Three ml of culture medium, containing the cells plus 1.5 ml of saline wash, were added to 4.0 ml of water-saturated phenol containing 0.1 per cent 8-hydroxyquinoline plus 4.0 ml of homogenizing solution while stirring rapidly in a water bath at $58^\circ \pm 1^\circ$. After 5 min of stirring, the mixture was poured into a centrifuge tube immersed in a melting ice bath and allowed to cool for 5 min. After centrifugation at 15,000 g for 3 min, the entire epiphase and interphase were removed, shaken with one-third volume of phenol, and centrifuged again. The epiphase was then removed and deproteinized a second time with phenol. The remainder of the procedure was

identical to Method I. All of the labeled RNA is found in the first supernate, but there is a small loss during the subsequent steps (Table 2). In the hot phenol extraction procedure, described by Scherrer and Darnell,¹⁴ further deproteinization with phenol was done at the same elevated temperature as the initial extraction. We find, however, that with marrow cells more labeled RNA is lost if the second and third phenol treatments are done at 58° than at 0-5°. If the heated extract of Method II is allowed to cool slowly, or is mixed during the rapid cooling, or if the interphase is not removed with the epiphase after the first centrifugation, the yield of pulse-labeled RNA is reduced to about 25 per cent. In contrast to Morrison and McCluer's¹⁵ findings with rat liver, no further labeled RNA can be obtained from marrow cells by treatment at 95° for 10 min after a prior 58° C extraction. We have found that although there is more label present, the yield of RNA is not affected by the presence of erythropoietin in the medium (Table 2).

Density gradient fractionation. This procedure was as previously described⁷ except that the rotor, speed, and time of sedimentation were as given in the figure legends, and the fractions were counted in a liquid scintillation spectrometer after the addition of 10 ml of Bray's solution.³⁷

Relative base composition. Relative base compositions of the RNA components were determined by pulsing cells with either (³H) adenosine and (¹⁴C) cytidine or with (³H) guanosine and (¹⁴C) uridine. The ³H/¹⁴C ratio was determined for each component separated on the gradients, and the relative amounts of each base were compared. This method does not allow determination of the actual base composition but does indicate relative differences in the base compositions of the several RNA components. Reversal of the labels of the same two nucleosides resulted in reversed ³H/¹⁴C ratios and the same relative base ratios, indicating that the different ratios did not reflect differential quenching of ³H and ¹⁴C at different levels in the sucrose gradient. The results obtained with this method agree with those obtained by others using ³²P (see Table 5).

RESULTS

The rapidly labeled RNA of rat bone marrow cells is rather quickly degraded after the cells are lysed in homogenizing solution and before phenol is added. The data in Figure 1 show that when compared with Method II, use of Method I caused a progressive shift toward smaller S with increase in time between lysis and phenol addition. We also found that the addition of phenol earlier than 20 seconds after lysis resulted in marked reduction in the yield of rapidly labeled RNA. This can be prevented by using Method II. To test whether the use of high temperature might cause aggregation of small-sized RNA, an aliquot from the initial emulsion of a Method I preparation was kept at 58° C for 5 minutes and then quickly cooled. This aliquot and a control kept at 0-5°, were then treated identically and analyzed on a sucrose gradient. We found that both samples had closely similar radioactivity profiles, indicating that the heat treatment does not cause aggregation.

Krantz and Goldwasser⁷ originally showed that erythropoietin increases the rate of RNA synthesis of cultured rat bone marrow cells as early as 15 minutes after its addition. The degree of response under these conditions was small, but a significant stimulation was seen in the 12-24 S region when the RNA was fractionated on a sucrose density gradient. Since we now know that rapid degradation of RNA occurred with the procedure used, the experiment was repeated using Method II for the extraction and isolation of the RNA, with the results shown in figure 2. In this experiment the cells were pre-incubated with (³H)-uridine for 10 hours before both erythropoietin and (¹⁴C)-uridine were added in a 15 minute pulse. The tritium labeling

Figure 1. Effect of time between lysis and phenol addition on the size of rapidly labeled RNA. Cultures contained 50×10^6 nucleated rat marrow cells/ml in 3.0 ml of medium with 0.10 units/ml of erythropoietin. After 30 min, the cells were pulsed for 10 minutes with $3 \mu\text{C}/\text{ml}$ of $[^3\text{H}]$ uridine. RNA was isolated by Method I, with intervals between detergent lysis and phenol addition of 20 sec (○—○), 40 sec (△—△), and 60 sec (×—×); or according to Method II (●—●). RNA was analyzed on a 5-20% linear sucrose gradient, centrifuged for 3 hr at 38,000 rev/min in the SW-39 rotor.

served as a measure of total RNA and, as we find with absorbancy measurement, shows no difference between control and stimulated samples throughout the gradient. The overall stimulation of RNA synthesis (shown with (^{14}C) uridine) at this time was rather small, as was noted originally, but, in contrast to the earlier finding, it was limited to the fraction at the bottom of the tube.

The rapid, erythropoietin-induced, stimulation of uridine incorporation into RNA might reflect an increased specific activity of the UTP pool due to increased uptake of the labeled nucleoside rather than increased RNA synthesis. We consider such a possibility unlikely, because the RNA fractionated on a density gradient shows increased incorporation due to erythropoietin only at a particular region of the gradient rather than a general increase. An additional test of whether the erythropoietin effect was caused by a change in the precursor pool was done as follows: cells were pre-labeled for one hour with (^{14}C) uridine, washed free of label, and then incubated with and without erythropoietin for 2-1/2 hours. Table 3 shows that the amount of uridine incorporated into RNA in the erythropoietin-treated cells was greater than that incorporated into control cells by about 16 per cent, and since both sets of cells had identically labeled pools the ef-

Figure 2. Density gradient profile of RNA after 15 minutes of erythropoietin action. Cultures containing 40×10^6 nucleated cells/ml in 3.0 ml of medium were pre-incubated with $[^3\text{H}]$ uridine ($0.01 \mu\text{c/ml}$) for 10 hours. 0.10 unit/ml of erythropoietin was added to one group and $[^{14}\text{C}]$ uridine ($1 \mu\text{c/ml}$) to both. After 15 minutes both sets were stopped, and RNA was isolated by method II and analyzed on sucrose gradients at 4800 RPM for 90 min in the SW 50 rotor. Open circles represent erythropoietin treated cells; closed circles, controls. The upper curve shows the distribution of RNA labeled with ^{14}C -uridine (pulse-labeled); the lower curve shows the distribution of RNA labeled with ^3H -uridine (pre-labeled).

fect cannot be ascribed to change in precursor specific activity. Although the results of this experiment do not completely eliminate the possibility that the early (15 minute) effect of erythropoietin is on the precursor pool, they strongly suggest that this is not the case.

The variation of the rate of stimulated total RNA synthesis (difference between control and erythropoietin-containing cultures) with time of incubation was measured, and the average of

Table 3

TEST OF EFFECT OF ERYTHROPOIETIN ON PRECURSOR POOL

cpm		
Control	Stimulated	Difference
11200 [$\pm$ 400]	13100 [$\pm$ 500]	1900 [$\pm$ 640] ($p < 0.05$)

50 x 10⁶ nucleated cells per ml in total volume of 3.0 ml were incubated with 2 μ c per ml of [¹⁴C] uridine for one hour. The cells were then washed free of label and diluted to 8 ml. 1.0 ml aliquots were then transferred to culture dishes. Erythropoietin, at 0.06 units per ml, was added to half of the dishes, and all were incubated 2.5 hours longer. RNA radioactivity was determined by the TCA method. The figure in brackets is the standard deviation of the mean.

three such experiments is shown in Figure 3. The effect of erythropoietin reaches a peak at about 11 hours and persists for 17-20 hours. In some experiments, however, stimulated synthesis may last for only 10 hours with a peak at 6 hours (see, for example, Figure 8).

We also studied the effect of increasing labeling time on the distribution of radioactivity in RNA from cells incubated with erythropoietin for three hours (Figure 4). After 5 minutes (Frame A) the RNA radioactivity was small and heterodisperse; the only significant difference between the control and stimulated groups was in the bottom fraction of the gradient. After 10 minutes of labeling (Frame B), the radioactivity distribution was still heterodisperse, but an increase due to erythropoietin was seen in the 35 S and greater region. In frames C, D, and E the radioactivity profile changed with time until it was parallel to the absorbancy profile, and the major difference due to erythropoietin was in the 28 S, 18 S and 6 S peaks. These results are in accord with many reports showing a shift from heterodisperse RNA (including 45 S) to ribosomal RNA.¹⁴ Chase experiments with stimulated marrow cells also show the conversion of 45 S RNA to 18 S and 28 S RNA. Figure 4 shows, in addition, that erythropoietin markedly increased the labeling of that RNA sedimenting to the bottom of the gradient at all labeling times.

In view of the effect of erythropoietin on the bottom fraction, the RNA from the 50 S and greater region was further characterized by re-running on another gradient for a shorter time (Figure 5). In these experiments RNA was analyzed after three different conditions of culture: (A) In frames A and C there were three hours of contact with erythropoietin, then a 30 minute pulse with labeled uridine; (B) In frames B and D, there were three hours of contact with erythropoietin, a 30 minute pulse with uridine, and a 3 hour chase; (C) And in frames E and F pre-incubation for 24 hours was followed by 3 hours with erythropoietin and a 30 minute uridine pulse. For each of these conditions, controls with no erythropoietin were also run. Frames C, D and F represent the re-run at shorter times of the heavy fractions from A, B and E respectively. The results in frame C show a small but definite radioactivity peak at 150 S, formed in response to erythropoietin, and no detectable amount of this component in the controls. The control cells had an appreciable peak of radioactivity in the 55-65 S region which was increased in the presence of erythropoietin. When cells were chased for three hours after the uridine pulse, and then analyzed, the 150 S peak was no longer in evidence but a broad peak centering about 45 S persisted (frame D). Cells that had been pre-incubated for 24 hours before the three hour erythropoietin treatment and 30 minute pulse showed a pattern of re-fractionated, labeled RNA

Figure 3. Effect of time after erythropoietin addition on stimulation of RNA synthesis. The results are a composite of three experiments. Triplicate cultures contained 15×10^6 nucleated cells/ml in 1.0 ml medium. Stimulated groups received 0.05 units/ml of erythropoietin at zero time. At the indicated times, control and stimulated groups were pulsed with $1 \mu\text{C}/\text{ml}$ of $[^3\text{H}]$ uridine for 30 min, and total radioactive RNA was determined by the TCA method.

(frame F) that was very similar to the one from cells without any pre-incubation. A definite 150 S component and a larger amount of a 55-65 S component were formed in response to erythropoietin.

We studied the stability of the RNA found in the 55-65 S portion of a sucrose gradient in a pulse-chase experiment (Table 4). Approximately two-thirds of the RNA (55-65 S) formed in the presence of erythropoietin persisted for 3 hours, while about one-third of the same fraction persisted in control cells. Since this region of the gradient probably consists of several different species of RNA and since the stimulated cells may represent a population distinct from the non-stimulated cells we cannot provide a convincing explanation for this difference in rates of decay. It might be due to the presence of longer-lived large RNA constituents in newly formed erythroid cells. Alternatively, the increased life-span may reflect a stabilizing effect of erythropoietin on the newly formed RNA.

Under conditions of incubation where the rate of formation of 150 S is maximal (see Figure 8) a pulse-chase experiment showed its half-life to be about 6 minutes.

In a double label experiment we examined the simultaneous incorporation of methyl groups from methionine and of uridine into the RNA of erythropoietin-treated marrow cells (Figure 6). The general pattern of RNA synthesis, as seen by uridine incorporation and analysis on a sucrose gradient, was heterodisperse and the only significant peak was at the bottom of the tube. When the bottom fraction was re-run on another gradient, as described above, most of its uridine radioactivity was in a peak at 150 S. In contrast, the methyl group incorporation showed

Figure 4. Effect of pulse time on the sedimentation profile of erythropoietin-stimulated RNA synthesis. Cultures contained 40×10^6 nucleated cells/ml in 3.0 ml of medium with (o—o) and without (●—●) 0.07 units/ml of erythropoietin. After 3 hours of incubation, $1 \mu\text{Ci/ml}$ of $[^3\text{H}]$ -uridine was added. RNA was isolated by Method II after 5 (A), 10 (B), 30 (C), 60 (D), and 120 (E) min of further incubation. Density gradient analyses were run for 120 min at 48,000 rpm in the SW50 rotor.

peaks at 45 S, 28 S and 4 S as well as at the bottom, while the 55-65 S region had no significant label.

We found that the sedimentation properties of the 150 S, 55-65 S and 45 S RNA fractions are not affected by DNase ($5 \mu\text{g/ml}$), pronase ($20 \mu\text{g/ml}$ at 25° for 15 minutes) urea (8 M) or by EDTA (0.01 M). Ribonuclease, however, causes rapid hydrolysis showing that these three fractions represent discrete RNA species rather than complexes of RNA with DNA, protein, or other RNA.

The results of Figure 4 indicated that erythropoietin also stimulates the synthesis of RNA

Figure 5. Refractionation of RNA from the 50 S and greater region. A. Cultures containing 60×10^6 nucleated cells per ml were incubated with (o—o) and without (●—●) 0.09 units of erythropoietin per ml for 3 hours, then pulsed for 30 minutes with $2 \mu\text{C}$ per ml of $[^3\text{H}]$ uridine. RNA was isolated by Method II and run on a sucrose gradient for 80 minutes at 48000 rpm in the SW50 rotor. B. Cells were incubated under the same conditions as in (A) but chased for 3 hours by addition of $8 \mu\text{g}$ per ml of actinomycin and $5 \mu\text{moles}$ per ml each of uridine and cytidine. C. and D. Fractions having S of 50 and greater from (A) and (B) respectively were pooled, precipitated with carrier RNA and run for 35 minutes at 48000 rpm. E. Cultures containing 25×10^6 nucleated cells per ml were incubated for 24 hours, then 0.20 units of erythropoietin (o—o) or an equal volume of medium (●—●) were added. After 3 hours more, all cultures were pulsed for 30 minutes with $2 \mu\text{C}$ per ml of $[^3\text{H}]$ uridine; RNA was isolated and analyzed as in (A) and (B). F. The 50S and greater region of (E) was re-run as in (C) and (D). All cultures had a total volume of 3.0 ml and six replicates of each were pooled.

sedimenting in the 4-10 S region. In addition, part of this RNA fraction accepts methyl groups from methionine (Figure 6), the peak of methyl-labeled RNA being consistently at 4 S whereas the uridine-labeled RNA sediments at 6 S. Recent work with other systems has shown that an

Table 4
STABILITY OF 55-65 S RNA

Control cpm after			Δ cpm due to erythropoietin after		
Pulse	Chase	%	Pulse	Chase	%
750	250	33	260	175	67

Cultures containing 50×10^6 nucleated cells per ml in a total volume of 3.0 ml were incubated with and without 0.10 units per ml of erythropoietin for three hours. They were then pulsed for 15 minutes with 1 μ c per ml of [3 H] uridine, and half of the cultures were stopped immediately using method II. The other half were chased for 3 hours as in Table 1, then stopped, and RNA was isolated by method II. Sucrose gradients were centrifuged at 48000 rpm for 80 minutes in the SW 50 rotor, and the 50 S and greater region was re-run for 40 minutes under the same conditions. The table shows the radioactivity in the 55-65 S region of the second gradient.

RNA species, different from transfer RNA, and sedimenting at 5 S or 7 S, is present in the "soluble" component of fractionated RNA.¹⁶⁻¹⁹ In the experiment represented in Figure 7, the 0-15 S fraction of RNA, synthesized in the presence of erythropoietin, was fractionated on a second sucrose gradient for a longer time. The results show that there were peaks (uridine labeled) at 4 S, 6S and 9 S. The synthesis of all three is stimulated by erythropoietin at this time (3 hours after addition). Only the 4 S RNA was appreciably methylated and presumably contains t-RNA. Under these conditions a negligible fraction of the methyl radioactivity is found as methionyl-t-RNA. The significance of the nonmethylated 6 S and 9 S RNA components will be considered later.

The relative base compositions of the different RNA fractions from erythropoietin-treated cells are shown in Table 5. In all cases erythropoietin caused an increase in RNA synthesis of 30-40 per cent. The relative base ratios of RNA in the 45 S peak* are intermediate between those of 18 S and 28 S RNA, and 28 S RNA has greater G/U and C/U ratios than 18 S RNA. This agrees with the results obtained by others using 32 P.^{20,21} The relative base composition of the chased 55-65 S RNA which is relatively free of contaminating 45 S RNA shows C/U and G/U ratios that are smaller than those found for the 45 S component. A greater AU and smaller GC content of the heterodisperse, high molecular weight nuclear RNA, when compared to 45 S RNA, has been described in other cell systems.^{9-11,22} The relative base ratios of the 150 S and 45 S components are rather similar, and those of the 6 S and 4 S RNA components are almost identical.

To determine how stimulation of the various RNA components changes with time of exposure to erythropoietin, cultures were pulsed for 15 minutes with 3 H uridine at different times after erythropoietin addition, and the isolated RNA was analyzed on a sucrose gradient (Figure 8). The rate of synthesis of 150 S RNA was maximal in the 0-15 minute period, decreased at 2-4 hours, rose to a small peak at 6 hours, and disappeared after 10 hours. In contrast, the

*We are aware that each peak in a sucrose gradient may contain several species of RNA and do not imply that the base composition represents that of a single component, but use the measurements to show differences and/or similarities among the various types of RNA.

Figure 6. Relationship between uridine labeling and methylation of RNA synthesized in the presence of erythropoietin. Two cultures containing 40×10^6 nucleated cells per ml (total volume 3.0 ml) were pre-incubated for 10 hours, after which 0.10 unit of erythropoietin per ml [^{14}C] uridine (o—o) ($1 \mu\text{c}$ per ml) and [^3H] methyl-methionine (●—●) ($10 \mu\text{c}$ per ml) were added to each. After a 15 minute pulse RNA was isolated by Method II, pooled and analyzed on a sucrose gradient at 48000 rpm for 90 minutes in the SW50 rotor.

stimulated rates of formation of RNA found in the 4-10 S, 45 S, and 55-65 S regions gradually increased to a maximum at about 6 hours and then sharply decreased after that time.

DISCUSSION

There is considerable evidence indicating that erythropoietin acts upon a primitive cell type to induce erythroid differentiation. The morphological and chemical changes accompanying the later stages of this developmental process have been extensively studied, but induction of these changes has come under investigation only recently. Previous work from this laboratory has shown that the addition of erythropoietin to rat bone marrow cells in vitro causes several biochemical changes including the stimulation of RNA synthesis in as short a time as 15 minutes. Data presented here have shown that the earlier characterization of this RNA as sedimenting between 12 and 24 S was faulty, because of some degradation of RNA during the isolation

Figure 7. Refractionation of RNA from the 0-15 S region. Four cultures containing 50×10^6 nucleated cells per ml, in 3.0 ml, were incubated in the presence of 0.08 units of erythropoietin per ml. After 3 hours, [³H] uridine (5 μ c per ml) and [¹⁴C] methyl methionine (1 μ c per ml) were added. After a 30 minute pulse RNA was isolated by Method II and fractionated on a sucrose gradient centrifuged at 48000 rpm for 80 minutes in the SW50 rotor. The 0-15 S portion was precipitated with carrier RNA and re-run on a sucrose gradient for 12 hours at 48000 rpm, ([¹⁴C] uridine o—o, [³H] methionine ●—●).

procedure. Other investigators have isolated RNA from different cell systems, using procedures similar to Method I and have not found the degradation pattern we observe with bone marrow.^{9,10,22} Minguell and Perretta,²³ however, have recently shown that rat bone marrow cells have an unusually high activity of nuclear ribonuclease when compared to other tissues. Since we find that the rapidly labeled RNA of bone marrow is restricted almost exclusively to the nucleus, the difference between marrow and other tissues may reflect this nuclear ribonuclease activity.

In the present report we have shown that erythropoietin stimulates the synthesis of rapidly labeled RNA fractions sedimenting at 150 S, 55-65 S, 45 S, 9 S, 6 S, and 4 S, and that the stimulated 45 S RNA is converted into 28 S and 18 S ribosomal RNA. Our observation that erythropoietin increases the rate of both ribosomal and transfer RNA synthesis is compatible with the fact that erythroid differentiation involves extensive hemoglobin formation. The induction of specific erythroid functions by erythropoietin indicates that some types of messenger RNA may also be formed.

The 6 S RNA component whose synthesis is stimulated by erythropoietin is unmethylated

Table 5

RELATIVE BASE COMPOSITIONS OF RNA COMPONENTS FROM
ERYTHROPOIETIN-STIMULATED CELLS

RNA component	G/C	G/U	A/C	A/U	G/A	C/U
150S	3.2	3.1	2.3	2.3	1.4	1.0
55-65S*	3.7	2.2	-	-	-	0.59
45 S	2.9	2.9	2.6	2.7	1.1	1.0
28 S*	3.4	3.6	-	-	-	1.1
18 S*	2.5	2.1	-	-	-	0.83
9 S	3.3	3.4	3.9	3.9	0.87	0.97
6 S	4.8	4.8	5.0	5.0	0.95	0.97
4 S	5.4	4.9	5.4	5.0	0.99	0.95

Cultures containing 50×10^6 nucleated cells per ml in a total volume of 3.0 ml with 0.10 units/ml of erythropoietin were incubated for 4 hours; half of them were then pulsed for 30 minutes with $1 \mu\text{C}$ per ml of [^3H] adenosine and [^{14}C] cytidine, the other half were similarly pulsed with [^3H] guanosine and [^{14}C] uridine. RNA was isolated by method II; sucrose gradient separation was for 80 minutes at 48000 rpm in the SW rotor. The 0-15 S region was re-run as described in Figure 7. These results are averages of 2 experiments. The base ratios of the components sedimenting at 55-65S, 28S, and 18S were determined in a similar manner but using [^{14}C] uridine and either [^3H] cytidine or [^3H] guanosine and chasing the cultures for 3 hours. These results are an average of 6 experiments, except those with an asterisk which are averages of two experiments.

and has relative base ratios almost identical to 4 S RNA. Our data also indicate that this component is synthesized in an amount approximately equimolar with 45 S RNA, and 28 S and 18 S ribosomal RNA. Many animal cell systems contain 5-7 S RNA species.¹⁶⁻¹⁹ This class of RNA lacks methylated bases and pseudouridine, but otherwise has a base composition almost identical to transfer RNA.¹⁶ It appears to bind to the 60 S ribosomal subunit in a one to one ratio.¹⁸ Hybridization and rate study experiments have indicated that this RNA is neither transfer RNA precursor nor messenger RNA, and it is considered to be a third class of ribosomal RNA.^{17,19} The 6 S RNA component in erythropoietin-stimulated marrow cells probably represents this type of RNA. A 5 S RNA component isolated from rabbit reticulocyte ribosomes,²⁴ may represent an RNA similar to the 6 S RNA of rat marrow.

We think that the possibility that 55-65 S RNA and 150 S RNA represent aggregates of smaller RNA molecules is minimal. Considerable evidence indicates that other similar rapidly sedimenting RNA molecules are single, linear, polynucleotide chains rather than aggregates. These classes of RNA can be treated with DNase, pronase, EDTA, or urea and refractionated without significant change in sedimentation properties.^{10,11} Such molecules appear as single threads 4-6 μ long when visualized under the electron microscope.¹¹ Bramwell and Harris²⁵ have recently suggested however, that all nuclear RNA may be composed of 16 S subunits. Wagner et al²⁷ have shown that ribosomal RNA can aggregate into larger sedimenting forms under certain conditions of high RNA concentration and hot phenol extraction. The latter results indicate that the exact chemical and physical nature of very rapidly sedimenting RNA may not yet be completely clear. We have found that the hot phenol extraction of Method II does not cause aggregation of the smaller-sized RNA isolated by the low temperature phenol. The 150 S, 55-65

Figure 8. Variations in rates of erythropoietin-stimulated RNA synthesis with time. Cultures containing 30×10^6 nucleated cells per ml in a total volume of 3.0 ml were pre-incubated for seven hours, and then given 0.25 units of erythropoietin per ml or an equal volume of medium. At each indicated time one control and one stimulated culture were pulsed for 15 minutes with $[^3\text{H}]$ uridine ($2 \mu\text{c}$ per ml) after which RNA was isolated by Method II. Density gradient analyses were done at 48000 rpm for 80 minutes in the SW50 rotor. The ordinate indicates the change in cpm due to erythropoietin for each of the components isolated.

S, and 45 S RNA components we find in marrow cells are also completely stable to treatment with EDTA, urea, pronase or DNase, but are degraded by treatment with RNase indicating that these components represent distinct RNA species.

The role of 150 S RNA in erythroid development is still completely unclear. Of all the RNA types studied, only this species, with a minimum molecular weight of about 18 million, seems to be unique to erythropoietin-stimulated cells of the marrow. In the first 15 minutes after erythropoietin addition, the formation of this component is stimulated to a much greater degree than that of the other RNA components. The fact that it is methylated might suggest a role for 150 S RNA as a precursor of 45 S RNA or of transfer RNA. These possibilities cannot be excluded on the basis of our present data. Since the 150 S RNA component disappears rapidly during a chase it cannot be the long-lived messenger for globin synthesis. It is possible, however, that it represents what Scherrer et al¹¹ have termed "a unit of transcription," only an undetectably small part of which is the messenger for whatever function has been initiated by erythropoietin. If, for example, the unit of transcription contained the messenger for globin, which was freed from all the other transcribed RNA in the 150 S unit by degradation within the nucleus, less than 1 per cent would be transferred to the cytoplasm. The rationale for transcribing a

very large RNA molecule in order to obtain a very small messenger has been discussed by Scherrer.²⁸ Whether 150 S RNA, or part of it, acts as a messenger; has a regulative function within the nucleus; or does something else, must await further investigation.

Extensive study of 9 S RNA isolated from rabbit reticulocytes indicates that it is long-lived, and suggests that it might be messenger RNA for globin synthesis.^{29,30} Burny and Marbaix³¹ have demonstrated that the 9 S RNA is associated with the polysomes of rabbit reticulocytes, is preferentially labeled after a 15 hour pulse in vivo, and is more sensitive than ribosomal RNA to degradation when reticulocyte polyribosomes are treated with a low level of RNase.²⁴ These properties are considered characteristic of messenger RNA. The base composition of rabbit reticulocyte 9 S RNA reported by Marbaix et al²⁶ agrees with the relative base composition we obtain for rat bone marrow 9 S RNA. It would be tempting to conclude that erythropoietin stimulation of the synthesis of 9 S RNA in the nucleus of rat bone marrow cells represents the induction of globin messenger RNA. The demonstration of messenger activity, however, must be functional and would require showing that this specific RNA codes for globin. Considerable effort has been made to demonstrate this relationship between rabbit reticulocyte 9 S RNA and hemoglobin synthesis.^{29,30,32} Recent work has indicated, however, that the added RNA in such experiments may affect endogenous messenger activity rather than act as a specific template.³³⁻³⁶ We have found that synthesis of both the 55-65 S RNA and the 9 S RNA are stimulated by erythropoietin, and that they are rapidly labeled, relatively long-lived, and are distinct from the known ribosomal RNA species. Assignment of a definite functional role for these two RNA components in developing erythroid cells, however, must await further study.

We have presented evidence that erythropoietin rapidly induces a complex set of changes in synthesis of RNA by cultured rat bone marrow cells. Erythropoietin increases the production of ribosomal and transfer RNA, and stimulates the synthesis of other RNA components whose role in erythroid differentiation still remains to be determined. This study indicates, however, that the simplified model of erythropoietin induced cytodifferentiation based solely on events at the transcriptional level¹ will require modification in order to incorporate the findings reported here.

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APPARENT TURNOVER OF MITOCHONDRIAL DNA AND MITOCHONDRIAL PHOSPHOLIPIDS IN THE TISSUES OF THE RAT*

By

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Mitochondria and other cell organelles such as chloroplasts have been shown to have a degree of autonomy.³⁻⁵ Mitochondria contain DNA that differs in molecular size, physical properties and, in certain cases, base composition from nuclear DNA.⁶⁻¹¹ The organelles also contain distinct species of ribosomal DNA¹² and transfer RNA's,¹³ and the enzymes necessary to synthesize RNA, DNA and protein.^{6,14,15} However it is apparent from the relatively small molecular size of mitochondrial DNA (about 1×10^7 daltons)^{8,9} that much of the information required for the assembly of the organelle is derived from the nuclear genome. Most of the mitochondrial proteins are probably synthesized on the ribosomes of the endoplasmic reticulum and subsequently assembled in the organelle, as has been shown to be the case for cytochrome *c*.¹⁶

The mechanism of destruction and turnover[‡] of mitochondria is still unclear. Several years ago Fletcher and Sanadi¹⁷ reported that the turnover rates of four components of rat liver mitochondria, i.e. cytochrome *c*, total phospholipid, soluble protein, and insoluble protein were nearly identical with half lives of about 8-10 days. They suggested that mitochondria are synthesized and degraded as a unit. Subsequent studies of Beattie et al.¹⁸ have shown that a variety of mitochondrial protein fractions turn over at nearly the same rate although some soluble proteins have a more rapid turnover. Similar turnover rates of mitochondrial protein and total phospholipid in normal rat liver were also confirmed by Bailey et al.¹⁹ Most of the components studied in the cited investigation are not exclusively mitochondrial so that their apparent turnover rate may in some measure reflect the purity of the mitochondrial preparations examined.

It is now clear that mitochondria are composed of at least two major subunits, the inner and outer membranes. It is quite possible that the two membranes follow entirely different pathways and patterns of synthesis and destruction. Furthermore inducible enzymes localized in the mitochondria such as δ -amino levulinic acid synthetase (ALAS)²⁰ and ornithine amino transferase and alanine amino transferase²¹ have substantially more rapid turnover rates than other mitochondrial components.

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[‡] It is evident from considerations of data presented in this paper that we cannot exclude the recycling of isotopic material through some of the mitochondrial components studied. We therefore use the terms turnover, half-time and half-life as really indicating apparent turnover, apparent half-time and apparent half-life.

The purpose of this study was to reevaluate the concept that mitochondria are synthesized and destroyed as a whole. The turnover rates of mitochondrial DNA and of individual mitochondrial phospholipids were studied in the liver, heart, kidneys and brain of the rat. Mitochondrial DNA can be considered to be one of the few structural elements of the mitochondrion which can be obtained in pure form. It is localized in the matrix region of mitochondrion²² and has been shown in yeast to be associated with a membrane mesosome-like structure.²³ The phospholipid cardiolipin or diphosphatidyl-glycerol is primarily localized in the inner membrane of the mitochondrion in liver.^{24,25} The validity of the turnover rates of mitochondrial DNA and of cardiolipin, at least in the liver, are therefore independent of the purity of the mitochondrial preparation employed for study. Turnover of other phospholipids isolated from purified mitochondria were also compared with turnover of mitochondrial DNA and cardiolipin. Since turnover of mitochondrial components varied greatly among different organs, more stringent criteria could be applied to test the hypothesis that the mitochondrion turns over as a unit when measurements were made in several organs.

MATERIALS AND METHODS

Labeling and DNA and phospholipids. Female Sprague-Dawley rats weighing 200 to 230 g were used. DNA was labeled with dThd-methyl-³H* (Schwarz BioResearch, 6.0 c/mMole); ³²P, carrier free (Mallinckrodt Nuclear), was used to label phospholipids.

Three separate experiments were performed; 24-60 rats were each given a mixture of 200-400 μ c tritiated dThd and 200-600 μ c ³²P by intraperitoneal injection. Preliminary experiments established that there was no difference in the specific activities of DNA and phospholipids if the isotopes were given in a single dose or by three equal injections separated by two hours. The doses of ³²P used did not produce anemia during the course of the experiment. Radioisotope was administered to all animals at the same time. Groups of 4-12 rats were sacrificed by decapitation at intervals 1 to 29 days after administration of the isotope.

Preparation of subcellular fractions. The livers, hearts, brains and kidneys were rapidly removed, rinsed and cooled in ice-cold sucrose Tris-EDTA (STE) buffer (0.3M sucrose, 1×10^{-3} M EDTA, 0.01M Tris-HCl pH 7.4), blotted dry, weighed, minced with scissors and processed as follows at 0-4°C.

Liver. The liver was homogenized in 10 volumes of the STE buffer, first in a Waring blender (top speed, 10 seconds) and then in a motor-driven Potter-Elvehjem homogenizer with a fitted teflon pestle (5 strokes). An aliquot was saved for determination of specific activity of DNA and the remainder centrifuged at 650 x g for 8 minutes in the GSA rotor of a Sorvall RC2-B. Nuclei were isolated from the pellet by the method of Widnell and Tata.²⁶ The supernatant was centrifuged at 10,000 x g for 12 min and the pellet resuspended with one stroke of the Potter-Elvehjem homogenizer with a loose fitting teflon pestle. The mitochondrial suspension was digested with DNase to remove contamination with nuclear DNA. MgCl₂ was added to a final concentration of 7×10^{-3} M and pancreatic DNase I (Worthington) added to a final concentration of 100 μ g/ml. The suspension was incubated at 37°C for 30 minutes. The reaction was stopped by addition of one tenth volume of 0.4M EDTA pH 9.0 and the suspension centrifuged at 10,000 x g for 12 minutes. The pellet was washed twice more in 0.3M sucrose, 0.01M Tris HCl pH 7.4,

* Abbreviation dThd = Thymine deoxyribonucleoside.

0.04M EDTA. The final pellet was divided into two portions for extraction of DNA and phospholipids. In the third experiment, the aliquot of mitochondria for phospholipid analyses was removed before treatment with DNase. Kidney, brain and heart mitochondria were used for phospholipid analysis without prior DNase treatment. The aliquots of mitochondria for phospholipid analysis were centrifuged once again at 800 x g for 10 min. The mitochondrial pellet was then washed twice more with STE buffer in the same way.

Kidney. Subcellular fractions were isolated as described for liver except that the initial homogenization was performed in the MSE homogenizer (intermediate size) and the centrifugations were all performed in the SS-34 Sorvall rotor.

Brain. Mitochondria were prepared by the discontinuous density gradient method of Eichberg, Whittaker and Dawson.²⁷ Mitochondrial preparations for DNA extraction were treated with DNase as described for liver. Nuclei were prepared from the pellet of the first low-speed centrifugation by the method of Widnell and Tata.²⁶

Heart. Hearts were homogenized in 10 volumes of STE buffer. Nagarse enzyme (Enzyme Development Corp., N. Y.) was added to a final concentration of 0.5 mg/ml and the mitochondria isolated by the method of Chance and Hagihara.²⁸ Nuclei were prepared from the pellet of the first low speed centrifugation by a modification of the method of Widnell and Tata.²⁹ Mitochondrial suspensions for DNA extraction were treated with DNase as for liver. Mitochondria for phospholipid analysis from kidney and heart were purified as described for the liver (third experiment).

Samples of homogenates, nuclei, and mitochondria were frozen and stored at -15°C until the samples from all time points had been collected. Several mitochondrial pellets from each organ were examined by electron microscopy to assess the amount of contamination.

Purification and measurement of specific activity of DNA. Two methods were used to measure the specific activity of DNA. Results were qualitatively similar with both methods although Method 2 gave data with less scatter.

Method 1. DNA was extracted by a modification of Schneider's method.³⁰ Samples were thawed and solubilized in cold 1 per cent sodium deoxycholate. After standing for 10 minutes at 0-4°C DNA was precipitated with cold perchloric acid added to a final concentration of 0.5N. The precipitate was vigorously dispersed with a Vortex mixer and collected by centrifugation at 2,000 rpm for 5 minutes in a model SVB International centrifuge. The pellet was extracted 3 more times with cold 0.5N perchloric acid, and then further extracted successively at room temperature with 95 per cent ethanol, ethanol: chloroform (3:1 v/v), and ethanol: ether (3:1 v/v). The pellet was dissolved in 3.0 ml of 0.3N NaOH and incubated for 2 hours at 80°C. After cooling the sample to 0-4°C sufficient cold 4N perchloric acid was added to neutralize the NaOH and bring the concentration of acid to 0.5N. After vigorous shaking the precipitate was extracted twice more with 0.5N perchloric acid. The pellet was resuspended in 1 or 2 ml of 0.5N perchloric acid, incubated at 85°C for 15 minutes, cooled and centrifuged as before. An aliquot of the supernatant was removed to determine DNA concentration by Burton's modification of the diphenylamine reaction³¹ using adenine deoxyribonucleoside as the standard. Duplicate aliquots were neutralized with 1N KOH, and counted in 10 ml of Bray's solution³² in a Packard TriCarb liquid scintillation spectrometer. For these and most radioactivity measurements a minimum of 500 counts above background were accumulated. Some few of the cardiolipin specific activity determinations to be described later were made on less than this number.

Method 2. DNA was purified by a modification of the method of Marmur.³³ Samples of homogenate, nuclei and mitochondria were thawed and solubilized in 1 per cent sodium deoxycholate. An equal volume of cold liquid phenol (Mallinckrodt) saturated with 0.15M NaCl, 0.1M Na EDTA, 0.05M Tris-HCl, pH 8.0 buffer was added. Samples were kept on ice and shaken gently and intermittently for one hour. After centrifugation at 2,000 rpm for 10 minutes the aqueous phase was removed and treatment with phenol was repeated 2 or 3 times until no precipitate was visible at the interphase. The phenol was extracted from the aqueous phase four times by shaking with an equal volume of ether; the ether was removed by aeration. Samples were digested with 200 μ g/ml of beef pancreatic RNase A (Worthington), previously heated to 90°C for 10 minutes, and 25 μ g/ml α -amylase (Worthington) for 30 minutes at 37°C. DNA from homogenate and from nuclear samples was precipitated by the addition of an equal volume of ice-cold isopropanol. After decantation of the supernatant the DNA threads were dissolved in one tenth standard sodium citrate, SSC (0.15M NaCl, 0.015M sodium citrate pH 7.0).

Nuclear DNA was subjected to a "denaturation-renaturation" procedure prior to purification by preparative Cs_2SO_4 ultracentrifugation. Approximately 50-100 μ g of nuclear DNA was dissolved in 2 ml of 0.1 x SSC and heated to 100°C for 10 minutes. The solution was rapidly cooled in ice, and 0.2 volume of 10 x SSC was added. Samples were then incubated at 65°C for 1 hour and allowed to cool slowly at room temperature. Following the addition of 0.1 volume of 2M glycine pH 10,^{*} solid Cs_2SO_4 (Schwarz BioResearch) was added to 1.5 ml aliquots of the nuclear and homogenate DNA. The density was adjusted to 1.4g/cm³ with an Abbe Zeiss refractometer using standard tables relating density to refractive index.³⁵ As many as 12 samples could be centrifuged simultaneously in the fixed angle Spinco rotor number 40³⁶ using 2 ml cellulose tubes with reducing adaptors. Ultracentrifugation was performed at 20°C in a model L2-65 Spinco ultracentrifuge at 33,000 rpm for 60 hours. The bottom of each tube was pierced and the samples collected in 10 drop aliquots. DNA concentration in each aliquot was determined from the optical density at 260 m μ using a Zeiss spectrophotometer. In the case of nuclear DNA the peak tube and tubes having a greater buoyant density were analyzed for radioactivity. In the case of homogenate DNA, all tubes containing DNA were combined and measured. After the addition of 25 μ g of carrier denatured salmon sperm DNA to each tube, DNA was precipitated with cold 5 per cent trichloroacetic acid and collected on millipore filters. The filters were washed with 5 per cent trichloroacetic acid and 80 per cent ethanol, dried, and counted in 10 ml of toluene scintillant (0.5 per cent PPO, 0.05 per cent POPOP in toluene) in the Tricarb liquid scintillation counter.

Mitochondrial DNA was purified by absorption on a 0.9 x 15 cm column of methylated albumin-kieselguhr using only the middle layer described by Mandell and Hershey.³⁷ The column was loaded at 0.3M NaCl, washed with 0.4M NaCl and the DNA was eluted with 0.8M NaCl. The DNA was dialysed for 2 hours against 0.1 x SSC and subjected to the denaturation-renaturation procedure described above except that renaturation at 65°C was continued for only 15 minutes. One tenth volume of 2M glycine buffer pH 10 was added as before. After addition of solid CsCl to a density of 1.7 g/cm³ the samples were centrifuged in the SW39 or SW65 rotor for 60 hours at 33,000 rpm. Samples were collected and the concentration of DNA and measurements of radioactivity were made as described for nuclear DNA. When significant contamination of the mito-

^{*}The buoyant density of denatured DNA increases at this pH³⁴ thus increasing the separation of renatured and denatured DNA in a density gradient.

chondrial DNA with nuclear DNA was present as judged from the A_{260} profile, aliquots of DNA from the lighter end of the gradient were combined and centrifuged in CsCl a second time.

Analytical ultracentrifugation. Purified samples of nuclear and mitochondrial DNA, which had been subjected to the "denaturation-renaturation" procedure, were examined by analytical ultracentrifugation on the Spinco model E ultracentrifuge to determine their purity. The method described by Schildkraut, Marmur and Doty³⁸ was followed using SPO-1 *B. proteus* phage DNA as a density marker ($\rho = 1.742 \text{ g} \cdot \text{cm}^3$). A Joyce-Loebl microdensitometer was used to scan the ultraviolet photographs.

Purification and analysis of phospholipids. Lipids were extracted from mitochondria as described by Dawson et al.³⁹ Extracts were subjected to a single wash with aqueous NaCl according to Folch et al.⁴⁰ Washed extracts were evaporated to dryness in a rotary evaporator at 40°C and the residue was dissolved in 2 ml of lower phase liquid⁴⁰ and transferred through a sintered glass filter to a column of Sephadex G-25 beads in lower phase liquid. The evaporating flask was rinsed out with a further 2-3 ml of lower phase liquid, these rinsings also being transferred to the Sephadex column. Lipids were purified by percolation through the Sephadex G-25 columns in 30 ml lower phase liquid according to Wuthier.⁴¹

In the case of extracts from heart, kidney and brain, these were evaporated to dryness and dissolved in a volume of chloroform/methanol 2:1 such that 500-1000 μg of phospholipid phosphorus was contained in 1 ml. These extracts were separated into their component phospholipids by two dimensional thin layer chromatography on neutral silica gel H (Analtech) employing a technique which will be described in detail elsewhere (Getz & Heywood). Chloroform: methanol: acetic acid: water (260:100:35:15) was the developing solvent in the first dimension. This was followed after washing the plates with diethyl ether by chloroform:methanol: 40 per cent methylamine: water (65:35:5:5) in the second dimension. This procedure permitted the separation of cardiolipin, phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl glycerol, lecithin, lysolecithin, sphingomyelin, lyso-phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositol from one another. After visualization of spots with iodine vapour, appropriate areas of gel were cut out and placed in pasteur pipettes containing washed glass wool. Phospholipids were eluted by percolation of the first developing solvent through the small column of silica gel in the pipette. Measured aliquots of the eluate were taken into glass counting vials, in which the eluting solvent was evaporated to dryness. One tenth ml of water and 10 ml Bray's scintillation fluid³² was added prior to counting in a Packard liquid scintillation spectrometer. Quench corrections were made by a channel's ratio method. An aliquot of the eluate was used for total phosphorus determination according to Bartlett.⁴²

In the case of liver mitochondria, the purified extract was chromatographed on basic silica gel (Camag DO) in the chloroform: methanol: $2\text{N NH}_4 \text{ OH}$ of Skipski et al.⁴³ in one dimension to separate cardiolipin and phosphatidyl ethanolamine from other phospholipids. Alternatively the whole lipid extract was separated into neutral and acidic phospholipid fractions on DEAE cellulose as described by Rouser et al.⁴⁴ The neutral fraction containing lecithin, phosphatidyl ethanolamine and sphingomyelin was separated into its constituents by one dimensional thin layer chromatography on silica gel H, basic (Analtech)⁴⁵ while the acidic lipids were further separated by one dimensional thin layer chromatography as outlined above.⁴³ Elution, counting and phosphorus determinations of the acidic phospholipids were made as for non hepatic mitochondrial phospholipids. In the case of the neutral phospholipids, elution was performed with meth-

anol: formic acid: H₂O (210:30:10). In other respects they were treated as for all other phospholipids.

Cytochrome c oxidase activity. This was determined by the method of Cooperstein and Lazarow⁴⁶ as modified by de Duve et al.⁴⁷

Calculation of results. Only data obtained from 8 days onwards were used in the calculations, except for brain and heart mitochondrial DNA in which case data from all time points were used. Half-lives were calculated from the first order decay constants. These were obtained using a modification of U.C.L.A. Computer Center program BIMED 34 which essentially fits the logarithmically transformed data to the best fit line by the method of least squares.

RESULTS

Incorporation of ³H-Thd into mitochondrial and nuclear DNA. One day after the administration of ³H-Thd, the specific activity of mitochondrial DNA of all tissues was 20-60 times greater than the specific activity of the corresponding nuclear DNA (Table 1). For tissues in a

Table 1
SPECIFIC ACTIVITY OF NUCLEAR AND MITOCHONDRIAL DNA
IN RAT TISSUES

Tissue	Specific activity of DNA		Ratio of specific activities (mitochondrial/nuclear)
	Nuclear	Mitochondrial	
	$\mu\text{Ci}/\mu\text{g}$		
Liver	59.6	1266	21.0
Kidney	19.9	975	49.0
Heart	24.0	908	37.8
Brain	3.3	191	58.0

³H-Thymine deoxyribonucleoside, 220 μCi , was given to each rat by intraperitoneal injection. The specific activity of the DNA was calculated by extrapolation of data obtained in Experiment III to the day of injection. Corrections for quenching were made with external standards.

steady state this suggests a more rapid turnover of mitochondrial DNA assuming that the specific activities of the mitochondrial and nuclear precursor pools are not vastly different. This is substantiated by the data presented below. The specific activity of DNA isolated from tissue homogenates was usually near but slightly higher than that of nuclear DNA. The variability of the results precludes an accurate calculation of the relative amounts of mitochondrial and nuclear DNA from the specific activity of the homogenate, nuclear and mitochondrial DNA. However, mitochondrial DNA appears to account for 2-4 per cent of the total DNA of the rat liver.

The yield of mitochondrial and nuclear DNA after purification was low. Compared to the amounts present in the homogenates the yield of nuclear DNA averaged 25 per cent with a range of 6-72 per cent. The yield of mitochondrial DNA averaged 8 per cent with a range of 2-24 per cent if it is assumed that mitochondrial DNA consistently comprises 4 per cent of the total DNA in each of the tissues studied.

Considering the variation in the yields of DNA, it was necessary to eliminate the possibility

that the isolation procedure selected a particular sort of mitochondria or mitochondrial DNA. To exclude this possibility liver mitochondria were isolated from a rat 24 hours after injection of ^3H -Thd. The mitochondria were separated on an isopycnic sucrose density gradient (1.2-2.0M), Figure 1. The ratio of ^3H incorporation into DNA, to the amount of mitochondrial DNA,

Figure 1. Isopycnic sucrose density gradient fractionation after labeling in vivo with ^3H -Thd. The 42-68% sucrose gradient was centrifuged at 48,000 x g for 2 hours. The tube was sliced into 7 fractions and each was analysed for total DNA ■, acid precipitable radioactivity □, and cytochrome oxidase activity ▨.

to the cytochrome *c* oxidase activity was the same in all parts of the gradient except for a minor variation in fraction 6. It therefore appears that the population of mitochondria was homogeneous with respect to labeling of mitochondrial DNA, at least so far as can be distinguished by equilibrium density gradient centrifugation. This result is consistent with other reports.⁴⁸

Turnover of mitochondrial and nuclear DNA. The turnover of mitochondrial and nuclear DNA was determined in liver, kidney, heart and brain in three separate experiments. In the first two experiments Method 1 was used for DNA purification. This method relies only on DNase digestion of intact mitochondria to eliminate contaminating nuclear DNA. The third experiment used Method 2 which involves methylated albumin-kieselguhr chromatography, a denaturation-renaturation cycle and CsCl density gradient centrifugation for purification of the DNA in addition to DNase treatment of mitochondria. Samples of mitochondrial DNA analysed by Method 1 contained an unknown amount of nuclear DNA. However, DNA extracted and purified by Method 2 contained no contaminating nuclear DNA when analysed by analytical ultracentrifugation after denaturation-renaturation procedure, Figure 2.

The turnover of the mitochondrial DNA in liver was similar in the three experiments, Figure 3. The only difference observed was that there was a little more scatter of the data from Method 1, presumably because of varying degrees of contamination with nuclear DNA. The scatter was greater in the other organs. In the third experiment it was possible to calculate the turnover rates from the ^{32}P label in liver, heart and kidney. The half life of mitochondrial DNA

Figure 2. CsCl equilibrium density gradient analytical ultracentrifugation of nuclear and mitochondrial DNA prepared by Method 2. The calculated buoyant densities are shown on abscissa. The cells have been deliberately overloaded to detect small quantities of contaminating DNA. 0.5 μ g SPO-1 DNA ($\rho = 1.742$) was added to each sample as a buoyant density marker.

Figure 3. Determination of the turnover of liver mitochondrial DNA in three different experiments using ^3H -Thd and ^{32}P as tracers. Experiments I (x—x) and II (•—•) show data obtained using ^3H -Thd incorporation into DNA; in experiment III data from both ^3H -Thd (Δ — Δ) and ^{32}P ($\blacksquare$ — $\blacksquare$) incorporation are shown. A freehand curve has been drawn through each set of data. The specific activity of mitochondrial DNA on the logarithmic scale of the ordinate is expressed in arbitrary units. Actual specific activities obtained at the earliest time points ranged from 88–175 cpm/ μ g for ^3H and was 1.5 cpm/ μ g for ^{32}P .

calculated from ^{32}P was the same as that from ^3H in each of these organs; Figure 3 shows the result for liver.

Figure 4 shows the specific activities of liver nuclear and mitochondrial DNA at each time point. The results from all experiments were normalized in order to combine them into a single presentation. The change in specific activity of mitochondrial DNA can be fairly well described by a single exponential function, although the decrement in its specific activity is somewhat more rapid at the beginning and slower toward the end of the experiment. The half life calculated from the data from day 8 to 29 is 9.4 ± 0.7 days. This agrees well with the figure of 7-10 days reported elsewhere.⁴⁸ Nuclear DNA, by comparison, has a much lower specific activity, and no turnover can be detected during the period of the experiment. The specific activity of the homogenate DNA is not shown in the figure but followed that of the nuclear DNA closely.

Figure 4. Turnover of nuclear and mitochondrial DNA from rat liver. Specific activity of DNA is shown on a logarithmic scale on the ordinate. The data from the three experiments using ^3H -Thd were normalized and combined. Mitochondrial DNA $\pm$ one standard deviation, $\bullet$; nuclear DNA $\pm$ one standard deviation, Δ . Freehand curves have been drawn through the data points.

Results from other organs also indicated a decline in specific activity of mitochondrial DNA which was almost exponential. The half lives over the period from 8 days to 29 days were: heart, 6.7 ± 1.0 days; kidney, 10.4 ± 1.2 days and brain 31.0 ± 16 days. As with liver, the specific activity of the nuclear DNA of liver and brain did not decline significantly during the course of the experiment. In the case of heart (Figure 5) a relatively rapid turnover of nuclear DNA was apparent. This was also reflected in the homogenate DNA. The half life of heart nuclear DNA calculated over the period of 8 to 29 days was 30.5 days from the results of all three experiments. The early more rapid decline in specific activity suggests a heterogeneous population for nuclear DNA.

Figure 5. Turnover of nuclear and mitochondrial DNA from rat heart. Specific activities of DNA are shown on logarithmic scales on the ordinates. The data for all three experiments using ^3H -Thd were normalized and combined. Mitochondrial DNA $\pm$ one standard deviation, $\bullet$; nuclear DNA $\pm$ one standard deviation, $\blacktriangle$. Freehand curves have been drawn through the data.

Comparison of turnover of mitochondrial DNA and phospholipids. The turnover of individual mitochondrial phospholipids of purified mitochondria were compared to the turnover of mitochondrial DNA in all 4 organs.

a) Liver. The data shown in Figure 6 are derived from the third experiment. The cardiolipin specific activity did not reach a maximum until 8 days after isotope injection. The decline in specific activity with time described a complex curve for phosphatidyl ethanolamine and lecithin. For these two phospholipids there appeared to be a component which rapidly declined in specific activity. The influence of this component was most obvious in the specific activity curves one to eight days after precursor isotope injection. This is similar to the pattern described by Bailey et al¹⁹ for the total phospholipids of rat liver mitochondria. In the interest of clarity, only the data obtained in experiment III from 5 days and beyond after precursor injection are shown in Figure 6. Data from 8 days and beyond from all experiments have been used for the calculation of turnover rates (Table 2). The radioactivity decay curves of these three phospholipids and of mitochondrial DNA are similar in rat liver. When the phospholipid data of the second experiment alone were compared by inspection with the DNA turnover, the similarity was even closer than that shown in the third experiment.

b) Kidney. The turnover of kidney mitochondrial DNA and phospholipids derived from the third experiment is seen in Figure 7. Clearly, a single exponential function does not adequately describe the turnover of these components. However, approximation of the turnover rates using the best exponential fit to the data shown, did yield very similar half lives of 10-11 days for all mitochondrial components (see Table 2).

Figure 6. Turnover of phospholipids and DNA in rat liver mitochondria. Specific activity is expressed in arbitrary units on a logarithmic scale on the ordinate. All data were obtained from experiment III. Cardiolipin (x—x), phosphatidyl ethanolamine (▲—▲), lecithin (●—●) and DNA (■—■). A freehand curve has been drawn through each set of points. The specific activity of DNA on day 5 was 175 cpm/ μ g and of phospholipids ranged between 0.2 - 0.77 cpm/ μ g of phosphorus (determinations of 32 P activity were made 17 weeks after isotope injection).

Figure 7. Turnover of phospholipids and DNA in rat kidney mitochondria. Specific activity is expressed in arbitrary units on a logarithmic scale on the ordinate. All data were obtained from experiment III. Cardiolipin (x—x), phosphatidyl ethanolamine (▲—▲), lecithin (●—●), and DNA (■—■). A freehand curve has been drawn through each set of points. The specific activity of DNA on day 5 was 133 cpm/ μ g and of phospholipids ranged between 1.1 - 4.0 cpm/ μ g phosphorus (determinations of 32 P activity were made 13 weeks after isotope injection).

Table 2

HALF-LIFE OF MITOCHONDRIAL CONSTITUENTS FROM VARIOUS RAT TISSUES

Organ and constituent	Half-life	Reference
	Days	
Liver		
Lipids	10.6	Fletcher and Sanadi ¹⁷
Soluble protein	10.8	
Insoluble protein	10.1	
Cytochrome c	9.7	Beattie, Basford, and Koritz ¹⁸
Whole mitochondrial protein	8.4	
Water-soluble proteins	8.6	
Cytochrome c	8.6	
Contractile proteins	8.4	
Structural proteins	8.8	
Other cytochromes	8.2	
H ₂ O-soluble protein	4.2-5.1	Swick, Rexroth, and Stange ²¹
0.9% KCl-soluble protein	4.5-5.0	
0.6 M KCl-soluble protein	4.6-5.3	
13% (NH ₄) ₂ SO ₄ fraction	4.3-5.3	
35% (NH ₄) ₂ SO ₄ fraction	3.9-4.8	
Residue	4.7-8.8	Bailey, Taylor, and Bartley ¹⁹
Insoluble protein	9.0	
Phospholipids	10.0	
Mitochondrial DNA	7-10	Neubert, Oberdisse, and Bass ⁴⁸
Mitochondrial DNA	No turnover	
Mitochondrial DNA	9.4 ± 0.7	Present study
Cardiolipin	11.5 ± 0.8	
Lecithin	10.0 ± 1.3	
Phosphatidyl ethanolamine	9.9 ± 1.0	
Sphingomyelin	11.4 ± 1.2	
Kidney		
Whole mitochondria	8.7	Beattie, Basford, and Koritz ¹⁸
Water-soluble proteins	6.0	
KCl-soluble proteins	7.6	
Structural proteins	9.0	Present study
Other cytochromes	8.4	
Mitochondrial DNA	10.4 ± 1.2	
Cardiolipin	11.4 ± 4.5	
Lecithin	10.0 ± 2.0	
Phosphatidyl ethanolamine	10.0 ± 1.5	
Heart		
Mitochondrial DNA	6.7 ± 1.0	Present study
Lecithin	10.5 ± 1.0	
Phosphatidyl ethanolamine	17.4 ± 3.1	
Brain		
Phosphatidyl ethanolamine	22, 34, 35	Cuzner, Davison, and Gregson ⁶³
Lecithin	11, 19, 23	
Cardiolipin + phosphatidic acid	73.7	
Phosphatidyl serine	27	Beattie, Basford, and Koritz ¹⁸
Phosphatidyl inositol	29, 34	
Whole mitochondria	26.3	
Water-soluble proteins	17.9	
Water-insoluble proteins	31.4	
Mitochondrial DNA	31.0 ± 16.0	
Cardiolipin	424	Present study
Lecithin	48.7	

Half-lives indicated for the present study are the mean values calculated from all experiments. Only data obtained from 8 days onward were used in the calculations, except in the case of brain and heart mitochondrial DNA in which case data from all time points were used.

c) Heart. In heart the turnover rates of individual mitochondrial phospholipids differed greatly and also differed from the turnover rate of mitochondrial DNA (Figure 8). The data are derived from the third experiment. The turnover of mitochondrial DNA was most rapid with a half life of 6.7 days; half life of lecithin was 10.5 days, and phosphatidyl ethanolamine 17.4 days. Exponential decline in specific activity of individual components was approximated in this experiment. The half time for cardiolipin appeared to be even slower, but could not be accurately calculated from the data.

Figure 8. Turnover of phospholipids and DNA in rat heart mitochondria. Specific activity is expressed in arbitrary units on a logarithmic scale on the ordinate. All data were obtained from experiment III. Cardiolipin (x—x), phosphatidyl ethanolamine (▲—▲), lecithin (●—●) and DNA (■—■). A freehand curve has been drawn through each set of points. The specific activity of DNA on day 5 was 66.7 cpm/ μ g and of phospholipids ranged between 1.1 - 6.2 cpm/ μ g phosphorus (determinations of 32 P activity were made 10 weeks after isotope injection).

d) Brain. The data for brain show more scatter, possibly because of the low level of incorporation of isotope into phospholipids and DNA (Figure 9). Although an accurate calculation of turnover rates cannot be made, it seems clear that turnover rates of mitochondrial DNA, cardiolipin and lecithin are all quite slow compared to turnover of these components in other organs.

DISCUSSION

Turnover of mitochondrial and nuclear DNA. In each of the four organs studied, liver, heart, brain and kidney, the specific activity of mitochondrial DNA was initially 20-50 times higher than that of the nuclear DNA. The subsequent decline in specific activity of mitochondrial DNA was almost exponential although in liver and kidney the rate was somewhat faster

Figure 9. Turnover of phospholipids and DNA in rat brain mitochondria. Specific activity is expressed in arbitrary units on a logarithmic scale on the ordinate. All data were obtained from experiment III. Cardiolipin (x—x), lecithin (●—●) and DNA (■—■). The best fit line has been drawn through each set of points. The specific activity of DNA on day 5 was 34.4 cpm/ μ g and of phospholipids ranged between 1.5 - 3.3 cpm/ μ g phosphorus (determinations of 32 P activity were made 8 weeks after isotope injection).

initially and somewhat slower later. In liver and kidney and brain there was no significant turnover of nuclear DNA during the experiment. This is consistent with the more extensive observations of others^{49,50} which show half-lives of 350 days or longer for nuclear DNA from rat liver. Nuclear DNA from heart however appears to turn over at an appreciable rate with some evidence of heterogeneity of cardiac nuclear DNA. The significance of this finding is not clear, but it probably represents mitotic division of connective tissue cells. Grove et al⁵¹ and Mor-kin⁵² have shown that the increased DNA synthesis in rat hearts following experimental cardiac hypertrophy is almost entirely due to replication of nuclear DNA of connective tissue cells.

The turnover of mitochondrial DNA differed greatly in different tissues; the turnover was most rapid in heart where aerobic metabolism and mitochondrial activity is very high, and slowest in brain where mitochondrial activity is slower relative to tissue mass. The turnover of nuclear DNA has usually been considered to reflect the turnover of the cell itself. If this is true for mitochondrial DNA, this component would be a most appropriate marker constituent with which to study the natural history of mitochondria in various organs. A possible alternative explanation for the relatively rapid turnover of mitochondrial DNA is that the turnover represents a repair mechanism rather than synthesis of new mitochondrial DNA. In an accompanying report⁵³ we present evidence that the incorporation of radioactive precursors into both nuclear and mitochondrial DNA of rat liver is not due to the repair of DNA.

The data for relative turnover rates of mitochondrial and nuclear DNA depend upon the efficacy of the separation of mitochondrial and nuclear DNA and the validity of measurement of specific activity. It would appear that valid results may be obtained whether or not the DNA is purified extensively by CsCl density gradient centrifugation. DNase digestion of mitochondria eliminates gross contamination of mitochondrial DNA with nuclear DNA. Incubation with DNase

results in the hydrolysis of most of the nuclear DNA but not of the DNA within the mitochondria.⁵⁴ Conversely the 25-200-fold preponderance of nuclear DNA over mitochondrial DNA, and the effective methods for the purification of nuclei from the tissues studied, ensure that the specific activity measurements on nuclear DNA were not significantly affected by contamination with mitochondrial DNA. The similar turnover rates derived from data in which DNA was or was not purified by CsCl density gradient centrifugation confirm that the simpler method yields valid results.

³H-Thd is an excellent precursor of DNA for these studies.⁵⁵ It is specific for DNA and the Thd turns over rapidly with a half-life of approximately 15 minutes. Injection of isotope therefore resembles pulse labeling and determinations of DNA specific activity for turnover calculation can be made as early as one day after injection of the isotope without complication from the persistence of radioactivity in the precursor pool. As a precursor for DNA, ³²P suffers from the theoretical disadvantage that damage to the DNA molecule from emission of beta particles may be repaired by excision of the regions containing isotope and therefore may result in an apparently more rapid turnover. The ³²P precursor pool also persists for a longer time therefore requiring the first useful specific activity measurements to be made at a later time after injection. Meaningful results can be obtained only if DNA is purified extensively since incorporation of ³²P into contaminating RNA or phospholipids would invalidate the results. Despite these difficulties the turnover of mitochondrial DNA using tritium and ³²P gave very similar values after 8 days.

Turnover of mitochondrial phospholipids. The determination of turnover of mitochondrial and nuclear DNA does not depend entirely upon the purification of the individual organelle, since the two types of DNA can be effectively separated and are localized exclusively in the nucleus and the mitochondrion respectively. Similarly cardiolipin is almost exclusively localized in the mitochondrion, at least in the case of the liver⁵⁶ and brain.²⁷ In these organs therefore purification of this phospholipid, which is efficiently accomplished by Sephadex and thin layer chromatography, will mitigate any possible contamination of mitochondria by other membranes. The extent to which the specific activity measurements of the other phospholipids and perhaps also of cardiolipin in heart and kidney reflect the turnover of mitochondrial phospholipids depends on the purity of the mitochondrial preparations employed. For example, contamination by endoplasmic reticulum could falsely increase the rate of turnover of mitochondrial phospholipids. Electron micrographs of mitochondria used in these experiments showed negligible contamination of mitochondrial preparations by endoplasmic reticulum.

The use of the turnover of ³²P phospholipids as valid indicators of the turnover of an important structural component of mitochondria requires the acceptance of several assumptions. First the precursor pool should not be delivering significant radioactivity to the structural component when the earliest measurement of its specific activity is made. Our calculation of the half-lives of mitochondrial phospholipids are based on specific activities determined at least 8 days after the injection of ³²P, on the assumption that at this time the ATP pool will be free of significant radioactivity. In the liver up to 3-4 days after isotope injection the specific activity of cardiolipin was still rising. It is still possible that recirculation, i.e., catabolic liberation of ³²P labeled components returns significant amounts of radioactivity to the precursor pool. This may vary in different organs and for precursors of different phospholipids and could cause the half-life measured by ³²P to appear longer than it actually is. It is also assumed that turnover

of the phosphorus-containing moiety of the phospholipids is representative of that of the entire molecule. In the case of microsomal phospholipids, there is evidence that different parts of these molecules turn over at different rates.⁵⁷ We have data suggesting that this may also be the case for mitochondrial phospholipids based on a comparison of the apparent turnover of phospholipids labeled with ¹⁴C glycerol and with ³²P. These data will be published in detail later.

The rapid decline of specific activities of lecithin, sphingomyelin and phosphatidyl ethanolamine observed in the first few days after labeling with ³²P may represent an exchange between the phospholipids of the mitochondria and those of some other subcellular membrane. In vitro exchange of this type has been reported between lipids of red cell membranes and plasma lipoprotein⁵⁸ and between plasma lipoprotein phospholipids and those of hepatic mitochondria and endoplasmic reticulum.⁵⁹ This type of in vitro exchange has also been observed between the phospholipids of the mitochondria and the endoplasmic reticulum of rat liver.⁶⁰ It is of interest that these latter authors did not observe any exchange of mitochondrial cardiolipin with any phospholipid of the endoplasmic reticulum. The significance of these in vitro exchange reactions for in vivo phospholipid transfers however is not yet clear. Another possibility is that the components turning over rapidly represent turnover of the outer mitochondrial membrane which may be faster than that of the inner membrane. Another factor must be borne in mind in this connection. In this study, we have measured the turnover of individual mitochondrial phospholipid classes, while most other authors studying membrane turnover have merely measured the decline in specific activity of total phospholipids. Even our measurements however surely estimate the average turnover of many molecular species within each phospholipid class some of which are known to differ metabolically from one another.⁶¹ Moreover we have not distinguished between diacyl phospholipids and plasmalogens in those tissues rich in these latter lipids, i.e., heart, kidney and brain.²⁴

Relevance of data to the concept of mitochondrial turnover as a unit. The data presented in this paper do not unequivocally support or negate the hypothesis that the mitochondrion turns over as a unit (Fletcher & Sanadi¹⁷). The turnover rates of mitochondrial components reported in the literature are summarized in Table 2. All the components of rat liver mitochondria with the exception of those from one report²¹ have half-lives within the range of experimental variation, 8.2 - 11.5 days. With the exception of the soluble proteins, the several components of kidney mitochondria also have similar half-lives of 8.4 - 11.4 days, a range that is probably within experimental error. These data all provide fairly strong support for the concept that the mitochondrion turns over as an entity. Nevertheless, the possibility that the turnover of mitochondrial DNA, proteins and phospholipids in liver and kidney were fortuitously similar cannot be completely discounted.

More recently, studies of mitochondrial proteins labeled with guanidino-¹⁴C arginine, which avoids the problem of re-utilization of precursor isotope, have yielded considerably shorter half-lives, 4 to 6 days.²¹ The discrepancy between these results and ours may be due to the use of a non re-utilized label by these workers. In the same study²¹ half-lives of 0.78 - 1.03 days, and 0.73 - 0.92 days were found for the mitochondrial enzymes ornithine aminotransferase and alanine aminotransferase respectively, following their induction with a high protein diet or injection of prednisolone. In another study,²⁰ following the administration of a porphyrogenic drug, a half-life of 72 minutes was observed for rat liver δ -amino levulinic acid synthetase (ALAS), a

particulate enzyme, which is either loosely associated with the inner mitochondrial membrane or with the mitochondrial matrix.⁶² Therefore, it appears to be necessary to modify the concept of unit turnover of mitochondria to allow the insertion and removal of some mitochondrial enzymes independent of the synthesis and destruction of the entire mitochondrion. Such a process may also account for the observation of a more rapid turnover of the water soluble proteins than of the other components of kidney mitochondria.¹⁸

The turnovers of phospholipids in the heart are substantially slower than the turnover of mitochondrial DNA. Of the components studied in heart mitochondria, cardiolipin had the longest half-life. Cardiolipin turnover of the brain as reported by Cuzner et al⁶³ and, as suggested by our data, was also considerably slower than that of mitochondrial DNA. It is possible that the relatively slow turnover of phospholipid and especially cardiolipin in heart and brain was due to extensive re-utilization in these organs of either whole labeled phospholipid molecules or labeled moieties. The latter possibility could also account for differences observed between the half-lives of liver phospholipids labeled with ³²P and with ¹⁴C glycerol. Such a possibility is consistent with the known reversibility of such enzymes as the phosphoryl choline glyceride transferase involved in lecithin biosynthesis.⁶⁴

Swift and Hruban have demonstrated by electron microscopy mitochondria in various stages of degradation inside lysosomes of rat pancreas, liver and prostate gland.⁶⁵ Administration of toxic agents increased the number of mitochondria visible within lysosomes. Digestion of mitochondria by lysosomes is a mechanism by which mitochondria would be destroyed in a unit. The capacity of lysosomes to digest mitochondria on in vitro incubation has been reported.⁶⁶ Although it is probable that this mechanism at least partly accounts for mitochondrial turnover, it is not clear whether it is the only or indeed the major mechanism responsible. It is conceivable that lysosomes might digest various mitochondrial components at different rates in different organs resulting in selective re-utilization of labeled components and apparently different half-lives. For example heart lysosomes may be deficient in acid phospholipase, which would explain the large discrepancy between mitochondrial DNA and phospholipid turnover in this organ. It is also possible that a whole phospholipid molecule such as cardiolipin is re-utilized as such in the synthesis of new mitochondrial membrane.

In conclusion, it appears that the hypothesis of Fletcher and Sanadi¹⁷ has some degree of validity. It seems probable that a major unit of the inner mitochondrial membrane does turn over as an entity. The outer membrane of mitochondria however may well be synthesized independently and be more analogous to the endoplasmic reticulum in which independent turnover of several membrane components has been demonstrated.⁵⁹ Indeed, we have evidence that the turnover of the proteins of outer mitochondrial membrane and endoplasmic reticulum is more rapid than that of inner mitochondrial membrane proteins.* It is also probable that some components of the inner membrane, especially those loosely associated with it, and matrix, can be inserted and deleted independently of the synthesis or destruction of the major structural unit of the inner mitochondrion.

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SYNTHESIS OF NEW STRANDS OF MITOCHONDRIAL AND NUCLEAR DNA BY SEMI-CONSERVATIVE REPLICATION*

By

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In a previous report¹ we presented evidence that there is turnover of mitochondrial DNA in rat liver and other organs, and that the nuclear DNA by comparison is relatively stable. Several questions are raised by these results: (1) Does the rapid turnover of mitochondrial DNA represent synthesis of new complete strands of DNA or is it due to repair and replacement of segments within existing strands of DNA? (2) If new strand synthesis is responsible for the observed turnover of mitochondrial DNA, what is the mechanism of replication of the DNA? (3) Does the incorporation of radioactive precursors into nuclear DNA represent synthesis or repair of nuclear DNA?

Previously reported experiments in micro-organisms² show that radioactive BUDR[§] can be used as a precursor of DNA to answer these questions. The principle of the method is as follows: when BUDR is substituted for thymidine, semiconservative replication of DNA results in a hybrid DNA with a buoyant density higher than DNA which contains no BUDR. If radioactive BUDR is used in the experiment the DNA may be resolved by CsCl density gradient centrifugation into an ultraviolet absorbing band of pre-existing DNA having a normal buoyant density, and a radioactive band of newly synthesized DNA having a density greater than normal. On the other hand, when DNA repair occurs BUDR is inserted into many separate regions of existing DNA and, consequently, radioactivity and absorbance are superimposed in the CsCl Gradient.

We have applied these techniques to the rat.

METHODS

Radioactive labeling of animals. Two adult female Sprague-Dawley rats weighing 220-250 g were used for each experiment. Preliminary experiments showed that the injection of 5-fluorouracil (FU) before and during the period of labeling with ³H-BUDR increases the incorporation of the latter approximately ten times. The probable mechanism of this action is by inhibition of the biosynthesis of dTMP.³

The rats were first given 15 mg FU (Roche Laboratories) in 0.2 ml isotonic saline by intraperitoneal injection. Two hours later they were given 100 μ C ³H-BUDR (Schwarz BioResearch, 2.0 c/mM), 500 μ g unlabeled BUDR (Calbiochem, grade A), and 10 mg FU in 0.2 ml isotonic saline by intraperitoneal injection. Injections of 0.1 ml of the same mixture were repeated every 15 minutes thereafter. In successive experiments the rats each received a total of 2, 12 and 19 injections of the above mixture.

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[§] BUDR, 5-bromo-2'-deoxyuridine.

Preparation of DNA. The rats were killed 24 hours after the first injection. Isolation of the nuclei and purification of nuclear DNA was performed as described for liver in a previous paper.¹ Mitochondria were prepared and their DNA extracted as described previously¹ up to and including purification by a column of methylated albumin keiselguhr (MAK).

Shearing and denaturation of DNA. Before CsCl density gradient centrifugation some samples of the nuclear DNA were sheared mechanically. The nuclear DNA was adjusted to a concentration of approximately 40 $\mu\text{g/ml}$ in $1/2 \times$ standard sodium citrate (SSC) (SSC 0.15 M NaCl 0.015 M NaCitrate, pH 6.7) and passed through a fine bore pipette from one to five times. Mitochondrial DNA was similarly treated only when it was to be denatured at a later stage. In some cases hydroquinone was used to facilitate subsequent denaturation and strand separation of mitochondrial DNA.⁴ Hydroquinone was added to a final concentration of 0.1 mM prior to denaturation of the DNA. Nuclear DNA was denatured by heating to 100°C for 10 min and cooling rapidly in ice. Mitochondrial DNA was first heated to 100°C for 10 min. The solution was cooled to room temperature, and NaOH was added to a final concentration of 0.1 N. After standing at room temperature for 5 min mitochondrial DNA samples were neutralized by adding glycine buffer pH 10.0 to a final concentration of 0.1 M; the final pH was between 10.2 and 10.4.

CsCl density gradient centrifugation. Solid CsCl was added to 2.5 ml samples containing 30-60 μg nuclear DNA or 5-12 μg mitochondrial DNA. The density was adjusted to 1.710 g/ml in the case of native DNA and 1.735 g/ml in the case of denatured DNA using a Zeiss refractometer. All samples were centrifuged in a Spinco SW39 rotor at 33,000 rpm at 20°C for 60 hr using a Spinco model L2-65 preparative ultracentrifuge. After centrifugation the bottom of the tube was pierced and the gradient was collected in fractions. The absorbance at 260 $m\mu$ and radioactivity of each fraction were measured as described previously.¹

Buoyant density calculation. Three μg SPO-1 DNA ($\rho = 1.742 \text{ g/ml}$) and 100 counts per min of ^{32}P labeled yeast mitochondrial DNA ($\rho = 1.685 \text{ g/ml}$) were added as buoyant density markers to a sample of native nuclear DNA and analyzed by preparative CsCl density gradient centrifugation. From these data the density gradient was calibrated to be 0.00049 g/ml/drop collected assuming that the gradient is approximately linear in the range of the densities measured. The result agreed well with the theoretical calculation of the density gradient⁵ based on initial density of CsCl, centrifugal force and the number of drops per cm of column.

Base analysis of DNA. To confirm that radioactivity was present only in the bromouracil residues, base analysis of the purified DNA was performed.⁶ The hydrolysis products were chromatographed on Whatman No. 1 paper in isopropanol-HCl⁷ with 5-bromouracil (Calbiochem) as an external marker. The chromatogram was cut into transverse strips 1 cm wide. The strips were eluted with dilute HCl and the absorbance at 270 $m\mu$ and radioactivity in Bray's solution⁸ were determined.

RESULTS AND DISCUSSION

Base analysis of 2 separate preparations of nuclear DNA showed that radioactivity was present only in bromouracil residues. Measurement of radioactivity was therefore a good indicator for DNA containing bromouracil. After administration of radioactive and unlabeled BUDR, and FU to rats, mitochondrial and nuclear DNA were isolated, purified and analysed by CsCl centrifugation. Figure 1 shows that for mitochondrial DNA there was partial separation of bands of absorbance at 260 $m\mu$ corresponding to pre-existing DNA, and radioactivity corresponding to newly

Figure 1. Equilibrium CsCl gradient centrifugation of native mitochondrial DNA labeled with ^3H -BUDR. Absorbance at 260 $\text{m}\mu$ (●—●), radioactivity (x---x), $\Delta\rho$ = buoyant density difference.

synthesized hybrid DNA. The separation of the peak of the bands is equivalent to a difference in buoyant density of 0.010 g/ml indicating that approximately 20 per cent of the thymine residues were substituted with bromouracil in one DNA strand.⁹ The results shown in Figure 1 were obtained when the rats received 19 injections of BUDR. In experiments where fewer injections were given the results were similar except that the separation between absorbance and radioactivity bands corresponded to buoyant density differences of 0.007 g/ml (after 12 injections) and 0.004 g/ml (after 2 injections). In all the experiments with native mitochondrial DNA both absorbance and radioactivity peaks were unimodal, suggesting that the radioactivity is only in new strands of NDA and that repair of DNA is absent. Since the degree of separation of DNA bands is a function of the number of injections of BUDR given, the incomplete separation is probably due to the incomplete replacement of thymine residues with bromouracil residues. The width of the radioactivity band was broader than that of the absorbance band (Figure 2). This is probably the result of a changing ratio of thymidine to BUDR in the precursor pool during the labeling period.

With nuclear DNA a greater separation between peaks of radioactivity and absorbance was obtained. Figure 2 shows the CsCl density gradient of native nuclear DNA from rats given 19 injections of BUDR. The radioactivity peak was single but it was not Gaussian. In this experiment there was no overlap between the two peaks. In general the degree of separation of peaks of radioactivity and absorbance was again a function of the number of injections of BUDR. The results are interpreted as suggesting that most if not all the BUDR incorporation was into new DNA. Here the radioactivity peak resembles the absorbance peak in width suggesting that the ratio of bromouracil to thymine is constant in the newly synthesized DNA. It therefore appears that the precursor pools from which mitochondrial and nuclear DNA are synthesized differ in this respect.

Nuclear DNA was denatured and analyzed by CsCl centrifugation (Figure 3). There was complete separation between radioactive and absorbance bands. The data shown in Figure 3 were obtained with the same nuclear DNA used in the experiment shown in Figure 2. After de-

Figure 2. Equilibrium CsCl gradient centrifugation of native nuclear DNA labeled with ^3H -BUDR. Absorbance at 260 mμ (●—●), radioactivity (x --- x), $\Delta \rho$ = buoyant density difference.

naturation the difference in buoyant density between radioactive and absorbance peaks was 0.039 g/ml as compared with 0.016 g/ml with native nuclear DNA. If replication of nuclear DNA is semi-conservative one would expect the buoyant density difference to increase by a factor of exactly 2. In six experiments with nuclear DNA the increase in density was always greater than 2 after denaturation. An explanation for this result may be that denaturation leads to a decrease of

Figure 3. Equilibrium CsCl gradient centrifugation of denatured nuclear DNA labeled with ^3H -BUDR. Absorbance at 260 mμ (●—●), radioactivity (x --- x), $\Delta \rho$ = buoyant density difference.

more than 50 per cent in average molecular weight because of the presence of single strand breaks in native DNA. The broadening of the radioactivity band following heat denaturation is consistent with this view. Smaller molecules would be less likely to contain segments having end-to-end association of newly synthesized and old DNA. Further support for this view is our observation that the separation of radioactivity and absorbance bands increased when native nuclear DNA labeled with ^3H -BUDR was subjected to varying degrees of shear.

The increase in the difference in densities between radioactive and absorbance bands by a factor of more than 2 following denaturation, together with the fact that there was no overlap between the 2 bands, indicates that native DNA was labeled in only one strand. It can be calculated⁵ from the width and position of the radioactivity band that the lower limit of the molecular weight of these strands is approximately 100,000 and that there is 40 per cent replacement of thymine with bromouracil residues. Repair that results in such extensive replacement of only one strand would be extremely unlikely. We conclude that most of the incorporation of radioactive precursors into nuclear DNA of rat liver represents semiconservative replication of new strands of DNA.

Similar results were obtained with mitochondrial DNA after denaturation. The experiments however were complicated by the difficulty in denaturing mitochondrial DNA. Mammalian mitochondrial DNA is circular.^{10,11} The majority of the circular molecules have a highly twisted supercoiled configuration showing that the molecules are covalently closed and have no single strand breaks. Electron microscopy of our samples of mitochondrial DNA showed that even one month after preparation 45 per cent of the circular molecules were highly twisted. Unless breaks are introduced into at least one of the strands the molecule rapidly "snaps back" to its double stranded configuration.¹² Since the two circular strands are covalently continuous it is impossible to separate the strands in a CsCl gradient after denaturation. For this reason the DNA was subjected to shear and treated with hydroquinone to introduce strand breakage before denaturation. When the treatment was extensive, however, it became impossible to band the DNA effectively in the CsCl gradient because of small molecular size. Figure 4 shows the results obtained under optimal conditions. The experiment was performed with the same preparation of mitochondrial DNA as that shown in Figure 1. Two peaks of absorbance and two peaks of radioactivity are seen. The difference in buoyant density between the heavier radioactive and absorbance bands is 0.020 g/ml, or exactly twice that obtained with native mitochondrial DNA. The difference in buoyant density between the lighter absorbance and radioactivity peaks is 0.010 g/ml, or exactly that found for native mitochondrial DNA. We believe the heavier peaks to be DNA which is fully denatured and in which topological separation of the strands has occurred. We interpret the lighter peaks of absorbance and radioactivity to represent DNA in which single strand breaks were not introduced. Therefore the topological restraints to strand separation still exist and renaturation or "snap back" has occurred. The difference in buoyant density between the heavier and lighter absorbance peaks was 0.024 g/ml, higher than the usual difference in density of 0.014 g/ml between native and denatured DNA. In this experiment the pH of the sample was adjusted to pH 10.3 to minimize renaturation. At pH 10 and above the difference in buoyant density between native and denatured DNA increases.¹³

The fact that the buoyant density difference between radioactivity and absorbance increased by a factor of two when the strands are denatured and separated indicates that radioactivity was present in only one strand, i.e. semiconservative replication. It is difficult to conceive of repair

Figure 4. Equilibrium CsCl gradient centrifugation of denatured mitochondrial DNA labeled with ^3H -BUDR. Absorbance at $260\text{ m}\mu$ (●—●), radioactivity (x---x), $\Delta\rho$ = buoyant density difference.

which occurs in only one of the two complementary strands. However, the possibility that there is a minor degree of repair in addition to semiconservative replication cannot be ruled out.

The very great increase in the widths of the bands of mitochondrial DNA after denaturation (Figure 4) suggests that the methods used to introduce single strand breaks have reduced the molecular weight of the DNA very considerably. Alternatively, the two complementary strands could have different buoyant densities as has been found in mouse satellite DNA¹⁴ and in bacterial DNA.⁹

That mitochondrial DNA replicates by a semiconservative mechanism was previously suggested by our electron microscopic demonstration of replicating molecules,¹⁵ and is consistent with the finding by Parsons and Simpson¹⁶ that mitochondria have the ability to incorporate precursors into mitochondrial DNA in vitro. It has also been shown that chloroplast DNA replicates in a semiconservative fashion.¹⁷ The probable absence of repair of mitochondrial DNA under the conditions of the experiment does not, of course, rule out the existence of repair mechanism in mitochondria. It does, however suggest that the turnover of mitochondrial DNA demonstrated previously¹ was not spurious by reason of extensive damage and repair of DNA.

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INTRAMITOCHONDRIAL LOCALIZATION OF δ -AMINOLAEVULINIC ACID SYNTHETASE AND FERROCHELATASE IN RAT LIVER*

By

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The metabolic pathway for heme synthesis in mammalian cells involves integration of mitochondrial and cytoplasmic enzyme activities.¹ The initial reaction, a condensation of glycine and succinyl-CoA to form δ -aminolaevulinic acid[¶] is catalyzed by the mitochondrial enzyme δ -aminolaevulinic acid synthetase (succinyl-CoA : glycine succinyl transferase).² Subsequent reactions that convert ALA to coproporphyrinogen occur in soluble cell fractions, while the final two enzymatic reactions of this pathway, catalyzed by coproporphyrinogen oxidase and ferrochelatase (protohaem ferrolyase, E.C. 4.99.1.1) take place within the mitochondrion.³ Although this compartmentalization has been suggested as a possible control mechanism in heme synthesis,¹ evidence that the mitochondrial enzymes are segregated from the cytoplasm is lacking. Permeability barriers to substrates imposed by the outer and inner mitochondrial membranes may be quite different. An enzyme bound to the external mitochondrial membrane may be freely accessible to substrates generated outside the mitochondrion, whereas a matrix or inner membrane locus may limit access to the substrate. It was therefore of interest to determine the intramitochondrial localization of ALA synthetase and ferrochelatase.

The intramitochondrial locus of ALA synthetase is of interest with respect to the biogenesis of this organelle. Based upon turnover rates for cytochrome c, soluble and insoluble protein, DNA, and phospholipids⁴⁻⁶ it has been suggested that mitochondria, or, more likely, the inner mitochondrial membrane, turns over as an entity with a half-life of 8 to 10 days. ALA synthetase, however, is known to be readily induced⁷ and to turn over with a half-life of 90 min.⁸ Inducibility and rapid turnover have also been reported for two other mitochondrial enzymes, alanine and ornithine aminotransferases.⁹ Exact localization of enzymes such as ALA synthetase would help to evaluate the hypothesis of synchronous turnover of the inner mitochondrial membrane. If ALA synthetase were an enzyme of either the matrix or outer membrane, then such an hypothesis would still be tenable. If, however, ALA synthetase were firmly bound to the inner membrane, the hypothesis would require radical modification, or would have to be discarded.

Development of techniques for isolation of mitochondrial components now permits examination of these questions. Inner and outer mitochondrial membranes have been characterized morphologically and biochemically.¹⁰⁻¹⁵ Marker enzymes for the inner membrane, matrix, and outer membrane have been identified. In this study we have used monoamine oxidase activity as a

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[¶] Abbreviation: ALA, δ -aminolaevulinic acid.

marker for the outer membrane,¹³⁻¹⁵ malic and glutamic dehydrogenase activities for the matrix,¹³ and cytochrome c reductase and cytochrome c oxidase activities to mark the inner membrane.^{10,11}

Although resolution of mitochondria into membrane components potentially constitutes a probe into the relation of structure and function, disruption of mitochondrial structure requires the assumption that structure-dependent changes in enzymatic activity are equal for all fractions. To justify this assumption, we have used two different methods of mitochondrial fractionation: chemical disruption with digitonin¹² and mechanical disruption by swelling, shrinking, and sonication.¹³

MATERIALS AND METHODS

To study ALA synthetase activity, we induced chemical porphyria in adult male Sprague-Dawley rats by tube feeding diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate, 400 mg/day/rat for 2 days.¹⁶ In untreated animals, ALA synthetase activity was negligible. The rats were fasted for 18-24 hr before sacrifice by stunning and exsanguination. Three to six rat livers were combined in 0.3 M sucrose for homogenization. Mitochondria were separated by differential centrifugation.¹⁷ Enzyme assays were completed on the same day as membrane isolation.

Mitochondria were fractionated after digitonin incubation as described by Schnaitman et al,¹² but in most experiments, 1 mg of digitonin was added for each 5 mg of mitochondrial protein. All pellets were suspended in 0.25 M sucrose for enzyme assays.

For disruption by swelling, contraction, and sonication (referred to as sonicated mitochondria in the text), mitochondria were initially suspended in 0.01 M Tris-phosphate, pH 7.5.¹³ After swelling for 5 min at 0°, the suspension, which contained 10-12 mg protein/ml, was diluted with one-third vol of 1.8 M sucrose containing 2 mM ATP and 2 mM Mg⁺⁺. This suspension was kept at 0° for 5 min before sonication (Branson, model S110, 3 amps for 10 sec). Six to 9 ml of sonicated mitochondria were layered on top of a discontinuous sucrose gradient; the bottom layer was 10 ml of 1.32 M sucrose; the middle layer was 5 ml of 0.76 M sucrose. Centrifugation for 3 hr at 90,000 g (Spinco SW 25.1) resolved 3 fractions: a brown pellet (heavy fraction); a band at the lower interface (light fraction); and a clear, yellow supernatant fluid (soluble fraction). Less concentrated material on either side of the interface band was collected as the intermediate fraction. The pellet (heavy fraction) was rinsed with 0.25 M sucrose and resuspended in 0.25 M sucrose for enzyme assays. The light and soluble fractions were removed from the gradient with J-shaped Pasteur pipettes and were assayed as collected.

Ferrochelatase activity was measured spectrophotometrically.¹⁸ Each assay mixture was incubated for 90 min at 35° with constant agitation. Before use, protoporphyrin IX was purified by precipitation from acidic and basic solutions.¹⁹ Triplicate samples were incubated under a stream of nitrogen. Blank samples were treated identically, but were incubated aerobically. After incubation, particulate material was removed by centrifugation at 12,000 g for 30 min at 4°. Difference spectra between reduced and oxidized pyridine hemochromogens were obtained with a recording spectrophotometer (Cary model 14).

To measure ALA synthetase activity, mitochondrial fractions were incubated for 60 min at 35° with constant agitation. Each milliliter of assay medium contained 100 μmoles of glycine, 10 μmoles of succinate, 5 μmoles of α-ketoglutarate, 1 μmole of pyridoxal phosphate, 0.5 μmole of ATP, 0.5 μmole of NAD⁺, 10 μmoles of EDTA, 20 μmoles of Mg⁺⁺, and 100 μmoles of Tris-

chloride adjusted to a final pH of 7.4. Three hundred micromoles of trichloroacetic acid were added to terminate the reaction. After centrifugation, ALA concentration was measured in the protein free supernatant solution.²⁰ For blank samples, trichloroacetic acid was added before incubation. Preliminary experiments showed that addition of succinyl-CoA increased ALA synthetase activity up to 20 per cent in internal membrane, external membrane, and soluble fractions. Hence, a total of 4.4 μ moles of freshly prepared succinyl-CoA²¹ was added to all assay tubes in equal aliquots at 15-min intervals. Succinyl-CoA concentration was measured by the hydroxamate method.²²

Succinate-cytochrome c reductase activity was measured at 37°. ²³ Albumin was omitted from the assay medium. Cytochrome c oxidase was assayed spectrophotometrically after 4-5 min preincubation at room temperature.^{24,25} Malic dehydrogenase activity was measured according to Ochoa's method.²⁶ Ninety micromoles of sodium amytal were added to each cuvette. Glutamic dehydrogenase was assayed according to Sottocasa et al.¹³ After preincubating for 3 min at 37°, monoamine oxidase activity was determined using benzylamine as substrate.²⁷

Digitonin, pyridoxal phosphate, and protoporphyrin IX were from Calbiochem. ATP, NADH, and coenzyme A were P-L Biochemical products. Benzylamine, cytochrome c, NAD⁺, α -keto-glutarate, and oxaloacetic acid were obtained from Sigma. Protein concentration was measured according to Lowry, Rosebrough, Farr & Randall,²⁸ or by the biuret reaction.²⁹

RESULTS

When observed by electron microscopy, isolated mitochondria appeared intact, free from nuclear material, and almost free from microsomal contamination. Treatment with digitonin, followed by differential centrifugation released four sub-mitochondrial fractions, as shown in Table 1. The pellet that sedimented at 9500 g (9.5-P) corresponded to the inner membranes

Table 1
COMPARISON OF MITOCHONDRIAL FRACTION PROCEDURES

Mitochondrial fraction	Digitonin [*]	Sonication [†]	Marker enzyme
Internal membrane	40-P (36-39)	Heavy (60-71)	Cytochrome c oxidase; succinate cytochrome c reductase
	9.5-P (32-35)		
Matrix	144-S (25-32)	Soluble (18-20)	Glutamic dehydrogenase Malic dehydrogenase
External membrane	144-P (3-5)	Light (2-8)	Monoamine oxidase

^{*} 9.5-P, pellet obtained after 10 min centrifugation 9500 g; 40-P, 15 min at 40,000 g; 144-P, 60 min at 144,000 g; 144-S, supernatant after final centrifugation.

[†] See text.

Numbers in parentheses indicate protein distribution calculated as ([mg/fraction]/[total mg recovered]) x 100.

plus some matrix; that at 40,000 g (40-P) to internal membranes without matrix; and the pellet recovered at 144,000 g (144-P) to external membranes. The 144,000 g supernatant (144-S) fraction contained solubilized and matrix enzymes. Density gradient centrifugation of sonicated mitochondria yielded three fractions, corresponding to internal membranes, external membranes, and matrix (Table 1).

Preliminary experiments with a digitonin:protein ratio of 1.3 mg/10 mg protein reproduced the distribution of monoamine oxidase activity and protein reported by Schnaitman et al.¹² Subsequently, more complete separation of the external membrane from the 9.5-P and the 40-P fractions was sought. At an increased digitonin:protein ratio (Table 2) the monoamine oxidase activity associated with inner membrane fractions was reduced from 50 to 14 per cent. Concomitantly, more monoamine oxidase activity and protein were released into the 144-S fraction. The appearance of a large proportion of monoamine oxidase activity in the 144-S fraction is ascribed to the higher digitonin:protein ratio employed.

Table 2

EFFECT OF DIGITONIN:PROTEIN RATIO ON THE DISTRIBUTION OF
MONOAMINE OXIDASE AND PROTEIN

Fraction	Digitonin:protein = 0.13		Digitonin:protein = 0.20	
	MAO* %	Protein %	MAO* %	Protein %
9.5-P	33	21	1	32
40-P	7	48	13	39
144-P	50	10	35	3
144-S	10	10	51	25

* MAO, monoamine oxidase activity.

The biochemical characterization of typical digitonin and sonication fractionations performed on mitochondria from normal rats is summarized in Tables 3 and 4 respectively. Cytochrome c oxidase activity, used as a marker for the internal membrane, was found with the 9.5-P, 40-P, and heavy fractions, while monoamine oxidase activity, used to trace the behavior of outer membranes, was limited to the 144-P, 144-S, and light fractions. In both fractionation procedures, ferrochelatase activity very closely paralleled that of cytochrome oxidase. Ferrochelatase is therefore an inner membrane enzyme.

With the digitonin fraction method, theoretical distribution for a matrix enzyme would be with the 9.5-P fraction or the 144-S fraction. Experimentally, malic dehydrogenase partitioned almost equally among 9.5-P, 40-P, and 144-S, and in no single fraction was there a marked enhancement of the specific enzyme activity. In the sonication procedure, malic dehydrogenase should have been found primarily in the soluble fraction, corresponding to its matrix locus. This activity, however, was found to be distributed between the heavy and the soluble fractions.

Representative data on ALA synthetase and marker enzyme distribution for fractionations of mitochondria from porphyric rats are shown in Tables 5 and 6. The distribution of marker enzymes was similar to that found in mitochondria from normal animals. ALA synthetase ac-

Table 3

ENZYME DISTRIBUTIONS IN MITOCHONDRIA FROM NORMAL RATS FRACTIONATION WITH DIGITONIN

Fraction	Ferrochelataase		Cytochrome c oxidase		Malic dehydrogenase		Monoamine oxidase	
	Sp.act. *	Yield†	Sp.act. *	Yield†	Sp.act. *	Yield†	Sp.act. *	Yield†
Intact mitochondria	60	-	303	-	1.3	-	11	-
9.5-P	54	37	442	46	1.1	29	7	15
40-P	76	55	402	45	1.3	35	4	9
144-P	62	8	341	5	1.0	3	56	16
144-S	0	0	38	4	1.3	32	29	60
Recovered activity of intact mitochondria‡	83		107		105		144	

* Sp.act. = specific enzyme activity: ferrochelataase, $m\mu$ moles heme formed hr^{-1} mg protein $^{-1}$; cytochrome c oxidase, μ moles cytochrome c oxidized min^{-1} mg protein $^{-1}$; malic dehydrogenase, μ moles NADH $^{+}$ reduced min^{-1} mg protein $^{-1}$; monoamine oxidase, $m\mu$ moles benzylamine oxidized min^{-1} mg protein $^{-1}$.

† Yield = (activity/fraction)/(activity in all fractions) x 100.

‡ Recovered activity of intact mitochondria = (activity in all fractions)/(activity in intact mitochondria) x 100.

Table 4

ENZYME DISTRIBUTIONS IN MITOCHONDRIA FROM NORMAL RATS, FRACTIONATION BY SONICATION,
SWELLING, AND DENSITY GRADIENT CENTRIFUGATION

Fraction	Ferrochelatase		Cytochrome c oxidase		Malic dehydrogenase		Monoamine oxidase	
	Sp.act.*	Yield†	Sp.act.*	Yield†	Sp.act.*	Yield†	Sp.act.*	Yield†
Intact mitochondria	94	-	106	-	2.7	-	16	-
Heavy fraction	306	94	197	94	2.9	69	5	26
Light fraction	78	3	72	4	0.3	1	83	53
Soluble fraction	32	3	10	2	4.9	30	12	21
Recovered activity of intact mitochondria‡	215		123		118		75	

* Sp.act. = specific activity; units as in Table 3.

† Yield = as in Table 3.

‡ Recovered activity of intact mitochondria = as in Table 3.

Table 5
 ENZYME DISTRIBUTIONS IN MITOCHONDRIA FROM PORPHYRIC RATS
 FRACTIONATION WITH DIGITONIN

Fraction	ALA-synthetase		Cytochrome c oxidase		Malic dehydrogenase		Monoamine oxidase	
	Sp.act.*	Yield†	Sp.act.*	Yield†	Sp.act.*	Yield†	Sp.act.*	Yield†
Control mitochondria	0.9	-	252	-	1.6	-	6	-
9.5-P	0.9	29	565	44	1.9	37	0.2	1
40-P	1.0	40	562	53	1.3	33	2	13
144-P	0.6	2	331	3	1.2	3	66	35
144-S	1.1	29	8	0	1.6	27	12	51
Recovered activity of intact mitochondria‡	98		157		94		101	

* Sp.act. = specific activity; units as in Table 3.

† Yield = as in Table 3.

‡ Recovered activity of intact mitochondria = as in Table 3.

tivity was recovered in the 9-5-P, 40-P, and 144-S fractions after digitonin fractionation (Table 5). The heavy and soluble fractions of the sonication fractionation experiments contained almost all of the ALA synthetase activity (Table 6). In both types of fractionation procedure the distribution of ALA synthetase activity was similar to that found for malic dehydrogenase.

Further release of ALA synthetase activity from inner membranes was explored by resonication of heavy fractions. After graded resonication, inner membrane suspensions were centrifuged at 144,000 g for 1 hr, and the pellets and supernatant fractions were then assayed for ALA synthetase, glutamic dehydrogenase, and cytochrome c oxidase activities. As shown in Table 7, with increasing length of sonication, ALA synthetase activity was released from the inner membrane fraction. The pattern of response is similar to that found with glutamic dehydrogenase, which has been designated a matrix enzyme.¹³ In contrast, only trace amounts of cytochrome c oxidase activity were released from the inner membrane even by prolonged sonication. Variable inactivation of malic dehydrogenase activity precluded its use in similar experiments.

The distribution of enzyme activities has been calculated as the percentage of the recovered enzymatic activity, using a sample of unfractionated mitochondria which had been either incubated with digitonin, or swollen, contracted and sonicated as a reference. Recovery data, shown in the bottom row of Tables 3-6, indicate that it was not always possible to recover the expected activity. As has been suggested^{12,13} this change probably reflects structural changes resulting from the fractionation techniques. Recovery of total protein in these experiments ranged from 95 to 106 per cent.

Membrane fractions isolated either by digitonin or by sonication were examined by electron microscopy. In both the 40-P and heavy fractions, inner membranes were found minimally contaminated by external membranes. The 144-P and light fractions, however, showed significant contamination by small fragments of the inner membrane.

DISCUSSION

Our data indicate that ferrochelatase activity is associated with the internal membrane of rat liver mitochondria. Both fractionation methods yield high recoveries and enhanced specific enzyme activities with internal membrane fractions. A small amount of ferrochelatase, albeit of high specific activity, is recovered with external membranes. This finding parallels our data on cytochrome c oxidase, or succinate cytochrome c reductase, and correlates with morphologic evidence of contamination of external membranes by fragments of the inner membrane. Neither ALA synthetase nor ferrochelatase, however, appear to be external membrane enzymes, and therefore this contamination does not affect our conclusions.

Segregation of ALA synthetase activity in a single mitochondrial fraction is less obvious. With both fractionation methods, the distribution of malic dehydrogenase and ALA synthetase is parallel. Neither ALA synthetase nor malic dehydrogenase, however, partitions uniquely to one component of the mitochondrion as does, for example, cytochrome c oxidase. In contrast to a previous report,¹² we find only 20 to 30 per cent of malic dehydrogenase activity in the soluble (144-S) fraction, while more activity (30 to 60 per cent) remains associated with inner membrane fractions. Significant enhancement of specific activity of malic dehydrogenase is not observed among these fractions.

The release of matrix marker enzymes and ALA synthetase from inner membrane fractions was studied further, as a function of progressive disruption of the heavy fraction. With increas-

Table 6

ENZYME DISTRIBUTIONS IN MITOCHONDRIA FROM PORPHYRIC RATS, FRACTIONATION BY SONICATION,
SWELLING, AND DENSITY GRADIENT CENTRIFUGATION

Fraction	ALA-synthetase		Succinate-cytochrome c-reductase		Malic dehydrogenase		Monoamine oxidase	
	Sp. act.*	Yield†	Sp.act.*	Yield†	Sp.act.*	Yield†	Sp.act.*	Yield†
Intact mitochondria	2.2	-	131	-	3.3	-	21	-
Heavy fraction	2.5	75	100	94	3.1	68	9	24
Light fraction	1.8	1	89	3	0.6	0	173	17
Soluble fraction	2.8	24	0	0	4.6	29	14	13
Intermediate fractions	-	-	26	3	1.2	3	115	46
Recovered activity of intact mitochondria‡	118		63		103		113	

* Sp.act. = specific activity: succinate cytochrome c reductase, μ moles cytochrome c reduced min^{-1} mgm protein^{-1} .
Other units as in Table 3.

† Yield = as in Table 3.

‡ Recovered activity of intact mitochondria = as in Table 3.

Table 7

RESONICATION OF INNER MEMBRANES: EFFECT ON SOLUBILIZATION OF ENZYMES

Length of resonication† (sec.)	ALA-synthetase		Glutamic dehydrogenase		Cytochrome c oxidase		Protein
	Supernatant* (%)	Sp.act.†	Supernatant* (%)	Sp.act.†	Supernatant* (%)	Sp.act.†	Supernatant* (%)
0 [§]	33	3.54	40	0.42	0.1	1	40.0
15	58	5.10	52	0.51	2.2	18	58.5
30	85	5.53	53	0.55	3.3	28	58.9
60	73	4.53	62	0.52	6.2	47	66.1
120	76	5.34	65	0.66	4.2	41	64.2

* Supernatant (%) = ([activity in supernatant]/[activity in total sonicate]) x 100.

† Sp.act. = specific activity. For glutamic dehydrogenase, $\mu\text{moles NADH}^+$ oxidized $\text{min}^{-1} \text{mg protein}^{-1}$. Other units as in Table 3.

‡ Sonication was performed on inner membranes prepared as described (Sottocasa et al 1967).¹³ For resonication, 15-sec bursts of energy were employed until the total required period of sonication was achieved. To insure against heating at least 2-min intervals of cooling were employed between bursts.

§ The results reported with zero resonication refer to the distribution of the original heavy fraction prepared as described in Methods and recentrifuged at 144,000 g for 1 hr.

ing duration of sonication of this latter fraction, the amount of ALA synthetase and glutamic dehydrogenase released into the supernatant increases in a similar manner; specific activities of the released enzymes also rise (Table 7). Negligible amounts of cytochrome c oxidase activity are released under the same conditions. Malic dehydrogenase partitions similarly, but with increasing sonication, recovery decreases, presumably from enzyme inactivation. Both the partition of ALA synthetase among isolated sub-mitochondrial fractions, and the release of this activity from heavy fractions are entirely parallel to the behavior of two enzymes generally characterized as biochemical markers of the mitochondrial matrix.

A matrix enzyme is operationally defined as an enzyme that is readily solubilized by osmotic lysis,¹² detergent treatment,¹² or sonication.¹³ Upon this basis malic dehydrogenase and glutamic dehydrogenase have been designated as matrix markers. In none of these solubilization procedures, however, have matrix enzymes been quantitatively released from sedimentable membrane fractions. Based upon these criteria, the behavior of ALA synthetase is entirely consistent with its being a matrix enzyme. Hackenbrock³⁰ has recently described the mitochondrial matrix as a reticular network, physically linked to the inner membrane. These observations provide an ultrastructural counterpart for the inability of ourselves and others to achieve quantitative solubilization of matrix enzymes by the procedures described above. Hackenbrock's observations also offer a basis for considering the localization of one isoenzyme of malic dehydrogenase within the mitochondria. Such a localization could be explained on the basis of the binding of this isoenzyme to the reticular matrix network. Munkres & Woodward³¹ have reported that a mutation involving structural protein of *Neurospora* alters the kinetic properties of malic dehydrogenase.

These data may be pertinent to problems of mitochondrial biogenesis and turnover. The known rapid turnover of ALA synthetase,⁸ together with its locus in the matrix, implies that synchronous turnover of a structural complex may be limited to the inner membrane unit. In yeast, ALA synthetase appears to be made outside the mitochondrion (Druyan, et al, unpublished data). It is possible that matrix enzymes are synthesized in the endoplasmic reticulum, and are then independently inserted into and deleted from the intact mitochondrion.

Heme synthesis in mammalian tissue is regulated via derepression of ALA synthetase synthesis.²⁰ The very short half-life of ALA synthetase may render it possible to achieve fine regulation via this mechanism alone.⁸ Product inhibition effected by heme is a second possible regulatory mechanism, and has been demonstrated in *Rhodopseudomonas spheroides*, where ALA synthetase occurs in soluble form.³² Kinetic experiments with tissue or mitochondria have failed to demonstrate feedback inhibition of ALA synthetase.^{20,33,34} In these studies, however, heme was added to intact cells or to intact mitochondria, and its actual concentration in the matrix is unknown. The proximity of the initial (ALA synthetase) and terminal (ferrochelatase) steps of this synthetic pathway makes it attractive to speculate that feedback inhibition may yet be demonstrated.

The intracellular compartmentalization of heme synthesis has itself been cited as a possible regulatory mechanism.¹ Of the seven enzymes requisite to heme synthesis, four are in the cytosol, and three are in the mitochondrion. Our data localize the first and final steps to the matrix and inner membrane, respectively, and provide structural evidence that may implicate the transport of intermediates across mitochondrial membranes as a factor in regulating heme synthesis.

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