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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1972

ALEXANDER GOTTSCHALK, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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Printed in the U.S.A.
Available from the National Technical
Information Service, U.S. Department of Commerce,
5285 Port Royal Road
Springfield, Virginia 22151

Price: Printed copy \$3.00; Microfiche \$0.95

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CLINICAL FEASIBILITY OF MYOCARDIAL IMAGING WITH $^{13}\text{NH}_3$ *

By

P. V. Harper, K. A. Lathrop, H. Krizek, N. Lembares,
V. Stark, and P. B. Hoffer

Hunter and Monahan's report¹ on myocardial localization of the 10-minute positron emitter nitrogen-13 as ammonia led us to investigate the clinical applicability of this phenomenon. The radionuclide was produced by the method of Tilbury et al² in which methane is rapidly circulated through a chamber where it is bombarded with 8 MeV deuterons. Under these conditions, ^{13}N resulting from the d,n reaction on ^{12}C in the methane appears principally as ammonia. A small percentage of the activity appears as HCN and this is largely removed by passing the gas from the bombardment chamber through a small soda lime column. The $^{13}\text{NH}_3$ is then almost completely removed from the methane carrier gas by bubbling it through 10 to 15 ml of sterile, pyrogen-free saline in which the $^{13}\text{NH}_3$ dissolves. This solution is then ready for intravenous injection after sterilization by membrane filtration. By this method, 20 to 30 mCi of radioactivity have been produced with a 5 μA beam in 20 minutes, and processed for intravenous injection in less than 5 minutes.

Animal distribution studies in mice indicated that the liver is the primary organ of localization, containing approximately 15 per cent of the intravenously injected radionuclide at two and ten minutes after intravenous injection. The human total-body absorbed radiation dose was computed (MIRD dosimetry) as 5 mrad/mCi assuming uniform distribution, and the liver dose was 25 mrad/mCi assuming 15 per cent of the dose in the liver. An image of the trunk of a normal human subject made after intravenous injection of $^{13}\text{NH}_3$ (Figure 1) showed no substantial localization of activity except in the liver, myocardium and bladder. Four and seven-tenths per cent of the injected dose was excreted at 35 minutes. The kidneys were briefly and faintly visualized. The blood disappearance curve was very rapid, 85 per cent of the radioactivity leaving the blood in the first minute, both in the mouse and in the human subject. Thus, the NH_4^+ ion behaves in a markedly different manner from the potassium ion and its analogues.

Detection and imaging of the positron annihilation radiation from the myocardium was accomplished with a Nuclear Chicago HP Anger Camera, recording the output on videotape for replay to image different phases of uptake and to obtain uptake curves over different regions. A newly available 550 KeV collimator (451 round parallel holes 1/4" x 3" on 7/16" centers) was used. This imaged well, but because of the septal thickness resulted in an overlying collimator pattern. It is possible to eliminate this pattern by moving the collimator during imaging.

Two volunteer subjects were studied, each receiving 10 mCi of nitrogen-13 ammonia intravenously in 15 ml of sterile saline. The myocardial images obtained are shown in Figures 2 and 3. The subject in Figure 2 was apparently normal and healthy. The left myocardium is well visualized and closely resembles the realistic heart phantom³ made from a plastic mold of a fixed specimen. The right myocardium is not visible. The subject in Figure 3 was also appar-

* This report is taken from a paper that appeared in J. Nuclear Med., 13:278, 1972.

Figure 1. Image of normal human trunk showing localization of ^{13}N from NH_3 in liver, myocardium, and bladder at 30 minutes using nuclear Chicago HP camera with diverging collimator 4-1/2 feet from subject.

ently healthy, but on questioning admitted to an episode of severe crushing chest pain lasting several days which occurred 8 years previously. The myocardial scan shows a defect in the inferior portion of the left myocardium which may well represent an old infarct.

Upon replaying of the video tape, activities over equal areas of myocardium, lung, mediastinum and liver were recorded in the normal subject and are presented as counts per minute per square centimeter per millicurie injected as corrected for physical decay (Figure 4). After the initial bolus leaves the heart chambers (about 30 seconds), the myocardium is immediately visible, indicating that the myocardial uptake is largely a first-pass phenomenon. The myocardial radioactivity falls slightly during the next few minutes and then stays virtually constant up to 30 minutes. Activity over the mediastinum is cleared rapidly, and over the lung more slowly, both reaching a level of about one-half that of the myocardium. The liver activity during the 6 to 8 minutes after injection builds up to about the same level over the left lobe as over the myocardium.

The most satisfactory images were obtained by recording between one minute and 30 minutes, in which time 1.5 to 2 million counts were obtained in the whole image ($\sim 3500 \text{ counts/cm}^2$ over myocardium). Figures 2 and 3 are recorded in this way. At this counting level, it would appear feasible to gate the camera with the electrocardiogram in order to reduce the blurring due

Figure 2. Myocardial image, anterior view, of normal subject using ^{13}N from NH_3 imaged with Nuclear Chicago HP camera with 550 KeV collimator. Ten mCi were administered intravenously and the image was recorded between one and 30 minutes.

Figure 3. Anterior myocardial image of human subject with history suggesting old myocardial infarction showing inferior myocardial defect.

Figure 4. Uptake curves over myocardium and adjacent regions following intravenous administration of $^{13}\text{NH}_3$.

to cardiac motion. Under these circumstances it might be desirable to increase the dosage of the radiopharmaceutical preparation several-fold, which could be done without excessive radiation absorbed dose.

The clinical indications for myocardial imaging must obviously evolve from more extensive experience. Some possibilities that immediately suggest themselves are: indeterminate ECG findings; screening candidates for coronary angiography; determination of extent of acute infarct with a view to aggressive therapy before cardiogenic shock appears. Studies of acutely ill patients in the nuclear medicine environment might not be feasible. The use of a portable imaging device under these circumstances has been suggested³ and might extend substantially the use of myocardial imaging.

SUMMARY AND CONCLUSIONS

The use of $^{13}\text{NH}_3$ as a scanning agent makes possible clinical myocardial images with high count density, good contrast, and low absorbed radiation dose using commercially available imaging equipment. The imaging equipment and technique are certainly capable of further refinement, and the available count densities should permit significant image processing, so that the possibility that this approach will provide a powerful diagnostic tool appears very real.

LITERATURE CITED

1. Hunter, W. Jr., and W. G. Monahan. J. Nucl. Med., 12:368, 1971.

2. Tilbury, R. S., J. R. Dahl, and W. W. Hunter. Submitted for publication as companion paper to this manuscript.
3. Harper, P. V., M. Kawana, and C. Athanassopoulos, et al. J. Nucl. Med., 12:436, 1971.

TRUNCATION ERRORS IN CALCULATING THE MTF OF RADIOGRAPHIC SCREEN-FILM SYSTEMS FROM THE LINE SPREAD FUNCTION*

By

K. Doi,[†] K. Strubler,[‡] and K. Rossmann

The modulation transfer function (MTF) has become a frequently used tool in assessing the optical characteristics of medical radiographic screen-film systems. It is, therefore, important to measure it as accurately as possible. The MTF of screen-film systems is often calculated from the line spread function (LSF) which is measured from slit exposures.¹⁻³ Although the LSF may have an infinite spatial extent, it is usually measured to approximately 0.01 of its maximum value for experimental reasons. This in effect truncates the LSF and introduces oscillating perturbations in the MTF, thereby limiting its accuracy.⁴

The present study was designed to investigate what effect truncating the LSF has on the computed MTF. The results are derived in three ways. First, a theoretical analysis to estimate the error due to truncation is described; second, experimental results in typical screen-film systems are shown; and finally, a correction procedure involving extrapolation methods is developed.

THEORETICAL ESTIMATION OF TRUNCATION ERRORS

Let $r(x)$ be the LSF of the screen-film system. This is the relative illuminance distribution in the image of a narrow slit with an x-ray input of unit intensity.[§] For simplicity, two assumptions are made: (1) the LSF is symmetric, that is, $r(x) = r(-x)$, and (2) the LSF is non-negative, that is, $r(x) \geq 0$. These conditions are usually satisfied in screen-film systems but are not necessarily satisfied in some other imaging elements in radiography. For example, condition (1) is not satisfied in the case of the focal spots of x-ray tubes.⁵

In practice, truncation of the spread function occurs at some distance d from the LSF center. This corresponds to a truncation level relative to the maximum of the LSF, which shall be called simply the truncation t . The "truncated" LSF, $r_r(x)$, and the "tail" LSF, $r_E(x)$, are then defined by

$$\begin{aligned} r_r(x) &= r(x) & |x| \leq d \\ &= 0 & |x| > d \end{aligned} \tag{1}$$

* This report is taken from a paper that appeared in *Phys. in Biol. and Medicine*, 17:241, 1972. The work was supported in part by a grant from the U. S. Public Health Services.

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[§] This definition of the LSF includes both energy conversion and optical diffusion properties of the system. The MTF is then defined by the Fourier transformation of the LSF divided by its area. In the literature, however, the LSF is sometimes defined differently, i.e., only in terms of the optical diffusion property of the system. In this case the area under the LSF is normalized to unity, and the MTF is given directly by the Fourier transformation of the LSF.

and

$$\begin{aligned} r_E(x) &= r(x) & |x| > d \\ &= 0 & |x| \leq d \end{aligned} \quad (2)$$

respectively, where $r(d) = t \cdot r(0)$ is defined. The truncated LSF is obtained experimentally, but the tail LSF, which is large in spatial extent and small in magnitude, is not ordinarily observed. From eqs. (1) and (2), the "true" LSF is described by

$$r(x) = r_r(x) + r_E(x). \quad (3)$$

Fourier transformation of both sides of eq. (3) results in

$$R(u) = R_r(u) + R_E(u), \quad (4)$$

where the Fourier transformation is defined by

$$R(u) = \int_{-\infty}^{\infty} r(x) \exp(-2\pi i u x) dx. \quad (5)$$

The MTF of the screen-film system is then given by $R(u)/R(0)$.

The deviation of the MTF, ΔR , which is the difference between the "truncated" and the "true" MTF, is the truncation error and is given by

$$\Delta R = \frac{R_r(u)}{R_r(0)} - \frac{R(u)}{R(0)} \quad (6)$$

Note that division by $R_r(0)$ and $R(0)$ is necessary for normalization of the respective MTF's.

Substituting eq. (4) for $T_r(u)$ and rearranging eq. (6), the truncation error becomes

$$\Delta R = \frac{R_E(0)}{R_r(0)} \left[\frac{R(u)}{R(0)} - \frac{R_E(u)}{R_E(0)} \right]. \quad (7)$$

Since $|R(u)/R(0)| \leq 1.0$ and $|R_E(u)/R_E(0)| \leq 1.0$ are satisfied by the assumption of a non-negative LSF, the quantity within brackets in eq. (7) is bounded by

$$2 > \left| \frac{R(u)}{R(0)} - \frac{R_E(u)}{R_E(0)} \right|. \quad (8)$$

An upper bound of the truncation error ΔR_{UB} can therefore be defined from eq. (7) by

$$\begin{aligned} \Delta R_{UB} &= \frac{2R_E(0)}{R_r(0)} \\ &= 2 \int_d^{\infty} r(x) dx / \int_0^d r(x) dx, \end{aligned} \quad (9)$$

where $\Delta R_{UB} > \Delta R$.

Eq. (9) shows that the truncation error is related to the area of the tail portion of the LSF, i.e., the portion that is not usually measured. This indicates that a significant effect may be involved in computing the MTF from an experimentally obtained LSF. Even if the truncation for the LSF is small, it is possible that the area under the unknown tail portion is large and, therefore, results in greater error in the MTF.

For a more detailed analysis of the truncation error, the upper bound deviations in eq. (9)

are calculated by using simple analytic functions for the LSF. Exponential, Lorentzian, and Gaussian distributions are used to approximate the LSF. The calculated results are listed in Table 1 and shown in Figure 1. The Gaussian distribution gives the smallest deviation of the

Table 1
UPPER BOUND DEVIATIONS OF MTF's CALCULATED FOR
THREE APPROXIMATED LSF's

	LSF, $r(x)$	MTF, $R(u)$	Distance, d	Upper bound deviation, ΔR_{UB}
Exponential	$\frac{1}{2c} \exp(- x /c)$	$\frac{1}{1 + (2\pi cu)^2}$	$-c \ln(t)$	$\frac{2t}{1-t}$
Lorentzian	$\frac{2c}{c^2 + 4\pi^2 x^2}$	$\exp(-c u)$	$\frac{c}{2\pi} \sqrt{\frac{1-t}{t}}$	$\frac{2(\frac{\pi}{2} - \tan^{-1} \sqrt{\frac{1-t}{t}})}{\tan^{-1} \sqrt{\frac{1-t}{t}}}$
Gaussian	$\frac{1}{\sqrt{2\pi} \sigma} \exp(-\frac{x^2}{2\sigma^2})$	$\exp(-2\pi^2 \sigma^2 u^2)$	$\sigma \sqrt{-2 \ln(t)}$	$\frac{2 \operatorname{Erfc}(\sqrt{-\ln(t)})}{1 - \operatorname{Erfc}(\sqrt{-\ln(t)})}$

t : truncation; $\operatorname{Erfc}(x) = \frac{2}{\pi} \int_x^\infty e^{-y^2} dy$; error function.

Figure 1. Truncation errors expressed by upper bound deviations of MTF's calculated for three approximated LSF's.

three. This is to be expected since it is the most rapidly decreasing function. The Lorentzian distribution, however, gives a large deviation which becomes 0.136 at a truncation level of 0.01. The exponential distribution gives an intermediate deviation. Since both the LSF and the MTF of screen-film systems can be approximated by an exponential distribution,^{6,7} the analytic results

indicate that there may be substantial truncation errors in evaluating MTF's of radiographic systems.

The actual deviation of the MTF cannot, in general, be calculated easily. The upper bound deviation defined by eq. (9) is, therefore, a convenient tool to estimate the truncation error because of its simple relationship to the LSF. The upper bound deviation is, however, a conservative measure in the sense that it is always greater than the actual deviation. It is, therefore, compared with the actual maximum deviation of a function which is easily computed to determine the difference between the two. For the exponential LSF in Table 1, the deviation of the MTF in eq. (6) is calculated as

$$\Delta R = \frac{t}{1-t} \left[\frac{1}{1+U^2} - \frac{1}{\sqrt{1+U^2}} \cos(TU + \Phi) \right], \quad (10)$$

where $U = 2\pi cu$, $T = \ln t$ and $\Phi = \tan^{-1}U$ are used. The maximum value of eq. (10) as a parameter of the truncation t is determined numerically by using a digital computer. The comparison between the upper bound and the maximum deviation of the MTF is shown in Figure 2. The dif-

Figure 2. Comparison of upper bound and maximum deviations of the MTF for an exponential LSF.

ference decreases as the truncation assumes smaller values. For the region encountered in practical MTF calculations ($t \sim 0.01$), this comparison indicates that the upper bound deviation can be used to evaluate the truncation error.

EXPERIMENTAL RESULTS

In practice, it is convenient to work with the LSF normalized to unity at the center of the LSF. For simplicity, therefore, all subsequent uses of the term LSF will refer to the function normalized in this manner.

To determine the truncation error in a screen-film system, the slit method for measuring the LSF was extended to permit evaluation of the LSF to less than 0.001 of its maximum value.

A LSF was obtained by using a multiple exposure technique by which five parts of the LSF were independently measured from five line images corresponding to different maximum densities. The entire LSF was then completed by connecting these partial LSF's. The validity of making these connections was examined by checking that the ratio of two partial LSF's in each overlapped region was almost constant. The multiple exposure technique was employed because the toe and shoulder regions of the characteristic curve cannot be used for precise measurements.

The x-ray tube voltage used was 80 kVP. The slit apparatus ($10\text{ }\mu\text{m}$ width) has been described in a previous paper.⁶ The processed slit images were converted to the effective exposure distribution by using the characteristic curve of the screen-film system measured by an x-ray sensitometer.⁸ The screen-film systems examined are denoted as the F system (Radelin TF-2 X-ray Intensifying Screens with Kodak Blue Brand Medical X-ray Film), the M system (DuPont Par-Speed X-ray Intensifying Screens with Kodak Royal Blue Medical X-ray Film), and the S system (DuPont Detail X-ray Intensifying Screens with Kodak Royal Blue Medical X-ray Film). The LSF's of the three screen-film systems are shown in Figure 3. The high

Figure 3. LSF's of three screen-film systems: F system (TF-2, Blue Brand), M system (Par Speed, Royal Blue) and S system (Detail, Royal Blue).

exposures for obtaining the longer tail region were 3.9, 7.9, 50 and 150 times larger than the lowest exposure used for the central part of the LSF in the F system.

The high exposure conditions necessary to evaluate the LSF tail to very low levels introduced several possible sources of error. X-ray transmission through the slit jaws, as well as scattered radiation, may contribute to the measured LSF. The effects were examined independently.

The slit jaws are 2 mm thick and made of a 75 per cent platinum - 25 per cent iridium al-

loy. Therefore, the penetration for monoenergetic x-rays of energy about 80 keV is calculated to be 4.5×10^{-5} , ignoring the absorption discontinuity near this energy. Actual penetration, of course, will be several orders of magnitude less for heterogeneous radiation. The scattered radiation from the slit was examined experimentally. No appreciable scattered radiation was observed.

The end effect, which is an error caused by the finite length of the slit, was also considered as a possible source of error in measuring an extremely wide LSF. The deviation of the LSF measurement was theoretically calculated by using the Lorentzian LSF. The relative error was under 2.5 per cent for the 18 mm long slit within a distance of 2 mm from the center of the LSF.

The highest exposures for obtaining the longest tail portions of the LSF's were made intermittently because of the capacity limits of the x-ray tube. The possibility that intermittent exposures would affect the characteristic curve of the screen-film system was examined. The results showed that there were no changes in curve shape within experimental error.

It was concluded that the errors caused by x-ray penetration, scattered radiation, end effect and intermittent exposures do not seriously affect the LSF measurements involved in this study.

The LSF's in Figure 3 were truncated at several positions. The corresponding MTF's were then calculated. Aliasing errors which are caused by the digital Fourier transformation of discrete LSF data were confirmed to be negligible in these calculations. The results for the F system are shown in Figure 4. The MTF with $t = 0.00033$ reduces the perturbing oscillations to a

Figure 4. MTF's calculated from truncated LSF's for the F system (TF-2, Blue Brand).

negligible level, but the MTF with $t = 0.085$ shows excessively large oscillations. The MTF with $t = 0.0094$, which nearly corresponds to the routine calculation in this laboratory, is approximately equal to the MTF with $t = 0.00033$ except in the low spatial frequency regions. The deviations of the truncated MTF's from the true MTF are shown in Figure 5 by regarding the true MTF as that calculated from the LSF with $t = 0.00033$. The maximum deviations occur at low

Figure 5. "True" MTF and truncation errors in the F system (TF-2, Blue Brand). Dotted portions show negative values for $t = 0.085$.

spatial frequencies. The envelopes of the truncation errors were nearly proportional to the MTF. This shows roughly that the relative deviations are almost constant over the entire spatial frequency range.

The maximum deviations of the MTF as a function of the truncation are shown in Figure 6 for the three screen-film systems. Based on these curves the LSF's of the three systems

Figure 6. Maximum deviations of MTF's in three screen-film systems: F system (TF-2, Blue Brand), M system (Par Speed, Royal Blue) and S system (Detail, Royal Blue).

should in some way be intermediate to the exponential and Lorentzian distributions. It should be stressed that the observed maximum deviations are quite large and range from 0.04 to 0.07 at the truncation level normally used in practice ($t \sim 0.01$).

The true MTF's of the three screen-film systems are shown in Figure 7, where the term "true" is used for the lowest truncations available in calculating the MTF's. Any resulting oscillations would be small compared with the precision (± 3 per cent) of the experiment⁶). Note that in the low spatial frequencies near the zero point the MTF's in Figure 7 are steeper than the MTF's measured previously.⁶

Figure 7. "True" MTF's of three screen-film systems: F system (TF-2, Blue Brand), M system (Par Speed, Royal Blue) and S system (Detail, Royal Blue).

CORRECTION PROCEDURE FOR TRUNCATION ERROR

It is apparent that one method of decreasing the truncation error is to measure the tail LSF as low as possible. This, however, is quite tedious and may, therefore, be impractical—particularly in routine MTF measurements. Another method of reducing the truncation error is to modify the Fourier transformation program in the computer. This is done by utilizing prior information about the tail LSF. A curve approximating the tail region can be obtained by extrapolating the truncated LSF. The Fourier transformation can then be made for both the truncated and tail LSF's. This would reduce the truncation error, because the area difference between the true LSF and the LSF to be calculated would be much smaller than without the extrapolation, and according to eq. (9), the upper bound deviation is directly related to the area.

If the tail LSF is given by the following exponential approximation as

$$\begin{aligned}
 r_E(x) &= a \exp(-|x|/b) & |x| > d \\
 &= 0 & |x| \leq d,
 \end{aligned} \tag{11}$$

then the Fourier transformation is derived as

$$R_E(u) = 2ab \exp(-d/b) \frac{\cos(2\pi du + \phi)}{\sqrt{1 + (2\pi bu)^2}}, \quad (12)$$

where $\phi = \tan^{-1}(2\pi bu)$. Since the tail LSF in eq. (11) is extrapolated from the truncated LSF which is directly obtained by experiment, the constants a and b can be determined by assuming that the exponential curve goes through the two points (x_1, t_1) and (x_2, t_2) on the truncated LSF. The point (x_2, t_2) is the final point of the truncated LSF, i.e., $x_2 = d$ and $t_2 = t$, where t_2 equals the truncation value. The other point (x_1, t_1) is somewhat arbitrary and was determined experimentally. The selection of this point is discussed later. By using $b = (x_2 - x_1)/\ln(t_1/t_2)$ and $t_2 = a \exp(-x_2/b)$, the Fourier transformation (F.T.) with the correction can be written as

$$\text{MTF} = \frac{(\text{F.T. of } \frac{1}{2} \text{ LSF}) + \frac{bt_2 \cos(2\pi x_2 u + \Phi)}{\sqrt{1 + (2\pi bu)^2}}}{(\text{area of } \frac{1}{2} \text{ LSF}) + (bt_2)}, \quad (13)$$

where the Fourier transformation is applied to only one half of the LSF because of the LSF symmetry. Equation (13) is derived from eq. (4) divided by a normalizing factor which is the two areas in the denominator.

Figure 8 shows the result corrected by this method. The deviations of the MTF's with and without the correction are shown in the S system at $t = 0.01$. The maximum deviation without

Figure 8. Comparison of deviations of the MTF with and without correction in the S system (Detail, Royal Blue) at $t = 0.01$.

the correction, which is 0.071, decreases to 0.022 with correction. The correction procedures were examined by using x_1 equal to $1/2$, $2/3$, or $5/6$ of x_2 . Among these fractions, the condition $x_1 = 5x_2/6$ resulted in the best correction, although the others gave nearly the same results. A similar result in the F system is shown in Figure 9. The maximum deviation is decreased from 0.136 to 0.031 by the correction. If the maximum deviations are evaluated by the equivalent truncations using Figure 6, these maximum deviations with the correction procedure

Figure 9. Comparison of deviations of the MTF with and without correction in the F system (TF-2, Blue Brand) at $t = 0.05$.

for the S and F systems correspond to approximately 0.0017 and 0.007 of the truncations, respectively. These results show that the correction procedure works well in decreasing the deviation of the MTF.

CONCLUSION

To reduce or eliminate the truncation error, at least two methods are applicable. One is to measure the LSF below 0.01 until the truncation error becomes negligible. This, however, requires tedious measurements. The other is to employ the correction procedure discussed in this investigation. Only a simple modification of the computer program used in calculating Fourier transformation is required. It is concluded that one of the two methods or a combination of them should be applied hereafter for measuring the MTF of a screen-film system accurately.

ACKNOWLEDGMENTS

The authors are grateful to Dr. C. E. Metz for suggesting the comparison of the upper bound deviation of the MTF with the maximum deviation.

LITERATURE CITED

1. Morgan, R. H., L. M. Bates, U. V. Gopala Rao, and A. Marinaro. Am. J. Roentgenol., Rad. Therapy and Nucl. Med., 92:426, 1964.
2. Rossmann, K. Phys. Med. Biol., 9:551, 1964.

3. Doi, K. Oyo Buturi, 35:559, 1966.
4. Sanderson, G. K. Phys. Med. Biol., 13:661, 1968.
5. Gopala Rao, U. V., and L. M. Bates. Phys. Med. Biol., 14:93, 1968.
6. Rossmann, K., G. Lubberts, and H. M. Cleare. J. Opt. Soc. Amer., 54:187, 1964.
7. Hiraki, M., and K. Doi. Kyokko X-ray Res. Repts., 21:13, 1967.
8. Haus, A. G., and K. Rossmann. Radiology, 94:673, 1970.

THE EFFECT OF AGE, FRACTIONATION AND DOSE ON RADIATION CARCINOGENESIS IN VARIOUS TISSUES OF MICE*

By

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Irradiation of various species of laboratory animals results in the development of neoplastic lesions, the incidence of which is dependent upon the dose rate and schedule of irradiation, as well as the biological system used. From the radiobiological viewpoint it has been of interest to explore the susceptibility of various physiological states to carcinogenesis by irradiation. Age at the time of irradiation has been one of these biological factors, and most such studies have been carried out on mice utilizing either single¹⁻⁶ or fractionated radiation exposures.^{7,8} The rat has been used less frequently,^{9,10} and mainly in studies of leukemogenesis and development of ovarian tumors. It has been shown that susceptibility to development of thymic lymphoma^{8,11,12} and ovarian tumors¹² decreased with adult age. Conversely, susceptibility to granulocytic leukemia increased during the first 6 months of life.¹² Lindop and Ratblat,³ however, observed an increase in the incidence of ovarian tumors following an irradiation with 650 R in 10- to 20-week-old animals.

There are fewer studies on the relation between neonatal age and susceptibility to radiation carcinogenesis than there are on chemical carcinogenesis. Kaplan⁸ found no difference in thymic lymphoma development between 2- and 4-week-old C57BL mice. Upton et al¹² demonstrated a higher incidence of myeloid leukemia at 5 weeks compared to newborn irradiated mice. Warren and Gates¹³ observed an increase in the number and variety of solid tumors in mice irradiated during their whole intrauterine life and/or a 21-day suckling period.

The present studies were designed to probe neoplastic capabilities of newborn (< 24 hours), infant (15 days), and young adult C57BL/6xC3H F₁ mice (42 days). Such mice were extensively used in our previous studies on chemical carcinogenesis.¹⁴⁻¹⁹ Our main objective was (a) to find out whether tissues in which cancers developed following exposure to chemical carcinogen, would also respond to irradiation, and (b) to compare patterns of tissue susceptibility. It was decided to probe the above problems by using either single irradiation exposures or several patterns of fractionated dose treatments. All experimental groups and one untreated control were terminated 86 weeks after initial irradiation. Two additional control groups were killed at 56 and 166 weeks of age. Irradiated animals that developed leukemia died before the termination of the experiment. Almost all other irradiated animals were alive at the time of sacrifice. The incidence of leukemias or malignant lymphomas, hepatomas, lung adenomas, Harderian gland cystadenomas, and ovarian tumors was dependent in various degrees upon the age of animals at treatment, their sex, and irradiation dose and/or schedule.

*This report is taken from a paper that appeared in Cancer Research 31:2133-42, 1971. The work was supported in part by grants from the American Cancer Society, and from the USPHS.

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MATERIALS AND METHODS

Mice. The experimental animals were bred in our laboratory, from Jackson Laboratory breeders. The C57BL/6 females, 8 to 10 weeks of age, were housed in triplets with a single C3HeB/Fe male of the same age in the plastic cages. Four hundred females and 133 males were utilized. Females were examined for pregnancy 2 weeks after breeding and at 2-day intervals thereafter. Pregnant mice were allocated at random to eighteen groups, of which fifteen were to be X-irradiated and three left non-irradiated, as outlined in the experimental design (Table 1). There were six experimental series (A to F), each consisting of three age groups ac-

Table 1

EXPERIMENTAL DESIGN

Group	Age at start (days)	Irradiation schedule					
		Series					
		A	B	C	D	E	F
1	1	Single exposure to 320 R	Two exposures to 160 R at 4-day intervals	Four exposures to 80 R at 4-day intervals	Four exposures to 80 R at 7-day intervals	Four exposures to 120 R at 7-day intervals	None
2	15						
3	42						

A total of eighteen experimental groups were used. All irradiated groups were sacrificed at 86 weeks of age. Non-irradiated controls, which were observed from birth, were sacrificed at 56 (Group 1), 86 (Group 2), and 166 (Group 3) weeks of age.

A G.E. Maxitron was the source of irradiation and was used under the following conditions: 40 R/min. at 230 KV, 25 MA, with a filter of 0.25 mm Cu and 1.00 mm Al, and source to target distance of 77 cm.

cording to the beginning of the experiment. At 30 days of age, mice were weaned, numbered, and sexes were separated. Throughout the experiment animals were kept in a temperature controlled laboratory, in plastic cages, fed Teklad Laboratory Chow and allowed to drink ad libitum water containing HCl at pH 2.0 to 2.5. Mice were weighed at intervals of 2 weeks, at which time they were inspected for exogenous neoplasms, signs of internal tumors, and other symptomatology. Daily inspections, and isolation of animals showing signs of illness, facilitated detection of moribund animals, which were later sacrificed when their condition became critical, to allow proper histological diagnosis of lesions. Dead animals were systematically autopsied, findings recorded, and tissues fixed in buffered 10 per cent solution of formalin, processed, stained with hematoxylin and eosin, and evaluated microscopically.

X-irradiation. A G.E. Maxitron 250 was the source of radiation. The desired dose was delivered at the rate of 40 R/minute at 230 KV, 25 MA, with a filter of 0.25 mm Cu and 1.00 mm Al. The distance from the source to target was 77 cm. The animals were confined singly in plastic perforated tubes and placed sixteen at a time on a rotating circular lucite holder. They were irradiated with a total dose of 320 R (Series A to D) or 480 R (Series E), as specified below and returned to their respective cages. The radiation dose was measured in air before each treatment.

Experimental design. Table 1 presents the experimental design. There were six experimen-

tal series, designated from A to F. Series A-E were exposed to specific irradiation schedules. Series F was left unirradiated, serving as negative controls. Series A to D were given a total dose of 320 R, either as single exposure (A), or fractionated in two (B, 2 x 160 R at 4-day intervals) or four treatments (C, 4 x 80 R at 4-day intervals; D, 4 x 80 R at 7-day intervals). Series E received a total dose of 480 R, administered in four sessions at 7-day intervals (4 x 120 R/7). Each of the irradiated series consisted of three age groups (1, 2, and 3), receiving their first irradiation on day 1, 15, or 42 after birth. Both sexes were represented by approximately the same number of animals. Survivors of all irradiated series were killed at 86 weeks, an age specifically selected to allow evaluation of their neoplastic expression before spontaneous tendencies of the tissues were fully manifested. Series F controls also consisted of three age groups, designated according to the age at which their survivors were sacrificed, namely at 56, 86, and 166 weeks of age, and provided us with a cross-section of development of "spontaneous" tumors to serve as a reference for irradiated experimental groups. The second group was sacrificed at the same time as the irradiated series, while the first and third groups supplied the early (at 56 weeks) and late (by 166 weeks) neoplastic expressions.

Animals that survived 30 days after irradiation served as the "effective" number for calculating incidence of lymphocytic leukemia, which occurred early. Animals not succumbing to this disease served as the basis ("effective" number) for calculating incidence of the other tumor types. It is noteworthy that the majority of X-irradiated animals survived to the time of sacrifice (86 weeks). Group 3 of the controls (Series F) began dying in the second half of their third year from various neoplasms emerging spontaneously late in life.

RESULTS

Our observations showed that both tumor incidence and the spectrum of tumors were positively affected by the X-irradiation. Five per cent of the non-treated controls (Series F, Group 2) that were sacrificed at the same age as the irradiated groups (86 weeks), developed tumors, while the animals of the X-irradiated series had an overall tumor incidence of 55 per cent and 87 per cent for males and females respectively. The higher incidence in females was due to development of ovarian tumors. Thirteen different primary neoplasms were found in the X-irradiated series while non-irradiated controls showed only seven tumor types. Hepatomas, leukemia, Harderian gland tumors, lung tumors, ovarian tumors, and probably pituitary (chromophobe) adenomas occurred with sufficient frequency to suggest that their causal relationship to irradiation was unquestionable. The overall incidence of the miscellaneous tumors was 8.34 per cent (93/1115) in irradiated and less than 1.0 per cent in the non-irradiated controls. Conclusions, based on the statistical evaluation of these data, will be made as to the modifying role of age and/or radiation factors in carcinogenesis of various tissues.

Malignant lymphomas. In neither sex did the incidence of lymphoma vary significantly with age under various irradiation schedules (Series A to E) (see Figure 1A). Irrespective of irradiation pattern, the incidence of leukemia ranged from 9 per cent to 13 per cent in males and from 11 per cent to 18 per cent in females. The overall incidence of leukemia in male mice (9.36 per cent) did not differ significantly from that in the females (13.30 per cent).

Radiation schedule and dose, however, had a differential effect on development of leukemia. Thus, a dose of 480 R (4 x 120 R at 7-day intervals, Series F) was significantly more leukemogenic than a dose of 320 R given either once (Series A) or in four exposures of 80 R at 4- (Se-

Figure 1. The incidence of leukemias, A, and ovarian tumors, B, is presented for irradiated animals (histogram) according to age at the beginning of the treatment, and for non-irradiated controls (solid curve extending to 166th week). Solid straight curve left of the histogram A presents actual age at which leukemias developed in the irradiated series; the numbers associated with "spontaneous" leukemia series represents age in weeks at which controls developed leukemia in incidences corresponding to irradiated series.

ries C) or 7-day (Series D) intervals ($P < 0.001$). In Series B, however, which received a total of 320 R given in two fractional exposures (2×160 R) at 4-day intervals, the incidence of leukemia was not significantly lower than in the groups exposed to 480 R (Series E). At the same time, the incidence of leukemia in Series B (2×160 R/4) was significantly higher than in Series A (1×320 R; $P < 0.02$), Series C (4×80 R/4; $P < 0.05$), and Series D (4×80 R/7; $P < 0.001$) (see Figure 4A).

A similar trend was observed in the female series. Series B (2×160 R/4) had a significantly higher incidence of leukemia than was observed in Series A (1×320 R; $P < 0.01$). The former series also showed a significantly lower incidence of leukemia than Series E (4×120 R/7; $P < 0.01$).

Hepatomas. Regardless of age and irradiation schedule, the male mice developed significantly more hepatomas than females (99/561 vs 30/554; $P < 0.001$). The overall sex difference

in hepatomas is exclusively due to a higher incidence of liver tumors in males irradiated either at 1 or 15 days of age than in those irradiated as young adults (48/190 and 38/183 vs 13/188; $P < 0.001$) (see Figure 2A). Females developed hepatomas with approximately the same frequency regardless of their age at irradiation (see Figure 2B).

Figure 2. The incidence of hepatomas in irradiated males, A, and females, B, is presented in the histogram form according to the animals' age at the time of treatment. The solid curves indicate hepatomas incidence throughout the life span for the nontreated controls. The numbers associated with the latter curve indicate age in weeks at which controls reach hepatoma incidences comparable to irradiated age groups.

The irradiation schedule was also a modifying factor in male animals. Thus, an exposure to 80 R at 4-day intervals (Series C) was significantly more effective than exposure to 2 x 160 R/4 (Series B; $P < 0.02$), 4 x 80 R/7 (Series D; $P < 0.05$), or 4 x 120 R/7 (Series E; $P < 0.01$) (see Figure 4B). When the dose was increased from 80 R to 120 R administered four times at 7-day intervals (Series D and E), there was no significant difference in the incidence of hepatomas.

Lung adenomas. Neither age nor sex of animals was efficient in modulating the incidence of lung adenomas (see Figure 3B). Although incidence of this tumor type was significantly higher

Figure 3. The incidence of Harderian gland cystadenomas, A, and lung adenomas, B, for combined sexes is presented in the histogram form according to the animals' age at the time of irradiation. The solid curves indicate the rate of "spontaneous" incidence of respective tumor types. The numbers associated with spontaneous lung adenoma development indicate age in weeks at which non-irradiated controls reached the incidence of these tumors corresponding to the irradiated animals.

in irradiated animals (151/1115; 13.5 per cent) than in non-irradiated controls (5/208; 2.4 per cent), $P < 0.001$, there was no difference between the radiation schedules used.

Harderian gland cystadenomas. Both sexes developed Harderian gland tumors with similar incidence (18.9 per cent and 18.0 per cent for males and females, respectively). The age of animals and the irradiation schedule modified tumor incidence. Thus, 1-day-old animals developed significantly fewer tumors of the Harderian gland than the infants or young adults ($P < 0.001$), when exposed to 320 R once (Series A), 2 x 160 R/4 (Series B), or 4 x 120 R/7 (Series E). Exposure of animals to 4 x 80 R/4 (Series C) or 4 x 80 R/7 (Series D) revealed no such phenomenon. The overall evaluation of the age effect shows decisively that infants and adults were more prone to develop Harderian gland tumors following ionizing irradiation than were the newborns (see Figure 3A).

Ovarian tumors. The most frequently observed neoplastic lesions were the ovarian tumors, tubular adenoma being the most frequent, followed by mixed tubular adenoma with granulosa

Figure 4. The incidence of leukemia, A, and hepatomas, B, is presented in the histogram form according to the irradiation schedule. The solid curves indicate the rates of "spontaneous" development of these tumors. The numbers associated with the latter curves indicate age in weeks at which non-irradiated controls developed respective tumor types comparable to the irradiated series.

cell tumors. The lowest incidence of these tumors was observed in animals that were irradiated with 2 x 160 R/4 (Series B), and the highest in animals of Series C and D (4 x 80 R/4 and 4 x 80 R/7, respectively). Series A (1 x 320 R) and E (4 x 120 R/7) showed a similar incidence, which was significantly lower than in Series C and D. As far as age was concerned, 1-day-old animals were less responsive than were infants or adults. Series C (4 x 80 R/4) was the only irradiation schedule that did not produce this effect. The overall incidence, regardless of irradiation schedule, shows that sensitivity increased with age (103/209 vs 114/153, $P < 0.001$; 103/209 vs 136/192, $P < 0.001$) (see Figure 1B).

Miscellaneous tumor types. Eight other tumor types, viz., tumors of the stomach, kidney, adrenal and brain, epitheliomas, sarcomas, mammary gland adenocarcinoma and pituitary adenoma, developed less frequently than the above-mentioned neoplasms. Females showed a significantly higher incidence of these tumors than males (10.6 per cent vs 6.1 per cent), apparently because of the higher frequency with which pituitary, adrenal, and mammary gland tumors devel-

oped in female mice. Other neoplastic lesions were observed in both sexes with the same frequency. Of 93 miscellaneous tumors, 44 (47.3 per cent) occurred in animals irradiated at birth, 30 (32.3 per cent) in infants, and only 19 (20.4 per cent) in young adults. This indicates the generally lower susceptibility of adult animals to irradiation carcinogenesis in these tissues.

Tumor development in non-irradiated controls. The following tumors were observed in non-irradiated controls (Series F) killed at 56 and 86 weeks of age or living to the 166th week of life: Leukemia, hepatomas, and lung adenomas developed with relatively high incidence (in about 1/3 of animals) in the last group, appearing at an average age of 145 weeks. Ovarian tumors were seen only in 2 per cent of females and at an average age of 131 weeks.

Comparison between irradiated and control groups is illustrated in Figures 1 to 5. The incidence of leukemia increased from less than 1 per cent in non-irradiated controls (Group 2, Series F) to 11.36 per cent in the X-irradiated animals. This increment represents a highly significant difference (143/1258 vs 1/199; $P < 0.001$). When compared with the third control group (sacrificed at 166 weeks of age), this difference in the incidence of leukemia appears reversed, since controls developed leukemia in 38 per cent and 49 per cent in males and females, respectively. However, the average age at death due to leukemia was 35 weeks in the irradiated series and 141 weeks in the non-treated controls. Thus irradiation mainly accelerated development of leukemias so that a significant difference in incidence was observed between irradiated and non-irradiated groups by the 86th week of life (Figure 1A).

The incidence of hepatomas was significantly higher in irradiated male animals than in controls of the same age. When a comparison is made with the third set of controls (166 weeks of age at sacrifice), no difference in incidence is observed. Figure 2 shows that non-treated controls developed identical tumor incidences much later than the irradiated groups. The incidence of lung adenomas was slightly enhanced by irradiation (Figure 3B). The development of Harderian gland adenomas, however, was both accelerated and augmented by irradiation (Figure 3A).

The incidence of ovarian tumors was strikingly enhanced by irradiation (64 per cent) (Figure 1B). A great majority of ovarian tumors were of epithelial origin, namely, tubular adenomas.

Figure 4 illustrates the role of the irradiation pattern in enhancing the occurrence of leukemia and hepatomas. Thus, 2 x 160 R given at 4-day intervals was most effective in leukemogenesis, while 4 x 80 R delivered at 4-day intervals resulted in the highest development of hepatoma. Figure 5 depicts the variation of tumor profile as it relates to the age of animals at the time of irradiation.

DISCUSSION

The present studies revealed several aspects of postnatal irradiation carcinogenesis which complement observations made by previous investigators. The positive effect of irradiation on tumorigenesis was further substantiated for leukemia, hepatoma, lung adenoma, Harderian gland adenoma, ovarian tumors, pituitary and adrenal neoplasms, epithelioma, sarcoma, stomach papilloma, and kidney adenomas, especially in the first five tissues.

Of five tissues or organs mentioned above, three varied in their carcinogenic sensitivity at different ages and one organ in relation to sex. The organs of the first group are the liver, Harderian glands, and ovaries, and, of the second group, the liver.

The liver was significantly less responsive to irradiation carcinogenesis in the adult than at birth or in the infant. The effect of irradiation on the adult was not significantly different from

Figure 5 A presents in histogram form tumor profile according to the age at which animals were irradiated. B depicts the change in the incidence of ovarian tumors, Harderian gland adenomas, lung adenomas, leukemias, and hepatomas with the irradiation age.

that on non-irradiated controls sacrificed at the same age (6.9 per cent vs. 3.0 per cent). This agrees with earlier studies on adult animals.^{20,21} It is of interest that male preweanling animals (newborns and infants) were highly prone to develop hepatomas during the first 86 weeks of life. Similar results were earlier observed in C57BLxC3H F₁ mice exposed to dimethylnitrosamine¹⁴ or urethan.¹⁶⁻¹⁹ It should be noted, however, that male and control animals developed hepatomas in relatively high incidence (33 per cent) during their third year of life. This indicates that irradiation accelerated an underlying neoplastic process, primarily when administered at an earlier age.

The increased development of Harderian gland adenomas following irradiation was also age-dependent, but in the opposite direction. Thus, newborn animals were less responsive than were infants or young adults, and no difference was observed between the sexes in response to irradiation. This is consistent with our earlier observations on the role of age in induction of Harderian gland tumors by urethan.¹⁷ At that time our morphologic studies showed that Harderian glands were functionally quiescent before the opening of the eyelids, which takes place at the end of the second week of life. Thus, functionally active glands were more responsive both to urethan and X-irradiation. The spontaneous incidence of Harderian gland tumors is very low, even late in life, so that in this case irradiation and urethan both accelerated and augmented neoplastic ex-

pression in those glands. Under present experimental conditions, irradiation of C57BL/6xC3H F₁ mice resulted in development of Harderian gland tumors in significantly higher incidence than observed by other investigators.^{21,22} Apparently genetic background and the irradiation schedule were decisive operating factors.

Ovaries showed a similar trend in sensitivity to irradiation carcinogenesis as was found for Harderian glands. Thus newborn animals were less prone to develop ovarian tumors than were infants or adults. This observation is in agreement with those of Lindop and Rotblat,³ but is at variance with Upton et al⁵ who observed significantly higher incidence of ovarian tumors in animals irradiated once with 300 R at 2 weeks of age than in groups exposed similarly at 4 or 6 weeks of age. Apparently the dose and rate of irradiation and the genetic background of mice used were conditions modulating the "age" factors.

Development of lung adenomas and leukemia (predominantly thymic lymphoma type) was not modified by the age of animals at the time of irradiation, nor by their sex, which is in agreement with our investigation on the effect of urethan on lung tumorigenesis. Conversely, the absence of lower sensitivity of adult mice to leukemogenesis following X-irradiation is at variance with studies using urethan.¹⁵ This, however, can be reconciled by evidence presented by Kaplan,²³ which indicates that leukemogenesis, regardless of the inciting agent (chemical or irradiation), requires a triad of effects: virus release, thymic injury, and bone marrow damage. Thus, urethan, when given to adult mice, "lacks the capacity to 'inject' virus in adult mice," which is not the case with irradiation.²³

Our observations regarding leukemogenesis are in agreement with those of Kaplan⁸ and Chen and Berenblum.²⁴ Upton et al,⁵ using RF mice and single 300 R exposures, observed a slightly higher incidence of thymic lymphoma in 6-week-old mice (34.2 per cent) than in 2-week-old infants (23.1 per cent), a difference short of being significant. Rudali et al¹¹ observed significantly higher incidence of leukemia in C57BL mice and C57BLxAKR F₁ hybrids when irradiation exposure began at birth as compared with groups first irradiated at 6 weeks of age.

Of the eight types of tumors which developed with low incidence in irradiated groups, only two (pituitary adenomas and mammary gland adenomas) were seen in non-treated controls. These appeared in 0.54 per cent of the control animals at 109 and 151 weeks, respectively. Irradiated animals developed pituitary tumors in 4.87 per cent (females) and 2.67 per cent (males) ($P < 0.05$) at an average age of 73 weeks. Thus, the development of pituitary tumors also was enhanced by X-irradiation. Mammary gland adenocarcinomas (0.72 per cent) developed in the irradiated animals before the 86th week of life.

Neoplasms of kidneys, stomach, adrenals, and brain (astrocytoma) each were observed in about 1 per cent of the X-irradiated animals, but none of the controls were killed concurrently. It is tempting to assume a causal relationship between the irradiation exposure and the neoplastic expression in these four tissues (combined frequency of 32/1115 vs 0/571, $P < 0.001$). Epitheliomas and sarcomas developed in 1.35 per cent of the irradiated animals (15/1115), which is a significantly higher frequency than the 0.0 per cent incidence observed in controls (0/571, $P < 0.05$).

In addition to the effect of age, the irradiation schedule and the dose administered had a modifying effect upon the incidence of the tumors. The modifying effect of the irradiation schedule was observed in development of leukemia, hepatomas, and ovarian tumors. The role of fractionation in leukemogenesis was studied originally by Kaplan.⁷ The interplay between the thymic

injury and its regeneration rate, bone marrow damage and the release of virus, which are essential for irradiation-triggered leukemogenesis, are dependent upon irradiation schedule. The fact that x-ray fractionation plays a role in liver and ovarian tumorigenesis has been observed, as far as we know for the first time.

The most striking effect of the irradiation dose was observed in leukemogenesis, where the incidence rose from 4.19 per cent to 29.91 per cent (Series D vs E, $P < 0.0001$). Development of hepatomas and ovarian tumors was generally lower after exposure to the higher irradiation dose, possibly because potential tumor-bearing animals (hepatomas and/or ovaries) had died earlier from leukemia.

The tumor profile (relationship between incidence in the five major tumor types), as shown in Figure 5, varied with the age at which animals were exposed to irradiation. The neoplastic profiles were mainly dependent upon variations in sensitivity of liver, Harderian glands, and ovaries to undergo carcinogenesis by X-irradiation. This does not imply that the same mechanism operates in carcinogenesis of different tissues. This is because X-irradiation, as a multicarcinogen, may be considered to operate either directly or indirectly depending upon the tissue, since there is no reason to assume the unitary mode or mechanism of its carcinogenic action. Actually, there is sufficient evidence to indicate (a) viral participation in leukemogenesis, (b) hormonal action in the ovarian tumorigenesis, (c) modifying effect of the hormonal environment on the rate of emergence of the liver tumors, and (d) direct ionizing irradiation effect on lung tumorigenesis. Therefore, the extent of the multicarcinogenesis due to postnatal X-irradiation is conditioned by the biological factors at the time of irradiation and by the interplay of its early and late manifestation of the original effects. Thus, depending upon the tissue in question, the treatment should be delivered at the age when the biologic conditions favor early inception of neoplastic process in that particular tissue.

The malignant lymphomas, hepatomas, lung adenomas, Harderian gland cystadenomas and ovarian tumors developed in the non-treated control groups on the average between the 124th and 153rd week of life. The origin of these spontaneously emerging tumors is unknown at present. They may represent an expression of the inherent capability of the cells revealed under specific physiological conditions. How much such neoplastic development may be due to vertically transmitted virus(es) and the exposure to naturally occurring carcinogens throughout the life span is impossible to evaluate at the current state of information. However, the important fact is that these tissues were capable of neoplastic expression even without deliberate administration of any "carcinogen." Therefore, the data showed that the exposure of animals to irradiation resulted in an increase in tumor incidence and/or their earlier emergence. Descriptively this represents an antedated development of spontaneous tumors. The question remains open whether this advanced emergence of tumors was due to an earlier onset of the neoplastic process, its increased rate of neoplastic expression, or a combination of both phenomena.

Our data show that tumor response is dependent upon the age of the animal at the time of exposure to ionizing radiation regardless of the capability of the tissues to develop tumors spontaneously. It is conceivable that the degree of specific reactivity to ionizing radiation varies with age due to the biochemical kinetics at the tissue level and the competence of the whole animal. Other factors, such as the degree of cell maturity, the size of the stem cell pool and the replicating compartments, and the relative synchrony of "sensitive" phase(s) of cell cycle to interact specifically in direct or indirect manner to irradiation as well as the immunological com-

petence of the host, change with the age and thus modulate differentially various phases of the carcinogenic process. It has been suggested previously that, by the analogy, it is possible that the inception and/or the development of the "spontaneously" emerging tumors occurred only when all necessary factors were complementary in their action.¹⁹ These factors represent possible operative components of mechanisms of various stages of the carcinogenic process and as such are worthy of further investigation.

ACKNOWLEDGMENTS

The competent technical assistance of James Keough and Branko Manojlovski has been greatly appreciated.

LITERATURE CITED

1. Deringer, M. K., and E. Lorenz. *J. Natl. Cancer Inst.*, 15:923, 1955.
2. Hollcroft, J., E. Lorenz, E. Miller, C. C. Congdon, R. Schweisthal, and D. Uphoff. *J. Natl. Cancer Inst.*, 18:615, 1957.
3. Lindop, P. J., and J. Rotblat. *Brit. J. Radiol.*, 35:23, 1962.
4. Lorenz, E., L. O. Jacobson, W. E. Heston, M. Shimkin, A. B. Eschenbrenner, M. K. Deringer, and R. Schweisthal. In Biological Effects of External X and Gamma Radiation, Part F, R. E. Zirkle, Ed. New York: McGraw-Hill, 1954, pp. 24-168.
5. Upton, A. C., T. T. Odell, and E. P. Sniffen. *Proc. Soc. Exptl. Biol. (N.Y.)*, 104:769, 1960.
6. Upton, A. C., F. F. Wolff, J. Furth, and A. W. Kimball. *Cancer Res.*, 18:842, 1958.
7. Kaplan, H. S. *Cancer Res.*, 7:141, 1947.
8. Kaplan, H. S. *J. Natl. Cancer Inst.*, 9:55, 1948.
9. Reincke, U., E. Stutz, and G. Wegner. *Zeitschrift für Krebsforschung*, 66:165, 1964.
10. Sikov, M. R., and J. E. Lofstrom. *Vgl. Loutit*: 1963.
11. Rudali, G., and J. Reverdy. *Académie des Sciences*, 253:590, 1961.
12. Upton, A. C., A. W. Kimball, J. Furth, K. W. Christenberry, and W. H. Benedict. *Cancer Res.*, 20:1, 1960.
13. Warren, S., and O. Gates. In Radiation Biology of the Fetal and Juvenile Mammal, M. R. Sikov and D. D. Mahlum, Eds. Oak Ridge, Tennessee: USAEC Division of Technical Information Extension, 1969, 17 AEC Series, pp. 419-37.
14. Vesselinovitch, S. D. *Cancer Res.*, 29:1024, 1969.
15. Vesselinovitch, S. D., and N. Mihailovich. *Cancer Res.*, 26:1633, 1966.
16. Vesselinovitch, S. D., and N. Mihailovich. *Cancer Res.*, 27:350, 1967.
17. Vesselinovitch, S. D., and N. Mihailovich. *Cancer Res.*, 27:1422, 1967.
18. Vesselinovitch, S. D., and N. Mihailovich. *Cancer Res.*, 27:1788, 1967.
19. Vesselinovitch, S. D., N. Mihailovich, and L. Itze. *Cancer Res.*, 30:2548, 1970.
20. Mewissen, D. J., and J. H. Rust. *Comptes rendus des séances de la Société de Biologie*, 157:680, 1963.

21. Upton, A. C., J. Furth, and K. W. Christenberry. *Cancer Res.*, 14:682, 1954.
22. Furth, J., R. Buffett, and N. Haran-Ghera. *Internatl. Union Against Cancer*, 16:138, 1960.
23. Kaplan, H. S. *Natl. Cancer Inst. Monogr.*, 14:207, 1964.
24. Chen, L., and I. Berenblum. *Israel J. Med. Sci.*, 4:1164, 1968.

LOSS OF THE Y CHROMOSOME IN MYELODYSPLASIA: THREE CASES STUDIED WITH QUINACRINE FLUORESCENCE*

By

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Chromosomal analysis of bone marrow cells from patients with various hematological disorders other than leukemia or polycythemia reveals that a small percentage, about ten per cent in our laboratory,¹ have chromosomal aberrations in a variable proportion of the cells examined. The variability in modal chromosomal number and group affected is similar to that observed in patients with acute leukemia, although C group chromosomes are frequently involved.² Males with 45 chromosomes, lacking either a G or Y chromosome have also been observed in both leukemic and non-leukemic populations.³⁻⁶ The differential fluorescence of the human Y chromosome which has been observed recently,⁷⁻⁹ can be used to distinguish the Y from the G group autosomes and thus permits the positive identification of males some of whose cells are 45,X.

The present paper reports on three males who possess a 45,X cell line, shows the importance of serial observations, and demonstrates the application of the new quinacrine fluorescence techniques.

MATERIALS AND METHODS

Chromosome preparations of bone marrow cells from 100 male patients were examined. Fifty-seven of these patients had some hematologic disease (excluding leukemia and polycythemia vera) while 43 did not. Forty-seven individuals were under 60 years of age while 56 were 60 years or older. A complete report of these 100 patients is in preparation.

Bone marrow specimens were processed using the direct method of Tjio & Whang¹⁰ with modifications.² Peripheral blood lymphocytes were cultured according to the technique of Moorhead et al.¹¹

Material from the spleen was processed in two ways:¹² In the direct technique, a sterile spleen sample was minced in Hank's Balanced Salt Solution to obtain a single cell suspension. About 10 ml of suspension was centrifuged, the supernatant was discarded, and the cells were resuspended in hypotonic KCl for 18 minutes. From then on the procedure was identical to that used for processing bone marrow cells. In the second method, spleen sample was minced in McCoy's medium with 30 per cent calf serum and incubated for 6 hours. One hour before terminating the culture, vinblastine sulphate (Velban), 0.2 ml (0.5 µg/ml) per 10 ml of suspension, was added and cells were processed as above.

Selected slides from each patient were examined with the quinacrine mustard technique of Caspersson¹³ as modified in our laboratory for re-examination of slides previously stained with Leishman-Giemsa.¹⁴

* This report is taken from a paper that appeared in *Brit. J. Haematol.*, 21:717, 1971. The work was supported in part by a grant from the Cancer Society of St. Joseph County.

CASE REPORTS

Case 1. G.T. is a 55-year-old white physician who noted increasing fatigue in March 1969. His PCV was 32 per cent. Two weeks later he developed "flu" and took at least 8 capsules of chloramphenicol. He was hospitalized elsewhere 28 March to 2 April, 1969 because of persistent weakness and malaise. Blood examination included: PCV 32 per cent, WBC 3200/ μ l, platelets 10,000/ μ l, reticulocytes 1.7 per cent, and a normal differential. Bleeding time was greater than 30 minutes. Direct and indirect Coombs test were negative.

Bone marrow aspiration showed a slightly hypocellular marrow with practically no megakaryocytes but with an apparently normal myeloid and erythroid series; the latter showed some shift to the left. Vitamin B₁₂ and folic acid and later prednisone, 40 mg/day and fluoxymesterone (Halotestin) 40 mg/day, resulted in a temporary rise in his platelets to 30,000/ μ l. The final diagnosis was pancytopenia of undetermined origin, possibly related to chloramphenicol.

The patient was admitted to Argonne Cancer Research Hospital on 7 July 1969 for further evaluation. Past history was noteworthy because of a duodenal ulcer that was treated by a Billroth II procedure in 1962. He is married and has 8 children.

Physical examination on admission was essentially negative except for numerous ecchymoses more prominent on the lower extremities. Blood counts on admission were: Hb 7.8 g/100 ml, PCV 25 per cent, RBC 2.74×10^6 / μ l, WBC 3350/ μ l; platelets 9000/ μ l; reticulocytes 1 per cent; neutrophils 48 per cent, small lymphocytes 46 per cent, monocytes 4 per cent, eosinophils 2 per cent. Extensive laboratory tests including serum folate, vitamin B₁₂ and serum iron concentration were essentially normal. Leukocyte alkaline phosphatase was 355 (normal: 40-113). Sections of a bone marrow sample obtained 8 July 1969 showed normal cellularity. The M:E ratio was 1:1 with some increase in erythroid elements. Maturation in both series was normal. Megakaryocytes appeared normal in number and configuration. Sections showed absence of stainable iron and no ring sideroblasts.

Blood counts showed a minimal response to oral iron and prednisone 60 mg/day; the patient was discharged 19 July, 1969. Although oxymetholone was added on 28 August 1969, there were no significant changes in his peripheral counts. He continued to have ecchymoses and stools for occult blood were 4+. A repeat bone marrow examination on 5 December 1969, showed little change, although cellularity was decreased to 20-30 per cent, and megakaryocytes were diminished in number. Six units of packed cells were administered in December 1969.

Because he failed to respond to medical management the patient was admitted on 22 February 1970 for splenectomy. Hematologic studies, including a bone marrow examination on 23 February, 1970 were similar to the previous results. No complications were encountered during the operation (3 March 1970) or the postoperative period. A low dose of prednisone was continued until 22 May when all medications except iron sulfate were discontinued. Recent blood counts have been normal. Except for increased cellularity (40 to 50 per cent), the marrow specimen obtained 14 September 1970 resembled previous samples.

Case 2. W.L. is a 61-year-old married white male who was first admitted on 30 July 1967 for hoarseness secondary to chronic inflammation of the vocal cords. His past history included peptic ulcer of the duodenum (1938). He has three children. He showed an elevated RBC 5.12×10^6 / μ l, Hb 18.1 g/100 ml, PCV 55 per cent, WBC 22,000/ μ l, platelets 120,000/ μ l, with a normal differential. The red blood cell mass and total blood volume were normal. In October, the

Rumple-Leed's test became positive, associated with a fall in the platelet count to $71,000/\mu 1$. Prednisone, 20 mg/day caused a rise in the platelet count to $130,000/\mu 1$ within one week. Prednisone was decreased and then discontinued after the second week. Platelet counts fluctuated between 75,000 and $125,000/\mu 1$. A left renal cyst was documented on renal arteriography in November, 1967.

He was readmitted in January, 1968 for evaluation of asymptomatic thrombocytopenia ($79,000$ platelets/ $\mu 1$), leukocytosis with eosinophilia (WBC $11,650/\mu 1$ with 14 per cent eosinophils), and elevated PCV (50-55 per cent). Physical examination on admission was normal except for blood pressure of 180/80. Extensive workup for collagen disorder, myeloproliferative disease, toxic exposure, and occult neoplasm failed to provide an adequate explanation for these hematologic abnormalities. The bone marrow contained adequate numbers of megakaryocytes and was otherwise normal, except for increased eosinophilic precursors. Abnormal studies included absent serum complement and anticomplementary Reiter protein complement fixation test. The presence of an anticomplementary factor, which caused rapid loss of assayable complement activity when blood clotting was initiated, was revealed.¹⁵

In September, 1969 there was a recurrence of duodenal ulcer disease which was treated successfully with a medical program. A second bone marrow examination (January, 1970) revealed no change except for a decreased number of morphologically normal megakaryocytes. A program of periodic phlebotomy was initiated to maintain the PCV at or below 50 per cent. Other current blood counts include WBC $13,400/\mu 1$ with persistent eosinophilia, and platelets $205,000/\mu 1$.

Case 3. I.F. is a 68-year-old unmarried white male first seen in March 1963 for a persistent sore throat which biopsy revealed to be due to squamous cell carcinoma.

Laboratory data on admission were as follows: Hb 14 g/100 ml, PCV 45 per cent, WBC $8400/\mu 1$, platelets $71,500/\mu 1$, and differential, neutrophils 79 per cent, small lymphocytes 15 per cent, monocytes 5 per cent, eosinophils 1 per cent. Bleeding and clotting times were normal.

The carcinoma was treated with 7100 Rads over six weeks, during which time the platelets fell to $15,000/\mu 1$. No petechiae or abnormal bleeding were noted. The bone marrow examination revealed a normocellular marrow with a normal M:E ratio and normal maturation. Megakaryocytes with satellite platelets were present in normal numbers but there was a paucity of platelets in the marrow smear. Iron stores were normal. A history of long use of hair dye led to shaving his head which was followed by a rise in platelets to $60,000/\mu 1$. The count subsequently fell to $16,500/\mu 1$ even though x-ray therapy had been completed and the patient had been receiving prednisone, 15 mg/day for three weeks. Prednisone was increased to 60 mg/day for two weeks but the platelet count did not exceed $38,000/\mu 1$.

X-rays in June 1963 revealed a gastric ulcer, presumably benign, which did not heal and prednisone was discontinued in August 1963.

A splenectomy was performed on 26 March, 1965 along with a partial (50 per cent) gastrectomy with a Billroth I anastomosis. Sections of the spleen showed evidence of extramedullary hematopoiesis and chronic congestion. His platelets fluctuated postoperatively between $35,000/\mu 1$ and $50,000/\mu 1$.

The patient did not return until April, 1969 when he continued to note chronic fatigue, but no bruising or bleeding.

Laboratory analyses were normal except for the platelet count which was 58,000/ μ l and his WBC which was 12,300/ μ l. A repeat bone marrow was histologically normal except for numerous clumps of giant platelets.

RESULTS

Chromosomal analyses done on four marrow samples from Case 1, summarized in Table 1, reveal a consistent change in the karyotype over 14 months. The first marrow in July 1969 contained less than 15 per cent normal diploid cells. The remainder were hypodiploid with a modal chromosome number of 45. Although the Y chromosome in the diploid cells could not be positively identified by its morphology, its presence could be confirmed by quinacrine fluorescence which demonstrated unequivocally that the small chromosome missing from the hypodiploid cells was the Y (Figure 1). The vast majority of cells with 43 and 44 chromosomes also lacked a Y chromosome. The second marrow sample five months later showed a remarkable change in that the modal chromosome number was now 46 and these cells had a normal karyotype. This normalization of the karyotype was confirmed by the third sample in February, 1970 when only 13 per cent of cells had 45 chromosomes and only one-half of these were 45,X.

Chromosomal analysis of the spleen, removed one week later, yielded only one 45,X cell of a total of 39 counted. The last marrow analysis in September, 1970 revealed not a single 45,X cell in 70 cells counted. A normal 46,XY karyotype was obtained from the cultured peripheral lymphocytes, studied two months after the first marrow. The correlation of the chromosomal findings with clinical events is summarized in Figure 2.

Case 2 had two marrow examinations two years apart. In the first sample, although 40 per cent of the cells were hypodiploid, with 25 per cent having 45 chromosomes, this condition appears to represent random loss as can be seen in Table 1. Analysis of cells with 45 chromosomes showed that five cells lacking a Y (confirmed by quinacrine fluorescence) were observed out of 16 karyotyped. A brightly fluorescent Y was observed in diploid cells. Results from the second sample were much more clearcut. Slightly more than 50 per cent of the cells were hypodiploid, and 49 per cent of all cells appeared to be G or Y minus; nine of these cells were scored for quinacrine fluorescence and all nine were 45,X. This suggested that most, if not all, of the 43 cells lacking a small acrocentric chromosome were Y minus, and implied, in turn, that the 45,X line comprised about 40 per cent of all mitotic cells in the sample. This compares with five out of 68 (7 per cent) in the first specimen. No 45,X cells were found in a peripheral blood culture obtained about three weeks after the second marrow sample.

Only one marrow sample was received for chromosomal analysis from Case 3 (Table 1). Ten per cent of the cells were 46,XY, whereas 75 per cent of them had 45 chromosomes (Figure 3) and 85 per cent of these were 45,X (confirmed by fluorescence). Results of the chromosomal analysis of cultured peripheral blood obtained five months later were difficult to interpret because of the high percentage of hypodiploid cells. Cells with 45 chromosomes constituted 18 per cent of the total counted, and half of the analyzed cells with 45 chromosomes were 45,X. These may reflect the presence of immature myeloid cells in the peripheral blood, or the loss of the Y chromosome noted by Jacobs & Court Brown¹⁶ in elderly males, or coincidental random loss.

Table 1

CHROMOSOMAL ANALYSIS OF CELLS OF THREE CASES

Source	Date	Total*	Chromosomal count					Tetra- ploid	Analysis of cells with 45 chromosomes								
			43	44	45	46	>46		A	B	C	D	E	F	G	Y	G/Y†
Case 1																	
Marrow	7.8.69	77(62)	8(4)	13(10)	42(40)	11(7)		3(0)				1	1	2	1	23	11
Blood	26.9.69	49(24)		4(4)	8(8)	37(12)					5	1	1		1		
Marrow	12.5.69	79(45)	5(4)	3(3)	17(17)	54(21)					3		2	1	2	7	
Marrow	23.2.70	256(71)	5(3)	13(10)	32(30)	194(26)	1(1)	11(1)			2	2	2	2	1	12	4
Spleen	3.3.70	39(29)	2(2)	2(2)	3(3)	32(22)					1		1			1	
Marrow	14.9.70	70(21)		4(4)	3(3)	63(14)				1	1		1				
Case 2																	
Marrow	1.4.68	68(34)	5(2)	6(4)	17(16)	39(11)		1(1)			3	1	2	1	2	5	
Marrow	27.1.70	105(56)		6(5)	51(31)	48(20)				1	1	1	2	1		9	15
Blood	16.2.70	50(24)		4(4)	8(8)	37(11)	1(1)				3		2	2	1		
Case 3																	
Marrow	10.4.69	65(49)		7(7)	51(36)	7(6)							1	1	3	29	
Blood	9.9.69	70(20)	3(1)	10(3)	13(6)	44(10)					1		1			3	

* Numbers in () indicates number photographed.

† Fluorescence not done.

Figure 1. (Upper) A cell in metaphase, prepared from the first marrow sample from Case 1. It contained four G chromosomes (†) and the Y chromosome (‡). The cell was stained first with Leishman-Giemsa and was photographed with bright field and then was restained with quinacrine dihydrochloride and was photographed with a UV fluorescent microscope (right). The distal portion of the long arm of the Y shows the typical bright fluorescence.

(Lower) A cell in metaphase, prepared from the first marrow aspiration, which contained four G chromosomes (†), stained as above. The brightly fluorescent Y is absent.

Figure 2. The relationship of the abnormal clone (45,X) to blood levels in Case 1. Androgen 1 is Halotestin and Androgen 2 is oxymethalone.

DISCUSSION

The three males presented in this paper have a mosaic karyotype 45,X/46,XY in a variable proportion of sampled marrow cells. Case 1 appears to have lost his abnormal clone in the 14-month observation period; the abnormal clone has become more prominent in Case 2; no statement regarding change is possible in Case 3. With the possible exception of Case 3, 45,X cells do not appear in the peripheral blood.

Cases 1 and 2 showed no obvious correlation between the degree of aneuploidy of marrow cells and their hematologic status. Thus Case 1 had his lowest platelet counts in January to March, 1970, when his marrow contained less than 10 per cent Y minus cells. The increase in hemoglobin at this time probably was due to the transfusion of packed RBC in December. The WBC, however, did show an increase with decreasing aneuploidy. The hematologic profile returned to normal only after splenectomy. In Case 2, only the platelet counts changed between the two marrow examinations, their numbers more than doubling during the period in which the percentage of Y-minus cells rose from eight to 40 per cent. This experience contrasts with that of Lawler¹⁷ who found a correlation between the blood level of Hb and the percentage of Y-minus cells in a polycythemic male.

These males all had some degree, often severe, of thrombocytopenia. In addition, Case 1 had evidence of anemia, either iron deficient or aplastic, and Case 2 had erythrocytosis and leukocytosis with eosinophilia in addition to the presence of an anticomplementary factor. It is interesting to note that all three had a history of peptic ulcer, as did the case reported by Goodall & Robertson.¹⁸

Figure 3. A cell in metaphase from Case 3. It contains 45 chromosomes including four G chromosomes (†) and lacks a Y chromosome. It is stained as in Figure 1.

The cause of the hematologic problems in these patients is obscure. It seems plausible to attribute some or all of the pancytopenia in Case 1 to chloramphenicol although his PCV was 32 per cent before he took the drug. Alternatively, he may have had idiopathic thrombocytopenia

Table 2

REPORTED CASES OF MALES LACKING A SMALL ACROCENTRIC CHROMOSOME IN BONE MARROW CELLS

Total no.	Total males	Age range	Disease	Abnormal males	Age	Hematologic diagnosis	Chromosome count				Comment	Reference	
							Total	45	Analysis				
9	>2	?	Pernicious anemia	1	?	Pernicious anemia	48§	13	8,	G-		Kiossoglou ¹⁹	
9	3	67-84	Sideroblastic anemia	1	68	Sideroblastic anemia	100§	89	Y-		Duodenal ulcer; died after 3 yrs. No transformation of anemia.	Goodall ¹⁸	
16	9	Mean age 60	Myeloproliferative	1	78	Thrombocythemia	57§	"most"	G-,	Cg-	G.I. bleeding; site not clear of sepsis. Died 5 yrs. later.	Engel ²⁰	
52	?	?	Various myelodysplasias	1	72	Anemia	54	17	11,	Y-	Refused treatment and died in 6 months.	Berry ²⁴	
13	6	8-61	Aplastic anemia	2	13	Pancytopenia	50§	9	9,	Y-	Died from cerebral hemorrhage 2 years later. Clinically improved.	Sakuri ²²	
					61	Pancytopenia	60§ 49§	23 9	23, 9,	Y- ¹ Y- ²			
Single case			Carcinoma larynx	1	69	Small A spike	37§	27	27,	Y-		Reported by Takatsuki ²¹	
32	?	20-87	Various myelodysplasias	1	62	Leukocytosis	?		70%	G-	Well 3 years later	Nowell ²³	
88	36 52	<60 >60	Various myelodysplasias & leukemia	0 4†								Died unexpectedly 1 month later. No clinical change 1 year later. Same result 4 months later.	Pierre ⁵
					77	Anemia	25	20	19,	Y-			
					89	Anemia	25	23	23,	Y-			
					86	Refractory anemia	25§	24	20,	Y-			
				60	Pernicious anemia	25§	11	11,	Y-				
Single case			Abdominal carcinoma	1	68	Normal	100§	54	All	G-	Died 4 weeks later.	Long & Pierre ²⁷	

Table 2 (continued)

Total no.	Total males	Age range	Disease	Abnormal males	Age	Hematologic diagnosis	Chromosome count				Comment	Reference
							Total	45	Analysis			
32	12	45-69	Bronchogenic carcinoma	3	60	Mild leuko- cytosis	50\$	12	11,	Y-	Died postoperatively. Eronchitis	O'Riordan ⁴
					64	Myeloid hyper- plasia	50\$	12	3,Y;	8,G/Y-		
					69	Normal	50	12	12,	G/Y		
	20	19-78	"Controls"	1	77	Normal	50\$	10	5,Y-;	2,G/Y-		
Single case			Squamous carcinoma	1	60	Pernicious anemia	50\$	10*	10,Y-			
79	33	48-82	Various carcinomas	2	73	Normal	50	7	7	G/Y-		Secker- Walker ⁶
					73	Normal	50	12	12	G/Y-		
100	57	20-79	Various myelodys- plasias	3	55	Thrombo- cytopenia	77\$	42	>23,	Y-	See Table 1.	Present report
					62	Thrombo- cytopenia & erythrocytosis	68\$	17	5,	Y-		
					74	Thrombocyto- penia	65\$	51	>29,	Y-		
	37 6	8-79 20-74	Secondary Control	0 0								

¹ January 1965.² August 1966.

† Does not include one patient with leukemia and another with lymphoma.

* Patient mosaic 46 X, mar +/-47, XY, mar +.

§ Peripheral blood 46, XY.

‡ First analysis only.

with occult gastrointestinal bleeding which resulted in an iron deficiency anemia. The clinical situation appears no clearer in Case 2 whose erythrocytosis has not been satisfactorily explained, except that values for whole blood volume and red blood cell mass are within the normal range, which makes a diagnosis of polycythemia vera less likely. No cause for the persistent eosinophilia or thrombocytopenia has been found. In Case 3, x-ray therapy appeared to precipitate the fall in platelets. The bone marrow findings and the rise in platelet count after the head was shaved implicate the hair dye. This case is classified as idiopathic thrombocytopenia because of the persistence of the thrombocytopenia after prednisone and splenectomy, and the normal platelet function tests.

The patients' response to treatment has also been dissimilar; all were treated with prednisone for varying periods ranging from two weeks to 10 months with dosages of from 20 to 60 mg/day. Case 2, with the lowest dose and shortest treatment period, is the only one to show improvement. Case 1 was also treated with androgens, including oxymethalone, with no clinically apparent improvement. Whether the improvement in the marrow chromosomal pattern noted in December 1969, is a result of oxymethalone begun in August, is an unanswered question. Cases 1 and 3 underwent splenectomy which appears to be a major factor in the clinical improvement of Case 1, although his chromosomal pattern showed a marked trend towards normality prior to splenectomy.

Is the presence of a Y minus cell line causally related to the patient's hematologic abnormality? There are at least three possible answers:

- 1) Males with hematological problems and a 45,X karyotype occur with higher than expected frequency;
- 2) Males with 45,X are found in various populations not associated with marrow dysfunction;
- 3) Males with 45,X are older and therefore loss of the Y chromosome is only, or primarily an aging phenomenon. It might then be asked: does loss of the Y carry an increased hazard of neoplasia?

Data from the present series would indicate that Y minus individuals are found with higher frequency in males with hematologic problems, since no males with 45,X cells were observed among the 43 patients without primary hematologic disease. Single cases of males, some of whose marrow cells lacked a small acrocentric chromosome, have been reported in a variety of hematologic diseases.¹⁸⁻²⁴ These cases are summarized in Table 2. In some instances, the missing chromosome appeared to be the Y, in others a G21-22, and in still other cases its identity was uncertain. The fluorescent technique clearly will be a very important tool for precise identification of the chromosome involved in these abnormalities.

In addition to the present report, more extensive surveys of patients, with and without hematologic diseases have been carried out. Pierre and Hoagland⁵ examined the marrow chromosomes of 88 males with various hematologic abnormalities including leukemia. They found no males with 45,X cells among 36 patients under 60 years, whereas they found four males with 45,X cells among 52 patients over 60 years of age. (Two males with Y minus cells who had leukemia and lymphoma have been excluded.) O'Riordan et al⁴ examined cells from 32 males and found three of 12 patients with bronchogenic carcinoma and one of 20 "controls" had about 25 per cent 45,X cells in their marrow. These four males were all over 60 years of age.

A more recent survey by Secker-Walker⁶ examined 39 hematologically normal males, most

of whom were hospitalized for carcinoma. Two 73-year-old males with carcinoma of the bladder were observed to have lost a small acrocentric chromosome in approximately 15 and 25 per cent of their marrow cells. Thus the loss of the Y chromosome appears to be much more frequent in older males. The fact that the percentage of 46,XY cells can increase is evidence that the loss of the Y is not, at least in some circumstances, an irreversible phenomenon.

Not all investigators have observed males with a Y minus cell line among their patients. Nava²⁵ studied marrow chromosomes in 27 males with dysplasias excluding leukemia or polycythemia, age 30 to 78, with 14 of them over 60 years. She did not identify any as 45,X/46,XY mosaics, although some of the patients had a rather high percentage of hypodiploid cells. Jensen²⁶ examined 41 patients of both sexes with various myelodysplasias without observing any such males. There is a need to clarify the frequency of aneuploidy in marrow cells of older patients with and without hematologic disorders. The use of males with carcinoma as a "control" population however may introduce serious bias. A survey of older males who are hospitalized for problems other than neoplasia is currently underway in our laboratory.

Does the loss of a Y chromosome in marrow cells carry any special hazard for patients with hematological disorders? Our Case 2 has been observed for three years. Admittedly in the first examination only a small percentage of Y minus cells were observed. The increase in the percentage of Y minus cells however does not seem to have been accompanied by any obviously deleterious effects. None of the cases reported in the literature showed any evidence of leukemic transformation even when observed for periods up to five years.

The percentage of aneuploid cells in patients with hematological disorders was quite variable, ranging from 15 to 90 per cent. In the majority of these patients, more than one-half of their cells showed a 45,X karyotype on at least one occasion. Thus, there seems to be no correlation between the degree of aneuploidy and clinical course. The instability of the degree of aneuploidy must be considered since most of the patients who had more than one marrow sample examined for chromosomes showed a change in the percentage of aneuploid cells. There is, at present, no clear evidence that loss of the Y chromosome from some marrow cells in this group of patients carries an unfavorable prognosis. This conclusion must be very tentative, since it is based on relatively few long-term observations.

The prognosis for males with aneuploidy and carcinoma is related to the latter disease. The presence in many patients, of a Y minus line in some bone marrow cells and carcinoma in another site may reflect the coincidental occurrence of two age-related phenomena in one individual. Since the sequence of occurrence of chromosomal mosaicism and the carcinoma is unknown at the present time, it is fruitless to speculate on whether an individual with a 45,X cell line is more susceptible to neoplasia, or conversely, whether some factor in males with carcinoma favors the development of a 45,X cell line.

ACKNOWLEDGMENTS

I am grateful for the cooperation of Drs. Stanley Yachnin, Joseph Baron, and John Ultmann of the Hematology Section, Department of Medicine, University of Chicago. I would also like to thank Miss Judith Mikuta and Mr. David Potter for their technical assistance.

LITERATURE CITED

1. Rowley, J. D. Myeloproliferative Disorders of Animals and Man. Proceedings of the Eighth Annual Hanford Biology Symposium at Richland, Washington. AEC Symposium No. 19, 1968, p. 556.
2. Rowley, J. D., R. K. Blaisdell, and L. O. Jacobson. *Blood*, 27:782, 1966.
3. Rowley, J. D. *Heredity*, 26, in press, 1971.
4. O'Riordan, M. L., E. W. Berry, and I. M. Tough. *Brit. J. Haematol.*, 19:83, 1970.
5. Pierre, R. V., and H. C. Hoagland. *Mayo Clinic Proceedings*, 46:52, 1971.
6. Secker-Walker, L. *Brit. J. Haematol.* In press. 1971.
7. Zech, L. *Exptl. Cell Res.*, 58:463, 1969.
8. Pearson, P. L., M. Bobrow, and C. G. Vosa. *Nature*, 226:78, 1970.
9. George, K. P. *Nature*, 226:80, 1970.
10. Tjio, J. H., and J. Whang. *Stain Technology*, 37:17, 1962.
11. Moorhead, P. S., P. C. Nowell, W. J. Mellman, D. M. Battips, and D. A. Hungerford. *Exptl. Cell Res.*, 20:613, 1960.
12. Balis, B. E. Personal communication.
13. Caspersson, T., L. Zech, C. Johansson, and E. J. Modest. *Chromosoma*, 30:215, 1970.
14. Rowley, J. D., D. Potter, and J. Mikuta. *Stain Technology*. 1971.
15. Gerwurz, H., J. Baron, O. D. Ratnoff, and S. Yachnin. *J. Clin. Invest.*, 49:33a, 1970.
16. Jacobs, P. A., and W. M. Court Brown. *Nature*, 212:823, 1966.
17. Lawler, S. D. Chromosomes Today, 3. Oliver & Boyd, in press.
18. Goodall, H. B., and J. Robertson. Myeloproliferative Disorders of Animals and Man. Proceedings of the Eighth Annual Hanford Biology Symposium at Richland, Washington. AEC Symposium No. 19, 1968, p. 253.
19. Kiossoglou, K. A., W. J. Mitus, and W. Dameshek. *Blood*, 25:662, 1965.
20. Engel, E., L. C. McKee, and M. E. Montmollin. *L'Union Médicale du Canada*, 97:901, 1968.
21. Takatsuki, N. *Acta Haematologica Japonica*, 31:636, 1968.
22. Sakurai, M. *Acta Haematologica Japonica*, 33:127, 1970.
23. Nowell, P. C. *Cancer*, 1971, in press.
24. Berry, E. W. Personal communication.
25. Nava, C. de. Monographics de Annales de Genetique, 1969, p. 47.
26. Jensen, M. K. *Acta Medica Scandinavica*, 183:535, 1968.
27. Long, D. L., and R. V. Pierre. *Amer. J. Clin. Path.*, 50:482, 1968.

POTENTIATION OF PHYTOHEMAGGLUTININ-INDUCED LYMPHOCYTE
TRANSFORMATION BY CELL-CELL INTERACTION;
A MATRIX HYPOTHESIS*

By

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Lymphocyte transformation or "derepression" may be classified into three categories. The first category includes those instances of lymphocyte activation which are restricted, in that they depend upon the selective recognition by specific lymphocytes of antigen by means of antibody-like molecules located on the cell surface.¹ A second category of lymphocyte activation involves antibodies directed against surface determinants of the lymphocyte membrane such as anti-lymphocyte serum,² anti-immunoglobulin sera,^{3,4} and, as a special instance of the latter, anti-allotype sera.⁵ Although these materials are immunologically specific they are not generally recognized as playing a role in the initiation or specificity of the immune response in the intact organism. A third category of lymphocyte activation depends upon the reaction of surface structures of the lymphocyte with transforming agents that lack immunological specificity. These agents include the phytomitogens such as phytohemagglutinin,⁶ Concanavalin A,⁷ and poke-weed mitogen,⁸ certain bacterial products,^{9,10} and simple inorganic salts such as mercuric chloride.¹¹ Although in the last analysis the specific activation of lymphocytes by antigen must remain central to a general conceptual formulation of the initiation and maintenance of the immune response, the other instances of lymphocyte transformation are useful as models for the study of the mechanism whereby signals arising at the lymphocyte cell surface lead to profound changes in metabolic and mitotic behavior.

Purification of a commercially available phytohemagglutinin derived from Phaseolus vulgaris (PHAP) yields two distinct mitogenic proteins.¹² These have been designated high-titer hemagglutinating PHAP (H-PHAP) and low-titer hemagglutinating PHAP (L-PHAP). Both are potent mitogens and are capable of leuko- and lympho-agglutination. H-PHAP is a much more potent red cell agglutinin than L-PHAP and is also capable of producing mixed red cell lymphocyte agglutination at very low concentrations. In highly purified lymphocyte preparations devoid of red cells, the transformation of lymphocytes by H-PHAP is markedly potentiated by the addition of autologous red blood cells (RBC), whereas the mitogenic potency of L-PHAP is unaffected. The mitogenic activities of both H- and L-PHAP are potentiated by autologous platelets or autologous mitotically inert X-irradiated lymphocytes. Preliminary reports of some of these findings have been presented,¹²⁻¹⁵ and have been confirmed by the work of Tärnvik.¹⁶

MATERIALS AND METHODS

L- and H-PHAP were isolated from Bacto-phytohemagglutinin P (Difco Laboratories, Detroit, Mich., Cat. No. 311D-57) as previously described.¹²

All experiments were performed with human peripheral blood lymphocytes, RBC and plate-

*This report is taken from a paper that appeared in Cellular Immunology, 3:569, 1972.

lets. Pure lymphocytes entirely free of RBC or RBC stroma, and containing 50-100 platelets/100 lymphocytes were obtained by gravity sedimentation of heparinized whole blood, nylon column passage, osmotic lysis of red blood cells, and centrifugation through a plasma-saline gradient.^{12,17}

RBC were prepared by washing the gravity-sedimented RBC four times in phosphate buffered saline, pH 7.4 (PBS), with careful removal of the buffy coat, followed by suspension at the desired cell concentration in Eagle's Minimal Essential Medium (MEM), Spinner modified, containing penicillin and streptomycin (Baltimore Biological Corporation, Cat. No. 71-016R). Red cell ghosts were prepared by hypotonic lysis of washed red blood cells in phosphate buffer (20 mOSm, pH 6.8) as described by Dodge et al.¹⁸ The final step of this procedure (suspension of ghosts in 0.1 N KOH) was omitted, and the RBC stroma was suspended in MEM for enumeration and addition to lymphocyte cultures. The supernatant stroma-free hemolysate derived from centrifugation of the hypotonic RBC lysate was diluted with 9 volumes of MEM for use in lymphocyte transformation experiments. The amount of hemolysate material utilized in any transformation experiment was expressed in terms of the number of intact RBC from which it was derived. RBC ghosts labeled with diisopropylfluorophosphate (DF^{32}P) (Amersham-Searle Corp.) were prepared by incubating 2 ml of packed washed RBC suspended to a hematocrit of 50 per cent in saline with 2.2 μg DF^{32}P (specific activity 400 $\mu\text{Ci}/\text{mg}$) for 1 hr at 37° C.¹⁹ Under these conditions, 90 per cent of the added label was taken up by the RBC, of which 20 per cent was residually bound to stroma after hypotonic lysis.

Human platelets were prepared from Na EDTA anticoagulated blood (0.3 ml 0.15 M Na_3EDTA per 10 ml blood) utilizing silicone treated glassware. The blood was spun at 200 g for 10 min, and the platelet-rich plasma was aspirated. The residual RBC were washed once with PBS under similar conditions, and both the supernatant and the platelet-rich plasma were again similarly centrifuged to remove contaminating RBC and white cells. The platelet-containing supernatants were now centrifuged at 1,500 g for 20 min, and the sedimented platelets were suspended in 4 ml of Na_3EDTA -PBS.¹⁷ One and three-tenths milliliter of this platelet suspension was layered carefully over 1 ml EDTA plasma in each of three 100 x 7 mm (I.D.) tubes and centrifuged at 100 g for 15 min. The platelets remaining above the plasma-saline interface were aspirated, pooled, and suspended in MEM. These procedures ensured that no RBC or white blood cells contaminated the platelet preparations.

White cells, RBC, and platelets were counted in standard hematocytometer chambers. RBC ghosts were counted by similar methods using phase microscopy or by a Coulter counter. The purity and homogeneity of lymphocyte preparations were checked by Wright-stained smears of the final lymphocyte preparations.

The methods used for culturing lymphocytes and determining their incorporation of 2- ^{14}C -thymidine into DNA have already been described.¹² Between $1.5\text{--}2 \times 10^6$ lymphocytes were cultured in 4 ml glutamine-fortified MEM containing 12.5 per cent autologous cell-free heparinized plasma. After 72 or 96 hours (chosen for convenience after it was established that the phenomena under study were not significantly altered by time), 0.1 μCi ^{14}C -thymidine was added, the cells were gently suspended, and the culture was continued for 16 hr longer. The cells were then harvested and their DNA was isolated and counted. Experiments were done in duplicate or triplicate; the amount of DNA synthesized in unstimulated cultures (usually less than 100 cpm) was subtracted from mitogen-stimulated cultures to achieve a measure of the lymphocyte transfor-

mation induced by mitogen. Mean values are given.

In assays of the effects of RBC or platelets on lymphocyte transformation, 3 ml of a lymphocyte suspension in MEM and plasma were added to each culture tube, followed by 1 ml of MEM or MEM containing the desired number of particles. The tubes were gently swirled and the appropriate dose of mitogen was added to each tube in volumes ranging from 2-100 μ l. The addition of RBC (or their derivatives) or platelets to unstimulated lymphocyte cultures had no significant effect upon baseline 2- 14 C-thymidine incorporation nor did these materials incorporate significant amounts of 2- 14 C-thymidine when cultured alone with or without PHAP.

The potentiation of lymphocyte transformation by RBC (or platelets) was expressed according to the following formula where CPM represents the amount of 2- 14 C-thymidine incorporated into lymphocyte DNA under the influence of mitogen (after subtraction of the amount incorporated into the control, unstimulated lymphocytes):

$$\text{Stimulation index (SI)} = \frac{\text{CPM} + \text{RBC (or platelets)}}{\text{CPM} - \text{RBC (or platelets)}} \times 100$$

The statistical significance of the stimulation indices was assessed by computing the significance of the difference between means using a two sample t test employing the Welch procedure for cases of unequal variance, with the aid of the University of Chicago 7094 IBM computer.

Both L- and H-PHAP were labeled with 125 I by the method of McConahey and Dixon.²⁰ Excess unbound 125 I was removed by passage over Sephadex G25 and the fractions containing 125 I-PHAP were concentrated by pressure ultrafiltration (Amicon Corporation, Lexington, Mass., filter UM-10). Radioiodinated PHAP retained its mitogenic activity, and, in the case of 125 I-H-PHAP, its hemagglutinating property as well. These labeled materials were incubated together with RBC, platelets and lymphocytes to study the binding capacity of each formed element for the two classes of PHAP. Incubation of graded numbers of formed elements with 125 I-PHAP was done at room temperature in 2 ml of PBS for 30' followed by three washings with diluent. The cell button was then dissolved in 2 ml 1 per cent Triton X-100 in PBS and the amount of PHAP remaining was determined by counting in a NaI crystal gamma ray scintillation counter. A small amount of 125 I-PHAP was found to adhere to the glass test tube walls, and the appropriate correction was applied.

In addition to our previously published methods of studying H- and L-PHAP-induced lymphocyte and red cell agglutination,¹² the following technique was used to study the same phenomena as well as the ability of both mitogens to cause mixed lymphocyte-RBC agglutination. A suspension of purified lymphocytes and autologous RBC in MEM containing 12.5 per cent autologous plasma was prepared. The RBC:lymphocyte ratio was 4:1 (6×10^6 RBC/ml, 1.5×10^6 lymphocytes/ml). Two-tenths ml of this cell suspension were added to 0.2 ml dilutions of both H- and L-PHAP in PBS, mixed, and allowed to stand at 25° C for 2 hr. The cell suspensions were then gently dispersed with a Pasteur pipette, and wet preparations were examined under phase microscopy for the presence of RBC clumps, lymphocyte clumps, and mixed lymphocyte RBC agglutinates. Selected samples were photographed after the addition of supravital dye (pinacyanol chloride) using bright field optics.

Lymphocytes were irradiated in vitro suspended in MEM + plasma in plastic tissue culture test tubes using a Maxitron 250 x-ray unit (General Electric) with the following specifications:

Filtration - 0.25 mm Cu + 1 mm Al, target distance 41 cm, 250 kvp, 30 ma, dose rate 290 R/min.

^{14}C and ^{32}P were counted in a Packard Tri-Carb Liquid Scintillation spectrometer (Model No. 3375). ^{125}I was counted in a Nuclear Chicago NaI crystal gamma ray scintillation counter. When two isotopes were present in the same sample, conditions of counting were arranged so that each was monitored free of interference from the other. The presence of RBC in $2\text{-}^{14}\text{C}$ -thymidine incorporation studies introduced no quenching or discernible effect upon the efficiency of counting. The counting error was 1 per cent or less in all experiments save those with DF ^{32}P RBC ghosts, where it was less than 5 per cent.

RESULTS

Interaction of H- and L-PHAP with formed elements of human peripheral blood. The serologic and mitogenic potencies of L- and H-PHAP have been reported.¹² H-PHAP is 200 times as potent a hemagglutinin, while L-PHAP is 10-30 times more potent as a leukoagglutinin, and is also the more potent mitogen. These same attributes emerge when the two mitogens are compared as agglutinins in a mixed suspension of RBC and lymphocytes (4 RBC/lymphocyte) (Table 1). Here, however, a further difference emerges; H-PHAP is by far superior in its ability to cause mixed agglutination of RBC and lymphocytes. In addition, the mixed agglutinates formed by L-PHAP at 81 and 40 $\mu\text{g/ml}$ consisted largely of lymphocytes, with only a few RBC inter-

Table 1

CELLULAR AGGLUTINATES FORMED IN A MIXED RBC-LYMPHOCYTE (4:1 RATIO) SUSPENSION BY L- AND H-PHAP

PHAP	Concentration of PHAP ($\mu\text{g/ml}$)	RBC clumps	Lymphocyte clumps	Mixed lymphocyte:RBC clumps
L-PHAP	81	0	4+	3+
	40	0	4+	2+
	20	0	4+	0
	10	0	3+	0
	5	0	3+	0
	2.5	0	2+	0
H-PHAP	71	4+	3+	4+
	36	4+	2+	4+
	18	4+	0	4+
	9	4+	0	4+
	4.5	4+	0	4+
	2.25	4+	0	4+
	1.1	3+	0	3+
	0.55	3+	0	3+
	0.3	1+	0	1+
	0.15	0	0	0

dispersed, while those mixed agglutinates formed by H-PHAP at concentrations as low as 2.25 $\mu\text{g/ml}$ were predominantly composed of many RBC interspersed with fewer lymphocytes (Figure 1). Since the concentrations of H-PHAP required for maximal lymphocyte transformation in our standard culture were far below that required to evoke gross lymphocyte agglutination, while optimum concentrations of L-PHAP required for lymphocyte transformation were well below those needed to evoke erythroagglutination or mixed agglutination, the two mitogens could be distinguished in the culture tubes with the naked eye after 24 hr of stationary culture. When lymphocytes were cultured alone, in the absence of mitogen or in the presence of H-PHAP, they formed a discrete button at the bottom of the culture tube. In the presence of L-PHAP they formed a carpet of cells. When lymphocytes and several times their number of RBC were cultured together in the absence of mitogen, both cell types formed discrete buttons at the bottom of the culture tubes, the lymphocytes overlying the RBC. In the presence of L-PHAP, lymphocytes were dispersed as a carpet, with the RBC button undisturbed in its center, while in the presence of H-PHAP, the cells formed a homogeneous carpet in which the naked eye could no longer distinguish discrete lymphocyte clumps.

The ability of ^{125}I -L- and H-PHAP to bind to lymphocytes is shown in Table 2. Paradoxically, despite its poor potency as a lymphoagglutinin, lymphocytes are able to bind a substantial amount of H-PHAP, and bind 2-3 times more H-PHAP than L-PHAP. When the ability of RBC and platelets to bind ^{125}I -L- and H-PHAP is examined, the results are less contradictory. H-PHAP binds almost completely to RBC, while RBC binding of L-PHAP amounts to less than 2 per cent. Platelets bind substantial amounts of both mitogens, with H-PHAP displaying the greater affinity (Figure 2). The mitogenic capacity of H-PHAP is almost totally removed by prior RBC adsorption, while the mitogenic capacity of L-PHAP is unaffected by prior exposure to RBC (Table 3).

Potential of lymphocyte transformation by red blood cells. During our early studies on comparative mitogenic potency, we noted that although L-PHAP was a more potent mitogen in the lower regions of the dose response curve, the plateau value of peak response was always higher with H-PHAP. In view of the marked interaction of H-PHAP with RBC and its potency in mixed lymphocyte-erythrocyte agglutination, and since our early experiments were done with lymphocytes contaminated with 3-5 times their number of erythrocytes, we decided to investigate the effect of RBC on lymphocyte transformation.

Figure 3 illustrates the transformation of lymphocytes by varying doses of mitogen with and without the addition of 20 million RBC. The presence of RBC does not substantially affect lymphocyte transformation when L-PHAP is the mitogen. The ability of H-PHAP to stimulate DNA synthesis in lymphocytes, however, is markedly enhanced by the presence of RBC. This effect is most marked at low and intermediate points on the dose response curve, and diminishes as the plateau of stimulation is approached. Figure 4 illustrates the effect of varying numbers of RBC on the transformation of lymphocytes in the presence of a constant amount of mitogen, which was calculated to produce from one-third to one-fourth of the peak response obtainable in the absence of RBC. Here again the presence of RBC did not alter the ability of L-PHAP to transform lymphocytes, although a RBC/lymphocyte ratio of as low as 1:1 increased lymphocyte transformation by almost 300 per cent when H-PHAP was employed. The plateau of stimulation by RBC was approached at a RBC/lymphocyte ratio of 10:1. In other experiments, as few as one red cell for every 10 lymphocytes resulted in a twofold increase in lymphocyte transformation

Figure 1. Mixed agglutination of RBC and lymphocytes (4:1) following 2-hr incubation with H-PHAP at a concentration of $2.25 \mu\text{g/ml}$. Bright field microphotograph, X400. (A) Two lymphocytes in mixed agglutination with RBC; (B) A large clump showing mixed RBC-lymphocyte agglutination.

Table 2
ADSORPTION OF ^{125}I -H- AND L-PHAP BY PURIFIED
HUMAN LYMPHOCYTES

PHAP	Concentration ($\mu\text{g}/\text{ml}$)	Number of lymphocytes/ml ($\times 10^{-6}$)	Adsorbed (%)
H-PHAP	2.36	6.3	26.1
	2.36	12.6	34.0
	2.36	18.9	38.7
L-PHAP	2.16	6.3	7.9
	2.16	12.6	13.2
	2.16	18.9	17.3

Figure 2. Uptake of ^{125}I -H- and L-PHAP by RBC and platelets. The concentration of ^{125}I -H-PHAP was $1.3 \mu\text{g}/\text{ml}$ and that of ^{125}I -L-PHAP was $1.8 \mu\text{g}/\text{ml}$.

by H-PHAP, while as many as 100 RBC/lymphocyte did not cease to be highly stimulatory, albeit less so than a more optimum RBC/lymphocyte ratio (Table 4).

The density of lymphocytes in tissue culture is known to affect their responsiveness to PHAP.²¹ In order to determine the effect of lymphocyte density on the RBC potentiation of lymphocyte transformation, we cultured lymphocytes over an eightfold density range with H-PHAP and RBC, testing both a constant number of RBC (20×10^6) and a constant RBC/lymphocyte ratio (10:1) with each lymphocyte density (Table 5). In both instances potentiation of lymphocyte transformation by red blood cells was achieved over the entire lymphocyte density range, although the effect was most marked in each case at low lymphocyte densities, and diminished as higher

Table 3

ADSORPTION OF MITOGENIC ACTIVITY OF PHAP BY RED BLOOD CELLS*

PHAP type	Packed RBC added (ml)	^{14}C -Thymidine incorporation (CPM)	Inhibition (%)
L-PHAP [†]		23,925	
	0.05	23,643	1
	0.10	23,377	2.3
H-PHAP [‡]		38,396	
	0.05	2,163	95.7
	0.1	1,182	98.3

* Following adsorption of mitogen in 0.5 ml PBS with the indicated volume of RBC for 30' at 25°, the red cells were removed by centrifugation and the supernatant assayed for mitogenic activity in the usual manner.

[†] 21 µg.

[‡] 17 µg.

Figure 3. Potentiation of PHAP-induced lymphocyte transformation by RBC. (A) 2×10^6 lymphocytes and 20×10^6 RBC were cultured together with varying doses of L- or H-PHAP; (B) The data presented in (A) are expressed in terms of the stimulation index at each mitogen dose. All points on the H-PHAP stimulation index curve with mitogen added have a $p < 0.05$.

lymphocyte densities were achieved.

To determine whether the potentiation of lymphocyte transformation by RBC could result from an alteration in the induction period and time course of DNA synthesis we studied the incorporation of ^{14}C -thymidine into H-PHAP-stimulated lymphocyte DNA at 24-hour intervals

Figure 4. Potentiation of lymphocyte transformation by RBC. In this experiment the dose of mitogen was kept constant, while 2×10^6 lymphocytes were cultured with varying numbers of autologous RBC. All points on the H-PHAP stimulation index curve with RBC added have a $p < 0.05$.

over a four-day period (Table 6). RBC potentiation of DNA synthesis was noted at each interval where significant incorporation of label occurred; in one experiment the presence of RBC resulted in the largest potentiation at an early time (48-52 hr) while in the other, potentiation was greatest at a later period (96-100 hr). In one of these experiments the time course of DNA synthesis in the RBC-potentiated cultures was similar to that achieved by a 7.5-fold increment in H-PHAP dosage without the addition of RBC.

As shown in Table 7, the ability of RBC to potentiate the transformation of lymphocytes by H-PHAP was limited to RBC ghosts derived from osmotic lysis of RBC; stroma-free hemolysate displayed no potentiating capacity. It is noteworthy that the potentiation efficiency of RBC stromal particles was only 1/40 that of intact RBC, since 84 million stromal particles were required to achieve the same potentiation as two million intact RBC. Studies with DF³²P-labeled RBC stroma, however, disclosed that a maximum of 1/12 of the added stromal particles were "sedimentable" and therefore capable of attachment to, or interaction with, the lymphocytes at the bottom of the culture tube; the remainder were found free in the supernatant medium (Table 8). RBC ghosts were rendered "sedimentable" only in the presence of H-PHAP; L-PHAP, even at much higher doses, did not achieve the same result. In no instance did the presence of lymphocytes increase significantly the proportion of "sedimentable" RBC ghosts.

Potentiation of lymphocyte transformation by X-irradiated lymphocytes and platelets. The potentiation of lymphocyte transformation by RBC solely in the presence of that mitogen capable of bringing red cells and lymphocytes into closely fixed apposition suggested that membrane-

Table 4

POTENTIATION OF LYMPHOCYTE TRANSFORMATION BY RBC*

Experiment IA. Constant RBC (10 x 10 ⁶); Varying H-PHAP Dose			
PHA dose (μg)	CPM ^{†,‡} RBC present	CPM ^{†,‡} RBC absent	Stimulation index
2.7	6,166 ± 499 [§]	416 ± 64	1,482 [‡]
5.4	32,373 ± 2,573	4,974 ± 717	651 ^{††}
13.4	38,909 ± 1,707	17,003 ± 1,357	229 ^{**}
26.8	39,377 ± 675	25,377 ± 545	155 ^{**}
40.2	39,899 ± 1,596	29,858 ± 1,749	134 [‡]
Experiment IB. Constant H-PHAP Dose (5.4 μg); Varying RBC			
RBC (millions)	CPM ^{†,‡}	Stimulation index	
0	4,974 ± 717		
10	32,373 ± 2,573	651 ^{††}	
5	22,618 ± 3,168	455 ^{**}	
2	18,917 ± 517	380 ^{**}	
1	13,989 ± 260	281 [‡]	
0.5	14,817 ± 970	298 ^{††}	
0.2	10,681 ± 2,674	215 [‡]	
Experiment II. Constant H-PHAP Dose; Varying RBC			
0	9,950 ± 231		
200	37,561 ± 7,706	378	
100	44,912 ± 1,565	451 [‡]	
50	43,887 ± 3,222	441 [‡]	
25	49,458 ± 3,004	497 [‡]	
12.5	48,866 ± 993	491 [‡]	
6.3	34,136 ± 4,850	343	

* Human lymphocytes (2×10^6) were cultured with and without RBC.

† CPM without mitogen: Experiment I, 173; Experiment II, 175.

‡ DNA synthesis as measured by 14 C-thymidine incorporation.§ $\pm$ one standard deviation.‡ $p < 0.05$.** $p < 0.01$.†† $p < 0.001$.

membrane interaction between lymphocyte and RBC, as well as between lymphocyte and lymphocyte, plays an important role in the stimulation of DNA synthesis and transformation by mitogen. If this were a general feature of lymphocyte transformation by mitogen then it should be possible to demonstrate a similar phenomenon with the L-PHAP mitogen. We therefore sought a membrane-bearing particle for use as stimulant, which like the RBC, was itself incapable of respond-

ing to L-PHAP by DNA synthesis. The particles chosen for these experiments were autologous X-irradiated lymphocytes, as well as autologous platelets. We determined that a dose of 5,000 R would be sufficient to reduce DNA synthesis to less than 3 per cent of the control value in the "nonresponding" lymphocyte particles.

Table 9 summarizes the effects of exposing one million normal lymphocytes to varying doses of both L- and H-PHAP mitogens in the absence of, and in the presence of, an equal number of nonresponding autologous irradiated lymphocytes. The results clearly demonstrate that the presence of these inert lymphocytes markedly potentiates the mitogenic capacity of both L- and H-PHAP. This potentiation resembles that obtained with RBC in the presence of H-PHAP, in that it is most marked at low and intermediate points on the dose response curve, and diminishes as the plateau peak response is approached.

Table 5

THE EFFECT OF VARYING LYMPHOCYTE DENSITY UPON THE POTENTIATION OF TRANSFORMATION BY RBC*

Lymphocytes per culture	RBC per culture	¹⁴ C-Thymidine incorporation (CPM)	Stimulation index
a. 4 x 10		15,218 ± 541 [†]	
	40 x 10	42,125 ± 303	277 [†]
	20 x 10	45,748 ± 5,368	301
b. 2 x 10		12,730 ± 2,276	
	20 x 10	40,170 ± 4,566	316 [§]
c. 1 x 10		9,781 ± 438	
	20 x 10	41,732 ± 7,163	427
	10 x 10	35,427 ± 3,694	362 [§]
d. 0.5 x 10		6,419 ± 2,566	
	20 x 10	40,270 ± 1,933	627 [§]
	5 x 10	33,777 ± 1,883	526 [§]

* The mitogen dose was 5.4 μg H-PHAP. Both a constant number of RBC (20 x 10⁶) and a constant RBC:lymphocyte ratio (10:1) were used.

[†] ± one standard deviation.

[‡] p < 0.01.

[§] p < 0.05.

The effect of autologous platelets on lymphocyte transformation by both H- and L-PHAP is shown in Table 10 and Figure 5. As with X-irradiated lymphocytes, and in contrast with RBC, the mitogenic activities of both L- and H-PHAP are potentiated by platelets. Again, in contrast with the potentiation of lymphocyte transformation by RBC in the presence of H-PHAP, both low and high doses of mitogen are substantially potentiated by the presence of platelets. The optimum platelet:lymphocyte ratio for the demonstration of potentiation, however, varies considerably with the dose of mitogen employed. At low doses of both L- and H-PHAP, high platelet:

lymphocyte ratios may actually inhibit lymphocyte transformation. In general, at any mitogen dose there exists an optimum platelet:lymphocyte ratio for the elicitation of maximal potentiation by platelets. Higher platelet:lymphocyte ratios are less stimulatory, and may actually inhibit lymphocyte transformation. This latter effect is in contrast with potentiation of lymphocyte transformation by RBC, where RBC:lymphocyte ratios as high as 100:1 were still capable of marked potentiation (Table 4).

Table 6
A STUDY OF THE TIME COURSE OF RBC-INDUCED POTENTIATION OF
LYMPHOCYTE TRANSFORMATION*

	Duration of culture (hr)	H-PHAP dose (μ g)	CPM -RBC	CPM +RBC (20×10^6)	Stimulation index
Experiment I	0-4	5.4	Nil	Nil	
	24-28	5.4	$77 \pm 14^\dagger$	156 ± 7	203^{**}
	48-52	5.4	$3,746 \pm 450$	$13,762 \pm 1,224$	367^J
	72-76	5.4	$3,782 \pm 289$	$11,923 \pm 1,943$	315^S
	96-100	5.4	$2,207 \pm 161$	$14,731 \pm 1,120$	667^J
Experiment II	0-4	5.4	Nil	Nil	
		40.5	Nil	N.D. [‡]	
	24-28	5.4	Nil	Nil	
		40.5	Nil	N.D.	
	48-52	5.4	228 ± 155	$1,597 \pm 257$	700^J
		40.5	1,266	N.D.	
	72-76	5.4	888 ± 140	$3,449 \pm 722$	388^S
		40.5	3,605	N.D.	
	96-100	5.4	$2,650 \pm 693$	$8,628 \pm 1,341$	326^J
		40.5	8,444	N.D.	

* 14 C-thymidine was added to a series of replicate cultures at 24-hourly intervals. After a further 4-hr period of incubation, the cultures were harvested and 14 C-thymidine incorporation determined. In Experiment II, a 7.5X increment in H-PHAP dose without RBC is shown for comparison. Non-stimulated cultures showed less than 100 CPM incorporation of label at all intervals.

[†] $\pm$ one standard deviation.

[‡] N.D. = not done.

^S $p < 0.05$.

^J $p < 0.01$.

^{**} $p < 0.001$.

DISCUSSION

Our understanding of the phenomena described in this paper is aided by the fortuitous fact that two potent mitogens, derived from the same source, and very closely related by immunologic²² and physicochemical properties,¹² differ so markedly with respect to the potentiation

Table 7

THE ENHANCEMENT OF LYMPHOCYTE TRANSFORMATION BY RBC
AND RBC STROMA

RBC or RBC equivalent added	Number of RBC or RBC equivalent added (millions)	H-PHAP dose (μ g)	14 C-thymidine incorporation (CPM)	Stimulation index
A. RBC	20		320	
B. None		5.4	$9,447 \pm 2,526^*$	
C. RBC	20	5.4	$52,284 \pm 2,004$	$553^\dagger$
RBC	2	5.4	$33,387 \pm 1,703$	$353^\dagger$
D. RBC stroma	84	5.4	$33,465 \pm 1,449$	$354^\dagger$
RBC stroma	42	5.4	$26,578 \pm 1,067$	$281^\dagger$
RBC stroma	21	5.4	$18,712 \pm 1,561$	$198^\dagger$
RBC stroma	10.5	5.4	$13,688 \pm 1,080$	145
RBC stroma	5.25	5.4	$10,039 \pm 1,147$	106
RBC stroma	2.6	5.4	$8,633 \pm 147$	91
E. RBC hemolysate	74	5.4	$8,376 \pm 2,193$	89
RBC hemolysate	37	5.4	$7,421 \pm 102$	79
RBC hemolysate	18.5	5.4	$6,988 \pm 525$	74

* $\pm$ one standard deviation. $^\dagger p < 0.01$. $^\ddagger p < 0.001$.

Table 8

CORRELATION BETWEEN RBC STROMA ENHANCEMENT OF LYMPHOCYTE
TRANSFORMATION AND SEDIMENTABLE 32 DFP-STROMA

PHAP type and dose	Lymphocytes (2×10^6)	RBC stromata (264×10^6)	14 C-thymidine incorporation (CPM)	Stimulation index	Sedimentable stromal particles	
					(%)	#($\times 10^{-6}$)
None	+	+	-	-	0.6	1.6
	-	+	-	-	0.4	1.1
H-PHAP (5.8 μ g)	+	-	$16,086 \pm 1,849^*$	-	-	-
	+	+	$41,960 \pm 2,741$	$261^\dagger$	8.3	21.9
H-PHAP (58 μ g)	-	+	-	-	7.3	19.3
	+	+	-	-	46	121.4
L-PHAP (2.4 μ g)	-	+	-	-	42.6	112.5
	+	-	$13,302 \pm 1,240$	-	-	-
L-PHAP (24 μ g)	+	+	$12,965 \pm 42$	97.5	0.4	1.1
	+	+	-	-	0.6	1.6
	-	+	-	-	0.5	1.3

* $\pm$ one standard deviation. $^\dagger p < 0.01$.

Table 9

POTENTIATION OF LYMPHOCYTE TRANSFORMATION BY
X-RAY KILLED LYMPHOCYTES*

PHAP type	PHAP dose (μ g)	¹⁴ C-thymidine incorporation (CPM)			Stimulation index
		Normal lymphocytes	Normal + x-ray lymphocytes (theoretical) [†]	Normal + x-ray lymphocytes (observed)	
H-PHAP	5.8	6,041	6,597 $\pm$ 561 [‡]	25,036 $\pm$ 317	380 [§]
	14.5	19,556	20,509 $\pm$ 1,286	29,612 $\pm$ 3,099	144
	58	31,464	32,417 $\pm$ 1,202	34,617 $\pm$ 545	107
L-PHAP	1.5	1,094	1,835 $\pm$ 53	8,062 $\pm$ 722	439 [§]
	3	3,734	4,475 $\pm$ 487	17,817 $\pm$ 1,687	398 [§]
	15.4	21,620	22,514 $\pm$ 713	24,166 $\pm$ 2,185	107

* Lymphocytes (1×10^6) were cultured with and without the addition of an equal number of autologous lymphocytes that had been exposed to a dose of 5,000 R.

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

[‡] $\pm$ one standard deviation.

[§] $p < 0.05$.

[§] $p < 0.01$.

by RBC of their ability to transform lymphocytes. Thus only that mitogen, H-PHAP, which to any significant extent binds to RBC while also binding to lymphocytes, is potent as a hemagglutinin, and, probably most important, causes mixed lymphocyte-RBC agglutination, can be potentiated in its activity by RBC. The most likely explanation suggested by the internal control provided by L-PHAP is that potentiation depends upon cell-cell or membrane-membrane interaction brought about by H-PHAP molecules acting as intercellular ligands. The notion that RBC, or RBC ghosts, in some way condition the medium so that lymphocyte DNA synthesis is enhanced, is rendered exceedingly unlikely by their failure to act similarly upon lymphocyte cultures that have been transformed by L-PHAP, especially if we assume that in all other particulars these two mitogens are acting upon lymphocytes through closely analogous mechanisms. Since two of the potentiating particles in the present series of observations lack genetic material in the form of RNA (RBC, RBC ghosts), while three of them lack genetic information in the form of DNA (RBC, RBC ghosts, platelets), and all four (including X-irradiated lymphocytes) are incapable of mitosis, the possibility that their ability to potentiate lymphocyte transformation depends upon information transfer seems quite remote. In all probability, the potentiation of DNA synthesis induced by mitogen which these formed elements invoke results, not from increasing the extent to which any single cell synthesizes DNA (which remains an all or none phenomenon), nor, as demonstrated, from an alteration in the period of induction for, or time course of, DNA synthesis, but rather through an increase in the efficiency with which individual cells are recruited into the transformation process at sub-optimal mitogen concentrations by a mechanism involving cell-cell, or more specifically, membrane-membrane interaction. These observa-

Table 10

POTENTIATION OF LYMPHOCYTE TRANSFORMATION BY AUTOLOGOUS PLATELETS

PHAP type	PHAP dose (μ g)	Number of platelets added* (millions)	14 C-thymidine incorporation (CPM)	Stimulation index
L-PHAP	3	36	1,217 $\pm$ 429 [†]	28.6
		18	3,445 $\pm$ 139	80.8
		9	8,897 $\pm$ 1,337	208.8
		0	4,261 $\pm$ 1,107	
	15	36	10,172 $\pm$ 292	105.9
		18	28,182 $\pm$ 3,436	293.4 [‡]
		9	37,932 $\pm$ 1,259	395 [§]
		0	9,604 $\pm$ 1,386	
H-PHAP	5.7	36	724 $\pm$ 147	20.9 [‡]
		18	1,476 $\pm$ 15	42.7 [‡]
		9	4,410 $\pm$ 32	127.6
		0	3,456 $\pm$ 366	
	28.5	36	25,480 $\pm$ 1,183	210.3 [§]
		18	30,881 $\pm$ 3,828	254.9 [‡]
		9	30,379 $\pm$ 2,547	250.8 [‡]
		0	12,115 $\pm$ 1,079	

* All cultures contained, in addition, 1×10^6 platelets residual from the lymphocyte isolation procedures.

[†] $\pm$ one standard deviation.

[‡] $p < 0.05$.

[§] $p < 0.01$.

tions may have some bearing on the experiments of Fisher and Mueller which indicate that lymphocyte membrane undergoes significant modulation of membrane phospholipids as one of the earliest steps in the process of transformation by PHAP.²³

It has long been appreciated that cellular density during in vitro culture plays an important role in determining the efficiency of lymphocyte response to PHAP-induced transformation. Thus the efficacy of suboptimal doses of PHAP is increased by increases in cell number.^{21,24} In addition, at optimal doses of PHAP which cause maximal DNA synthesis in lymphocyte cultures, the average amount of DNA synthesized per lymphocyte present at zero time increases as cell numbers increase, plateaus at an optimal cell concentration, and then declines²¹ (S. Yachnin, unpublished observations). This implies that, up to a certain point, increased cell density aids in the recruitment of cells for transformation, and that in cultures of highly purified viable lymphocytes cell-cell interaction may serve to potentiate the response. This conclusion is also supported by the observation that PHAP-induced transformation of a constant number of lymphocytes in stationary culture diminishes in roughly linear inverse proportion to the surface area occupied by the cells at the bottom of the culture vessel.²⁴ Thus, when lymphocyte numbers

Figure 5. Potentiation of lymphocyte transformation by autologous platelets. Each dose of mitogen was tested with varying numbers of platelets. The figures in parentheses represent the amount of ^{14}C -thymidine incorporated into lymphocyte DNA at each particular mitogen dose in the absence of platelets.

The following stimulation indices are not significant ($p > 0.05$):

A. L-PHAP	Mitogen dose	# of platelets added ($\times 10^{-6}$)	B. H-PHAP	Mitogen dose	# of platelets added ($\times 10^{-6}$)
	3 μ g	20		5.7 μ g	5, 10
	7.5 μ g	40		14.3 μ g	5, 10, 20
	15 μ g	10, 40		28.5 μ g	5, 40
	30 μ g	5, 10, 20, 40		57 μ g	40

are kept constant, and their opportunity for interaction is diminished by spreading them over a larger surface area during culture, transformation is less efficient. If we accept, then, that cell-cell interaction between lymphocyte and lymphocyte, or between lymphocyte and other cell types, potentiates PHAP-induced lymphocyte transformation, it behooves us to consider in some detail how this may be brought about.

In constructing an hypothesis to explain the potentiation of PHAP-induced lymphocyte transformation by cell-cell interaction, we have sought to take account of, and to explain, the following observations:

1. The potency of H-PHAP as hemagglutinin and, by comparison with L-PHAP, its poor lymphoagglutinating capacity;
2. The potency of H-PHAP in mixed RBC-lymphocyte agglutination;
3. The potency of L-PHAP as lymphoagglutinin, and conversely, its extremely weak capacity for hemagglutination;
4. The strong binding of H-PHAP to RBC; the weak binding of L-PHAP to RBC;
5. Substantial binding of both L- and H-PHAP to platelets;
6. Substantial binding of both H- and L-PHAP to lymphocytes (H-PHAP > L-PHAP);
7. The ability of RBC to potentiate the mitogenic activity of H-PHAP but not L-PHAP; the lack of inhibition of this potentiation, or of H-PHAP-induced transformation, by extremely high RBC:lymphocyte ratios;
8. The ability of platelets and X-irradiated lymphocytes to potentiate both L- and H-PHAP;

inhibition of potentiation and transformation by high platelet:RBC ratios (5-20:1, depending on mitogen dose);

9. The homogeneity of L-PHAP;^{12,25} the heterogeneity of H-PHAP as judged by acrylamide gel electrophoresis,¹² isoelectric focusing,²⁵ CM Sephadex chromatography,^{12,22} mitogenic capacity of CM Sephadex column purified fractions eluted at various ionic strengths,²² and IR C-50 chromatography;²⁶

10. The dissociation of PHAP molecules into subunits by urea²⁷ or guanidine HCl;²⁵

11. The isolation from RBC trypsin digests of a glycoprotein (M.W. 2,000) capable of inhibiting H-PHAP hemagglutination and mitogenicity.²⁸

The subunit structure of PHAP is fairly well defined although the precise number of subunits is in dispute;^{25,27} for purposes of our discussion it will be convenient to assume that in fact both L- and H-PHAP are made up of four subunits.²⁵ Subunits may carry a binding site with high affinity for a RBC membrane glycoprotein determinant and weak affinity for a lymphocyte membrane glycoprotein determinant (R subunit) or, alternatively, subunits may have exactly the converse affinities (L-subunit). L-PHAP is a symmetrical molecule composed of four L-subunits; H-PHAP is a mixture of molecular species, one of which may bear four R subunits, and the others varying numbers of L and R subunits (1R + 3L, 2R + 2L, etc.). RBC bear on their surface only R determinants, lymphocytes bear on their surface only L determinants, while platelets carry L (and perhaps R determinants) on their membrane (or alternatively, a single determinant capable of relatively strong reaction with both L and R subunits). Given these restrictions one can largely account for and explain the observations listed above.

With respect to PHAP-induced transformation it is not possible with certainty to dissociate agglutinating activity (that is multivalency) from transforming ability, with the possible exception of low dose H-PHAP induced transformation of pure lymphocytes. Certain mitogens of a nonspecific nature, however, such as staphylococcal filtrate,⁹ a product of streptococci,¹⁰ and possibly HgCl₂,¹¹ can transform lymphocytes without leukoagglutination. In addition, univalent fragments, Fab', of sheep anti-rabbit IgG have been reported to cause rabbit lymphocyte transformation,⁴ although a recent study has failed to confirm this finding.²⁹ Despite the lack of firm documentation then, we will assume for purposes of discussion that once a critical number of PHAP molecules have attached themselves to a lymphocyte,³⁰ regardless of whether that cell was bound through the same molecules to another, it could undergo transformation.

If we turn to RBC potentiation of PHAP-induced lymphocyte transformation at low and intermediate mitogen doses, the significance of the potentiating mechanism as a manifestation of the concentration and presentation of H-PHAP molecules to the lymphocyte membrane emerges. At these H-PHAP concentrations, such threshold levels of H-PHAP binding by individual lymphocytes may not be reached; the addition of RBC to this system, however, with their strong affinity for the R subunit on the hybrid H-PHAP molecules, and their ability, after cosedimentation, to present to the lymphocyte surface a matrix of directionally oriented L subunits for simultaneous and consequently more irreversible binding, allows the lymphocyte to pass its threshold for stimulation at concentrations of soluble mitogen which might otherwise be insufficient to attain it. Furthermore, it is not inconceivable that the resultant asymmetry of lymphocyte membrane deformation by surface matrix H-PHAP is more efficient in evoking cellular response than the presumably geographically random and symmetrical attachment that would occur if H-PHAP were to attach directly from the fluid phase. The necessity for "homing" or finding the

appropriate partner for synergism is emphasized by the relatively poor efficiency, compared to intact RBC, which the buoyant RBC ghosts display; the study using $DF^{32}P$ suggests that only particles that are co-sedimentable with lymphocytes can take part in the potentiating process. Not to be dismissed is the possibility that the close apposition of membrane surfaces may itself play a role in the triggering of lymphocyte derepression.

The persistence of potentiation of H-PHAP-induced lymphocyte transformation at extremely high RBC:lymphocyte ratios (100:1) is consistent with the matrix theory, since RBC bind only weakly to L subunits, which consequently remain available, despite large increments in RBC number, for presentation to, and interaction with lymphocytes. Examination of mixed aggregation, even at RBC:lymphocyte ratios of a lower order of magnitude (4:1), where potentiation and transformation are nearly maximal, reveals that most lymphocytes occur as single units embedded in, and surrounded by, a lattice of agglutinated RBC. Surely, at higher RBC:lymphocyte ratios, the opportunities for lymphocyte-lymphocyte interaction must be even more restricted. We can interpret these findings to mean that isolated lymphocytes not in contact with other cells of their class can in fact undergo transformation, and that the origin of the membrane involved in matrix formation and subsequent interaction with a lymphocyte, lends no special quality or power to lymphocyte transformation. All the cell membranes studied play a purely passive role in potentiation. The only feature of the matrix potentiation scheme which is unique to viable lymphocyte interaction is that the signal is reciprocal, in that both partners (i.e., viable lymphocytes) are capable of a response; in all other instances (RBC, platelets, X-irradiated lymphocytes) response and potentiation are unidirectional. The fact that small numbers of platelets are potentiating, while large numbers, in contrast to RBC, are inhibitory to lymphocyte transformation, is also consistent with our matrix theory. Platelets potentiate the action of both H- and L-PHAP largely by membrane matrix construction with L-subunits; consequently a large excess of platelet L-binding sites would serve to bind all available L-subunits and prevent their interaction with lymphocytes especially at low mitogen doses, as is in fact observed. One would predict that a large excess of X-irradiated lymphocytes would act in a similar fashion, although this experiment was not performed. Some of these principles are illustrated in Figure 6,

These observations, then, strongly suggest a cell-surface matrix theory of mitogen concentration and presentation as a rationalization of the phenomenon of cellular synergy in the PHAP response. Since PHAP-responsive lymphocytes are regarded as being largely, if not exclusively, T lymphocytes,³¹ our experiments suggest that synergy is in fact possible amongst T cells, and that in such interaction antigen may in fact play a role similar to PHAP.

It is now widely recognized that cooperation between thymus derived (T) and bone marrow derived (B) lymphocytes is necessary in order for certain antigens to evoke an optimum primary or secondary immune response.³²⁻³⁵ Study of the "carrier" effect in the hapten-carrier system has been a powerful tool in elucidating the mechanism by which T-B lymphocyte synergism operates,³⁶ and has led to the proposal that T lymphocytes play a role in this synergy by a process of passive specific adsorption and concentration of antigen in the form of a cell-surface antigen matrix.³⁷ A close analogy exists between the matrix formation concept in RBC potentiation of PHAP-induced lymphocyte transformation, and the matrix theory of T-B lymphocyte synergism in the immune response. The two different epitopes on the bridging immunogen in T-B cell synergy are here represented by the R-L subunits of the H-PHAP molecule.

Finally, since many methods of lymphocyte collection and preparation yield lymphocytes

Figure 6. Schematic representation of the subunit structure of H- and L-PHAP. Only 2 of the 4 PHAP subunits are shown. In a-e single RBC and/or lymphocyte membrane receptor sites are shown as the heavily shaded areas: (a) L-PHAP-induced lymphoagglutination (strong); (b) L-PHAP-induced RBC agglutination (weak); (c) H-PHAP-induced RBC agglutination (strong); (d) H-PHAP-induced lymphoagglutination (weak); (e) H-PHAP (hybrid molecule)-induced mixed RBC-lymphocyte agglutination (strong).

significantly contaminated with RBC and/or platelets, comparative studies of PHAP-induced lymphocyte transformation in various human disease states which neither remove these contaminants nor control their number are subject to the most tentative interpretation. Similar constraints are imposed upon lymphocyte transformation studies with other mitogenic stimuli such as pokeweed mitogen, Concanavalin-A, and mixed lymphocyte culture, where the presence of RBC and/or platelets may profoundly affect the results.³⁸

ACKNOWLEDGMENTS

The authors are grateful to Mrs. Janine Raymond for expert technical assistance.

Computation was accomplished with the assistance of the Biomedical Computation Facilities, University of Chicago, under a USPHS grant from the National Institutes of Health. We are indebted to Mr. Richard Blough for his kind assistance.

We thank Dr. N. A. Mitchison for helpful discussion and review of the manuscript.

LITERATURE CITED

1. Permain, G., R. R. Lycette, and P. H. Fitzgerald. *Lancet*, 1:637, 1963.
2. Gräsbeck, R., C. Nordman, and A. De la Chapelle. *Lancet*, 2:385, 1963.
3. Sell, S. J. *Exptl. Med.*, 125:393, 1967.

4. Sell, S. *J. Immunol.*, 98:786, 1967.
5. Sell, S., and P. G. H. Gell. *J. Exptl. Med.*, 122:423, 1965.
6. Nowell, P. C. *Cancer Res.*, 20:462, 1960.
7. Douglas, S. D., R. M. Kamin, W. C. Davis, and H. H. Fudenberg. In Third Annual Leucocyte Culture Conference. W. O. Rieke, Ed. New York: Appleton-Century-Crofts Inc., 1969, p. 607.
8. Chessin, L. N., J. Borjeson, P. O. Welsh, S. D. Douglas, and H. L. Cooper. *J. Exptl. Med.*, 125:873, 1966.
9. Ling, N. R., E. Spicer, K. James, and N. Williamson. *Brit. J. Haematol.*, 11:421, 1965.
10. Taranta, A., G. Cuppari, and F. Quagliata. *J. Exptl. Med.*, 129:605, 1969.
11. Schöpf, E., K. H. Schulz, and I. Isensee. *Arch. Klin. Exptl. Dermatol.*, 234:420, 1969.
12. Allen, L. W., R. H. Svenson, and S. Yachnin. *Proc. Nat. Acad. Sci. U.S.*, 63:334, 1969.
13. Yachnin, S., R. H. Svenson, L. W. Allen, and J. M. Baron. *J. Clin. Invest.*, 48:91a, 1969.
14. Yachnin, S., L. Allen, and R. Svenson. *Blood*, 34:825, 1969.
15. Yachnin, S., L. W. Allen, J. M. Baron, and R. H. Svenson. In Fourth Leukocyte Culture Conference. O. R. McIntyre, Ed. New York: Appleton-Century-Crofts Inc., 1971, p. 37.
16. Tarnvik, A. *Acta Path. Microbiol. Scand. Sect. B.*, 78:733, 1970.
17. Thompson, J. S., C. D. Severson, A. R. Lavender, M. Forland, and H. P. Russe. *Transplantation*, 6:728, 1968.
18. Dodge, J. T., C. Mitchell, and D. J. Hanahan. *Arch. Biochem. Biophys.*, 100:119, 1963.
19. Hjort, P. F., H. Paputalis, and B. Cheney. *J. Lab. Clin. Med.*, 55:416, 1960.
20. McConahey, P. J., and F. J. Dixon. *Int. Arch. Allergy Appl. Immunol.*, 29:185, 1966.
21. Leventhal, B. G., and J. J. Oppenheim. In Third Annual Leucocyte Culture Conference. W. O. Rieke, Ed. New York: Appleton-Century-Crofts Inc., 1969, p. 13.
22. Yachnin, S., and R. H. Svenson. *Immunology*, 00:000, 0000.
23. Fisher, D. B., and G. C. Mueller. *Proc. Nat. Acad. Sci. U.S.*, 60:1396, 1968.
24. Moorhead, J. F., J. J. Connolly, and W. McFarland. *J. Immunol.*, 99:413, 1967.
25. Weber, T. H. *Scand. J. Clin. Lab. Invest. Suppl.* 111, 1969.
26. Rigas, D. A., E. A. Johnson, R. T. Jones, J. D. McDermed, and V. V. Tisdale. *Assoc. Greek Chemists*, 3:151, 1966.
27. Rigas, D. A., and C. Head. *Biochem. Biophys. Res. Commun.*, 34:633, 1969.
28. Kornfeld, S., and R. Kornfeld. *Proc. Nat. Acad. Sci. U.S.*, 63:1439, 1969.
29. Fanger, M. W., D. A. Hart, J. V. Wells, and A. Nisonoff. *J. Immunol.*, 105:1484, 1970.
30. Foerster, J., J.-P. Lamelin, I. Green, and B. Benacerraf. *J. Exptl. Med.*, 129:295, 1969.
31. Doenhoff, M. J., A. J. S. Davies, E. Leuchars, and V. Wallis. *Proc. Roy. Soc. Ser. B., London*, 176:69, 1970.
32. Mitchison, N. A. *Cold Spring Harbor Symp. Quant. Biol.*, 32:431, 1967.

33. Miller, J. F. A. P., and G. F. Mitchell. Transplant. Rev., 1:3, 1969.
34. Davies, A. J. S. Transplant. Rev., 1:43, 1969.
35. Taylor, R. B. Transplant. Rev., 1:114, 1969.
36. Mitchison, N. A., K. Rajewsky, and R. B. Taylor. In Developmental Aspects of Antibody Formation and Structure. New York: Academic Press, 1970, p. 547.
37. Mitchison, N. A. In Proc. Sixth International Immunopathology Symposium, Grindelwald. P. Miescher, Ed. Basle: Schwabe, 1971.
38. Yachnin, S. Clin. Exptl. Immunol., 11:109, 1972.

POTENTIATION AND INHIBITION BY AUTOLOGOUS RED CELLS AND
PLATELETS OF HUMAN LYMPHOCYTE TRANSFORMATION
INDUCED BY POKEWEEED MITOGEN, CONCAVALIN A,
MERCURIC CHLORIDE, ANTIGEN, AND MIXED
LEUKOCYTE CULTURE*

By

S. Yachnin

In vitro lymphocyte transformation has provided an important tool for studies of the immune response at the level of the immunologically competent cell. In particular, it has focused attention on those events at the cell surface which can provoke a proliferative response from a more or less specifically defined group of lymphocytes. Quantitation of the response utilizing the technique of measuring lymphocyte DNA synthesis by means of a radioactive DNA precursor such as thymidine, has proved to be a discriminating way of measuring a parameter relating to the immunocompetence of the individual from whom the reactive lymphocytes are derived; for example, careful quantitation of such a response in the mixed leukocyte culture (MLC) has been used as an index of the histocompatibility differences between two subjects.¹ Similarly, the response of lymphocytes to a wide variety of mitogenic materials has been studied in a wide variety of disease states in an attempt to gain some insight into the immunological competence of the individual. In humans particularly, because of its easy accessibility, the peripheral blood lymphocyte has been the main target for such studies.

The isolation of high titer hemagglutinating (H-PHAP) and low titer hemagglutinating (L-PHAP) mitogens from the phytohemagglutinin derived from Phaseolus vulgaris² has helped to define the role of cell-cell and membrane-membrane interaction in the potentiation of lymphocyte transformation as measured by [¹⁴C]thymidine incorporation. The presence of autologous red blood cells (RBC) leads to a marked increase in lymphocyte transformation when H-PHAP is used as mitogen, but has no effect on lymphocyte transformation by L-PHAP. Potentiation of lymphocyte transformation by H-PHAP is also effected by RBC stromal particles. Potentiation has been shown to depend upon the formation of a mixed agglutination lattice between RBC and lymphocytes by H-PHAP, a property not shared by L-PHAP. In contrast to the selective potentiating action of RBC, the mitogenic capacities of both L- and H-PHAP are potentiated by the simultaneous presence of mitotically inert autologous X-irradiated lymphocytes, or by the presence of autologous platelets. The effects of platelets can be shown to be bimodal, their presence in lower numbers potentiates lymphocyte transformation, while higher numbers may be inhibitory.^{3,4}

These studies were extended to the effects of autologous RBC and platelets upon human peripheral blood lymphocyte transformation induced by a variety of mitogens including pokeweed mitogen (PWM), concanavalin-A (CON-A), mercuric chloride, specific antigen (PPD tuberculin) and the one way MLC. The results form the body of this report.

* This report is taken from a paper that appeared in *Clinical and Experimental Immunology*, 11:109, 1972.

MATERIALS AND METHODS

Mitogens. PWM was a gift from Lawrence N. Chessin, M.D. of the University of Rochester. It was isolated from roots of the plant *Phytolacca americana*.⁵ CON-A was obtained from Dr. Myron A. Leon, St. Lukes Hospital, Cleveland, Ohio. It was prepared by a modification of the method of Agrawal and Goldstein.⁶ Purified Protein Derivative (PPD) was obtained from the Parke Davis Company (Lot #978822W). Mercuric chloride was commercially obtained (Reagent Grade). All mitogens were dissolved in sterile saline or tissue culture medium prior to use and were added to lymphocyte cultures in appropriate amounts in volumes ranging from 1-50 μ l. Mitomycin C was a product of Nutritional Biochemicals, Cleveland, Ohio (Lot #8799).

All the experiments were performed with human peripheral blood white cells, lymphocytes, RBC, and platelets. Lymphocytes, entirely free of RBC, or RBC stroma, and containing 50-100 platelets/100 leukocytes were obtained by gravity sedimentation of heparinized whole blood, nylon fiber column passage, osmotic lysis of RBC, and centrifugation through a plasma-saline gradient.² In MLC experiments the passage through a nylon fiber column was omitted. The preparation of autologous washed RBC and platelets, each free of other formed elements of blood, has been described elsewhere.⁴

The methods used for culturing lymphocytes and determining their incorporation of 2[¹⁴C]thymidine into DNA, as well as for assessing the effects of RBC or platelets on lymphocyte transformation have already been published.^{2,4} Exactly similar methods were used in assessing the effects of RBC or platelets on PWM-, CON-A-, or PPD-induced lymphocyte transformation. Where lymphocyte transformation was induced by antigen (PPD), lymphocytes were obtained from patients displaying delayed hypersensitivity to intracutaneous injections of the same antigen. PPD lymphocyte cultures, with or without the addition of RBC or platelets, were incubated for 5 days. One-tenth μ C[¹⁴C]thymidine was then added, and the cultures were harvested after a further 16 hr growth.

In studies of the effects of RBC or platelets on lymphocyte transformation induced by one way MLC the method of Amos and Bach was used.⁷ Four milliliter cultures containing 20 per cent autologous plasma (obtained from the blood of the responding lymphocyte population donor), 2×10^6 responding mononuclear cells, and from $1.5-6.0 \times 10^6$ mitomycin-treated stimulating leukocytes were incubated with or without RBC or platelets for 7 days. [¹⁴C]thymidine was then added and the cultures were harvested 16 hr later. Control cultures received an equivalent number of autologous mitomycin-treated leukocytes in place of the allogeneic cells; in each case where a given dose of autologous RBC or platelets was being studied for its effect on the MLC transformation response, control cultures containing autologous mitomycin-treated leukocytes together with the appropriate number of RBC or platelets were prepared. This was necessary because the amount of precursor incorporated into DNA in such control cultures was occasionally quite substantial and was found to be subject to variable inhibition by the addition of RBC or, more markedly, platelets.

Mercuric chloride-induced lymphocyte transformation was studied by the procedures outlined by Schöpf, Schulz, and Isensee.⁸ 2×10^6 purified lymphocytes were cultured in 2 ml TC 199 containing 25 per cent autologous plasma. [¹⁴C]thymidine was added after 72 or 96 hr of culture and harvesting was performed 16 hr later.

Except for the MLC already described, the addition of autologous RBC or platelets to un-

stimulated lymphocyte cultures had no significant effect upon baseline lymphocyte [^{14}C]thymidine incorporation, nor did these formed elements incorporate significant amounts of [^{14}C]thymidine when cultured alone with or without the mitogenic agents.

As a convenient means of expressing the potentiation or inhibition of lymphocyte transformation by RBC or platelets a stimulation index was devised according to the following formula where CPM refers to the amount of [^{14}C]thymidine incorporated into lymphocyte DNA under the influence of mitogen (after subtraction of that amount incorporated into the control, unstimulated lymphocytes):

$$\text{Stimulation index (SI)} = \frac{\text{CPM} + \text{RBC (or platelets)}}{\text{CPM} - \text{RBC (or platelets)}} \times 100$$

Experiments were done in duplicate or triplicate. Mean values are given.

RESULTS

Pokeweed mitogen. The optimum dose of PWM for the transformation of 2×10^6 pure lymphocytes was $2 \mu\text{g}$. Transformation of 2×10^6 lymphocytes by varying doses of PWM in the presence of 10 times their number of autologous RBC resulted in 2- to 3-fold potentiation of lymphocyte response. Potentiation by RBC was greatest at high and low points on the dose response curve of purified lymphocytes, and was slightly less pronounced at the optimum. In effect, the optimum dose of PWM for lymphocyte transformation was shifted from $2 \mu\text{g}$ to $10 \mu\text{g}$ (mode of 5 experiments) by the addition of RBC. One such titration is illustrated in Figure 1,

Figure 1. Potentiation of PWM-induced lymphocyte transformation by RBC. 2×10^6 lymphocytes were cultured with varying doses of PWM with and without 20×10^6 RBC. Unstimulated lymphocytes incorporated 9 cpm $2[^{14}\text{C}]$ thymidine. ●-----● - RBC; O———O + RBC.

while Table 1 summarizes the results of five separate experiments. If the dose of PWM was kept constant and varying numbers of RBC were added to 2×10^6 lymphocytes, optimum potentiation was found at an RBC:lymphocyte ratio of 10:1, although potentiation was frequently discernible at much lower ratios (Table 2).

Table 1

POTENTIATION OF PWM-INDUCED LYMPHOCYTE TRANSFORMATION BY RBC

	PWM dose (μg)					
	0.2	1	2	4	10	20
Experiment 1 (S.I.)*	N.D.†	N.D.	176	224	309	N.D.
Experiment 2 (S.I.)	N.D.	N.D.	116	115	131	127
Experiment 3 (S.I.)	162	130	114	N.D.	195	239
Experiment 4 (S.I.)	295	258	241	314	282	320
Experiment 5 (S.I.)	202	182	170	255	247	255
MEAN (S.I.)	220	190	183	227	233	235

2×10^6 lymphocytes were incubated with and without 20×10^6 RBC in the presence of varying doses of PWM.

* S.I. = Stimulation Index.

† N.D. = Not done.

Table 2

POTENTIATION OF PWM-INDUCED LYMPHOCYTE TRANSFORMATION BY VARYING NUMBERS OF RBC

RBC added (millions)	[^{14}C]thymidine incorporation (cpm)	Stimulation index
0	2460	
0.5	4068	165
1	4858	198
2	5921	241
5	7108	289
10	7462	303
20	8294	337
40	6746	274

2×10^6 lymphocytes were cultured with a constant dose of PWM ($4 \mu\text{g}$) and varying numbers of RBC. Unstimulated lymphocytes incorporated 8 cpm of labeled thymidine.

The density of lymphocytes in culture is known to affect their responsiveness to mitogenic stimuli.^{4,9} In order to determine the effect of lymphocyte density on the RBC potentiation of PWM-induced lymphocyte transformation, we cultured lymphocytes over an 8-fold density range with PWM and RBC, testing both a constant number of RBC (20×10^6) and a constant RBC:lymphocyte ratio (10:1) with each lymphocyte density (Table 3). RBC potentiation of PWM-induced lymphocyte transformation was seen at each lymphocyte density although potentiation was most

Table 3

EFFECT OF LYMPHOCYTE DENSITY UPON POTENTIATION OF PWM-INDUCED
LYMPHOCYTE TRANSFORMATION BY RBC

Lymphocytes per culture	Red cells per culture	[¹⁴ C]thymidine incorporation (cpm)	Stimulation index
a. 4×10^6		9,814	
	40×10^6	18,668	190
	20×10^6	20,380	208
b. 2×10^6	20×10^6	7,615	
		18,145	238
c. 1×10^6		4,695	
	20×10^6	13,182	281
	10×10^6	10,620	226
d. 0.5×10^6		2,447	
	20×10^6	5,073	207
	5×10^6	3,929	161

The mitogen dose was 4 μ g. Both a constant number of RBC (20×10^6) and a constant RBC:lymphocyte ratio (10:1) were used. 4×10^6 unstimulated lymphocytes yielded 292 cpm labeled thymidine incorporation.

marked at intermediate lymphocyte densities, and diminished at both the highest and lowest lymphocyte densities tested.

The effect of autologous platelets upon PWM-induced lymphocyte transformation was studied in four experiments wherein lymphocytes were cultured with varying doses of PWM alone and in the presence of graded numbers of platelets: The results of a representative experiment are shown in Figure 2. Both low and high doses of mitogen are potentiated by the presence of platelets, the optimum platelet:lymphocyte ratio for potentiation being 5-10:1. Higher platelet:lymphocyte ratios are less stimulatory, and may actually inhibit lymphocyte transformation. These results are very similar to those obtained in a study of the potentiation of phytohemagglutinin-induced lymphocyte transformation by platelets.⁴

Concanavalin-A. In contrast to our present findings with PWM, and to those previously described for phytohemagglutinin,^{3,4} the presence of autologous RBC during CON-A-induced lymphocyte transformation had no potentiating effect. In fact, at all RBC:lymphocyte ratios tested (up to 10:1), the presence of RBC inhibited CON-A-induced lymphocyte transformation in a straightforward dose-dependent fashion. Table 4 shows a single representative experiment as well as the mean results of four such experiments. It is noteworthy that the degree of inhibition obtained by any single RBC:lymphocyte ratio is independent of the dose of mitogen employed.

Autologous platelets, on the other hand, were able to potentiate lymphocyte transformation with CON-A. On occasion, within a single experiment, intermediate doses of platelets were most effective, with higher platelet numbers yielding less potentiation, or even being inhibitory. When the mean values for six experiments are examined, however, this phenomenon is not apparent,

Figure 2. Potentiation of PWM-induced lymphocyte transformation by autologous platelets. 2×10^6 lymphocytes were cultured with varying mitogen doses in the absence of, and in the presence of, graded numbers of platelets. Unstimulated lymphocytes incorporated 18 cpm labeled thymidine into DNA.

and the highest potentiation is found with the highest (20:1) platelet:lymphocyte ratios employed (Table 5).

Antigen (PPD)-induced lymphocyte transformation. When tested at various antigen doses or with varying RBC:lymphocyte ratios at a constant antigen dose, RBC had no significant effect on lymphocyte transformation. In a single experiment, platelets caused only a slight potentiation of lymphocyte transformation at optimum antigen doses; but in the supraoptimal dose range, where transformation by purified lymphocytes was substantially diminished, the presence of platelets resulted in much more pronounced enhancement. In the presence of platelets, then, the optimum antigen dose for lymphocyte transformation was significantly altered (Table 6).

Mixed leukocyte culture. Before embarking upon a study of the effects of autologous RBC and platelets (i.e., those obtained from the donor of the responding lymphocytes in the MLC) we studied the ability of RBC and platelets themselves to evoke transformation of allogeneic lym-

Table 4
INHIBITION OF CON-A-INDUCED LYMPHOCYTE TRANSFORMATION
BY AUTOLOGOUS RBC

Mitogen dose (μ g)	Number of RBC added (millions)	[14 C]thymidine incorporation (cpm)	Stimulation index	Stimulation index (mean of 4 experiments)
a. 10	0	4,036		
	2.5	2,806	70	81
	5	2,225	55	70
	10	1,992	49	56
	20	824	22	44
b. 20	0	5,874		
	2.5	4,203	72	79
	5	3,575	61	68
	10	1,339	23	43
	20	1,006	17	35
c. 50	0	10,637		
	2.5	9,637	91	84
	5	7,968	75	73
	10	2,575	24	42
	20	1,864	18	29
d. 100	0	13,387		
	2.5	12,634	94	87
	5	9,983	75	72
	10	5,164	39	48
	20	3,108	23	34

2×10^6 lymphocytes were used in each culture tube. The results shown in the far right column are the means obtained from four individual experiments, including the experiment shown in detail.

phocytes, as well as their ability to alter the response evoked by the stimulating leukocytes from the homologous donor. Neither RBC nor platelets were capable of provoking lymphocyte transformation of allogeneic lymphocytes, while their presence during the course of a normal MLC response proved to be highly inhibitory (Table 7).

When MLC was performed in the presence of varying numbers of autologous RBC or platelets, substantial inhibition was observed. With RBC, inhibition was not dependent upon RBC dose (range $40-4 \times 10^6$ RBC) and transformation was roughly 50 per cent of control values (mean of three experiments, Table 8). With platelets, however, inhibition was clearly related to platelet dose and at 40×10^6 platelets was quite profound ranging from 75-100 per cent inhibition (mean of three experiments 88 per cent, Table 9). Both RBC and platelet inhibition of the MLC reaction were studied at optimal and suboptimal doses of stimulating leukocytes, and the results were similar.

Table 5

POTENTIATION OF CON-A-INDUCED LYMPHOCYTE TRANSFORMATION BY
AUTOLOGOUS PLATELETS

Mitogen dose (μ g)	Stimulation index with varying numbers of platelets			
	40×10^6	20×10^6	10×10^6	5×10^6
10	247 (5)	237 (6)	228 (6)	149 (4)
20	126 (2)	159 (2)	143 (2)	133 (2)
50	342 (3)	309 (4)	266 (4)	187 (4)
100	214 (5)	209 (5)	185 (5)	173 (3)

2×10^6 lymphocytes were used throughout. Six experiments were performed using varying numbers of platelets and varying mitogen dose. In certain experiments some mitogen doses or platelet additions were deleted. Mean values are shown. The number of determinations contributing to each mean value is shown in parentheses.

Mercuric chloride. In 11 out of 14 experiments employing HgCl_2 as mitogen, the optimum concentration for lymphocyte transformation was $4 \mu\text{g/ml}$ culture medium. The presence of RBC in RBC:lymphocyte ratios of from 1-5 had no significant effect upon HgCl_2 -induced lymphocyte transformation. At low mitogenic doses of HgCl_2 platelets did not affect lymphocyte transformation. At optimum mitogenic doses of HgCl_2 , however, platelets in platelet:lymphocyte ratios of from 5-10:1 had a modest potentiating effect (Table 10).

DISCUSSION

The potentiation phenomena observed in our earlier studies on the potentiation of phytohemagglutinin-induced lymphocyte transformation by autologous RBC, X-irradiated lymphocytes, and platelets were interpreted as resulting from the ability of potentiating particles to construct a concentrated matrix of PHA molecules at their membrane surfaces, for presentation to, and more irreversible binding with, sites on the surface of responsive lymphocytes. Specifically rejected as explanations for the potentiation of PHA-induced lymphocyte transformation by RBC or platelets were the notions that potentiating particles in some way condition the culture medium so that lymphocyte DNA synthesis is enhanced, or that potentiating particles function by means of some form of information transfer.⁴

The present report deals with a more diverse group of mitogenic agents and makes no attempt to investigate in detail the mechanism whereby autologous RBC and platelets potentiate, or in certain instances, inhibit, mitogen-induced lymphocyte transformation. Two of the mitogenic agents under study (PWM and CON-A), like PHA, are glycoprotein lectins possessing both erythro- and leuko-agglutinating activities^{10,11} which have not yet been dissociated from the ability to transform lymphocytes. Such activities suggest that with respect to their ability to in-

Table 6

EFFECT OF AUTOLOGOUS RBC OR PLATELETS ON PPD-INDUCED
LYMPHOCYTE TRANSFORMATION

	PPD dose (μ g)	Platelets added (millions)	RBC added (millions)	[14 C]thymidine incorporation (cpm)	Stimulation index
Experiment A	1			2,603	
	1		20	1,977	76
	2			3,499	
	2		20	3,142	91
	4			5,309	
	4		20	4,221	80
Experiment B	1			10,709	
	1		4	9,644	90
	1		10	9,884	92
	1		20	9,020	84
	1	8		11,532	107
	1	20		14,528	136
	1	40		14,871	139
	4			6,729	
	4		4	8,888	132
	4		10	4,714	70
	4		20	6,974	104
	4	8		14,751	219
	4	20		16,531	246
	4	40		15,007	223

2×10^6 lymphocytes were used in all experiments.

Cpm control unstimulated lymphocyte cultures: Experiment A, 28; Experiment B, 71.

teract with cell surfaces, both PWM and CON-A are at least bivalent molecules* despite the fact that PWM has been shown to be resistant to dissociation by 5 M guanidine-HCl¹¹ (indicating the lack of non-covalently linked polypeptide subunits). Of all the mitogens currently under study, the findings with PWM are most strongly analogous to those with PHA. Both mitogens are subject to potentiation by RBC and platelets, and the pattern of platelet potentiation in particular is identical with that found with L- or H-PHAP; optimum numbers of platelets enhance lymphocyte transformation, while larger numbers of platelets are less stimulatory, and may actually be inhibitory.^{3,4} The progressive inhibition of CON-A-induced lymphocyte transformation by increments of added RBC might be an extreme example of the same mechanism, if one postulates that the binding affinity of CON-A for RBC membrane sites is markedly great-

* That CON-A is in fact a bivalent molecule has been confirmed by equilibrium dialysis studies with α -D mannopyranoside.³¹

Table 7

RESPONSE OF LYMPHOCYTES TO ALLOGENEIC LEUKOCYTES,
RBC, AND PLATELETS

Stimulating leukocyte dose	RBC (millions)	Platelets (millions)	[¹⁴ C]thymidine incorporation (cpm)	Stimulation index
6 x 10 ⁶	40,20,10 or 4	100,50,25, or 10	13,666	
3 x 10 ⁶			9,747	
1.5 x 10 ⁶			5,899	
6 x 10 ⁵			3,300	
			0	
	20	50	0	
3 x 10 ⁶	20		1,819	19
3 x 10 ⁶		50	3,996	41

6.8 x 10⁶ responding leukocytes (2.0 x 10⁶ mononuclear cells) were incubated with varying numbers of stimulating leukocytes (mitomycin treated). RBC, platelets, or mixtures thereof (all derived from the same donor).

Table 8

INHIBITION OF ONE-WAY MLC LYMPHOCYTE TRANSFORMATION BY AUTOLOGOUS
RBC (RBC DERIVED FROM THE RESPONDING LYMPHOCYTE DONOR)

Stimulating leukocyte dose (millions)	RBC (millions)	[¹⁴ C]thymidine incorporation (cpm)	Stimulation index	Stimulation index (mean of 3 experiments)
3		4,898		
3	40	684	14	58
3	20	1,234	25	41
3	10	1,285	26	49
3	4	2,081	43	58
1.5		5,479		
1.5	40	831	15	43
1.5	20	1,043	19	63
1.5	10	1,804	33	53
1.5	4	1,775	32	65

The results shown in the far right column are the means obtained from 3 individual experiments, including the experiment shown in detail.

Table 9

INHIBITION OF ONE-WAY MLC LYMPHOCYTE TRANSFORMATION BY
AUTOLOGOUS PLATELETS

Stimulating leukocyte dose (millions)	Platelets (millions)	[¹⁴ C]thymidine incorporation (cpm)	Stimulation index	Stimulation index (mean of 3 experiments)
3		7,033		
3	40	0	0	13
3	20	2,326	33	40
3	10	2,309	33	51
3	4	3,545	50	62
1.5		4,248		
1.5	40	464	11	11
1.5	20	417	10	26
1.5	10	1,475	35	41
1.5	4	1,569	37	44

The results shown in the far right column are the mean obtained from 3 individual experiments, including the one shown in detail.

er than its binding affinity for lymphocyte membrane sites. Thus, even relatively small numbers of RBC would compete effectively for the available CON-A molecules, and CON-A would preferentially form RBC-RBC cross linkages. In proposing that RBC and platelet potentiation of PHA-induced lymphocyte transformation might be viewed as a model for understanding the role of the matrix theory of antigen concentration and presentation in thymus- and bone marrow-derived lymphocyte cell synergy in the immune response, it was acknowledged that the ability to respond to PHA in vitro was limited to thymus-derived lymphocytes.⁴ PWM-transformed lymphocyte cultures, in contrast to those transformed by PHA, contain large numbers of cells resembling plasmablasts¹² and, in prolonged PWM cultures, cells resembling mature plasma cells.¹³ These findings suggest that PWM may be capable of inducing transformation in bone marrow-derived lymphocytes; recent studies utilizing mouse lymphocytes are in keeping with this hypothesis.⁹ Thus, the analogies previously drawn from our observations on the potentiation of PHA-induced lymphocyte transformation by cell-cell interaction may now be tentatively extended to the bone marrow-derived lymphocyte as well. Clearly, more information is required before such a formulation may be confidently asserted.

The mechanism by which mercuric ions induce lymphocyte transformation is not known. Mercuric ions, in common with other heavy metal cations, are capable of combining with sulfhydryl groups. Heavy metal ions, such as Cr⁺⁺⁺ and to a lesser extent Hg⁺⁺, can cause intermolecular bridging and thus couple protein molecules to cell membranes, or can cause erythroagglutination.¹⁴ The possibility, therefore, exists that mercuric ions may effect lymphocyte transformation via intercellular bridging through mixed disulfide or other linkages. Caron, Poutala, and Provost¹⁵ however, have found that HgCl₂ in mitogenic doses is not capable of erythro- or leuko-agglutination. Using [²⁰³Hg]Cl₂ these authors were unable to show specific

Table 10

EFFECT OF AUTOLOGOUS RBC (A) OR PLATELETS (B) ON HgCl_2 -INDUCED
LYMPHOCYTE TRANSFORMATION

HgCl_2 ($\mu\text{g/ml}$)	Platelets added (millions)	RBC added (millions)	^{14}C thymidine incorporation (cpm)	Stimulation index	Stimulation index (mean of 3 experiments)
A. 0.4			908		
0.4		2	812	89	110
0.4		5	955	105	100
0.4		10	911	100	101
1			1,644		
1		2	1,452	88	96
1		5	1,498	91	122
1		10	1,314	80	97
4			2,911		
4		2	3,608	124	103
4		5	3,084	106	125
4		10	3,060	105	119
B. 0.4			937		
0.4	4		1,051	112	93
0.4	10		1,128	120	92
0.4	20		1,105	118	86
1			2,098		
1	4		2,899	138	132
1	10		3,299	157	121
1	20		2,674	128	115
4			1,015		
4	4		1,691	167	135
4	10		1,318	130	179
4	20		1,117	110	158

2×10^6 lymphocytes were used throughout. CPM in control unstimulated cultures ranged from 37-209. The results shown in the far right column are the means obtained from 3 individual experiments, including the one shown in detail.

cellular labeling of lymphocytes after prolonged incubation, and concluded that firm chemical binding between lymphocytes and mercuric ions does not occur.¹⁵ It is, therefore, perhaps not surprising that HgCl_2 -induced lymphocyte transformation is not appreciably affected by RBC or platelets.

The in vitro response of lymphocytes from tuberculin-positive individuals to PPD depends upon the clonal proliferation of a small proportion (circa 1 per cent) of lymphocytes which bear specific receptors on their surfaces for the antigen molecules.¹⁶ Although detailed studies of possible adsorption of PPD by RBC or platelets were not carried out, the lack of potentiation of PPD-induced lymphocyte transformation by RBC is consistent with the presumed lack of PPD receptors of a specific or nonspecific kind on RBC membranes.

Although platelets did not affect PPD-induced lymphocyte transformation at optimal concentrations of antigen, the "sparing" effect which platelets displayed when lymphocytes were cultured in the presence of a supraoptimal, toxic concentration of PPD cannot be explained on the basis of our present knowledge. Such a phenomenon, however, would clearly alter the results

of any dose-response studies in transformation systems of this kind. The fact that human platelets can be made to aggregate in the presence of antigen-antibody complexes¹⁷ may be pertinent to those observations, since such complexes might be formed with antibody present in the autologous plasma used in the tissue culture medium. This possibility is intriguing because it suggests that antigen-antibody complex matrices formed by adsorption onto platelet membranes might enter into the potentiating or "sparing" action of platelets at high antigen doses.

The most complex of the transformation systems studied in the present report is the MLC. We did not use purified lymphocyte preparations because of the known dependence of efficient MLC lymphocyte transformation upon the presence of macrophages.^{18,19} As a result, our starting cell population was much more complex, including large numbers of monocytes and granulocytes. In man, MLC transformation is known to depend exclusively upon differences in cell surface antigens defined by the major histocompatibility locus HL-A.⁷ Although immature red cell precursors and reticulocytes carry HL-A antigens on their cell membranes, mature human RBC lack these antigenic determinants.^{20,21} Thus, the failure of allogeneic RBC to function as a lymphocyte transformation stimulus is not surprising. Human platelets on the other hand, do carry at least some antigens specified by the HL-A locus,²²⁻²⁵ and their failure to stimulate allogeneic lymphocytes is not so easily understood. Perhaps this behavior can be better rationalized if it is recalled that MLC reactions are only weakly, if at all, elicited by lymphocyte antigen preparations that have been fragmented by freezing and thawing or by ultrasound.²⁶ The inability of platelets and RBC to elicit lymphocyte transformation was noted earlier by Bain, Vas, and Lowenstein.²⁷ More recently, dissociated epidermal cells have been found to be potent elicitors of allogeneic lymphocyte transformation, so that this property is by no means exclusively reserved for lymphocyte HL-A surface antigens.²⁸

Since the MLC demonstrates those characteristics required for an immunological response, namely selectivity and specificity, the large number of cells (1-3 per cent)²⁹ involved in histocompatibility reactions of this kind as well as in *in vivo* graft-versus-host reactions³⁰ has prompted the suggestion that the thymus-derived cells involved may in fact be multipotential.³⁰ Möller, however, has offered the alternative explanation that the thymus-derived lymphocytes involved in the MLC response are for the most part cells of very low affinity for the stimulating antigens, and that it is only by virtue of special, and highly concentrated antigen presentation on the cell membranes of intact stimulating cells that such low-affinity cells can be made to respond.²⁶ One could then postulate that the inhibition of MLC lymphocyte transformation by both syngeneic and allogeneic RBC and platelets depends upon the ease with which cell surface interactions of low affinity might be disrupted by competing low-affinity interactions based on surface structural differences, such as those that are believed to cause the phenomenon of "allogeneic inhibition."³⁰ Alternate theories to explain these phenomena, such as the "preoccupation" of macrophages by RBC or platelets, are of course possible.

Lastly, an important point of these studies can be made purely on empirical grounds. There have by now been countless studies of phyto mitogen-induced blood lymphocyte transformation in various human disease states, carried out in the search for some clue to the state of immunological reactivity or responsiveness of the individual. In addition, quantitative use of one-way MLC transformation has been proposed as an index of individual HL-A phenotypes.¹ Since many methods of lymphocyte collection and preparation yield lymphocytes significantly contaminated with RBC and/or platelets, comparative studies of human peripheral blood lymphocyte transfor-

mation in health and disease which do not remove these contaminants or control their number are subject to the most tentative interpretation.

ACKNOWLEDGMENT

The author would like to thank Mrs. Janine Raymond for expert technical assistance.

LITERATURE CITED

1. Albertini, R. J., and F. H. Bach. *J. Exptl. Med.*, 128:639, 1968.
2. Allen, L. W., R. H. Svenson, and S. Yachnin. *Proc. Nat. Acad. Sci. (U.S.)*, 63:334, 1969.
3. Yachnin, S., L. W. Allen, J. M. Baron, and R. H. Svenson. Fourth Leukocyte Culture Conference, O. M. McIntyre, Ed. New York: Appleton-Century-Crofts, Inc., 1971, p. 37.
4. Yachnin, S., L. W. Allen, J. M. Baron, and R. H. Svenson. *Cell. Immunol.*, 00:000, 0000.
5. Börjeson, J., R. Reisfield, L. N. Chessin, P. Welsh, and S. D. Douglas. *J. Exptl. Med.*, 124:859, 1966.
6. Agrawal, B. B. L., and I. J. Goldstein. *Biochem. J.*, 96:23c, 1965.
7. Amos, D. B., and F. H. Bach. *J. Exptl. Med.*, 128:623, 1968.
8. Schöpf, E., K. H. Schulz, and I. Isensee. *Arch. klin. exptl. Derm.*, 234:420, 1969.
9. Janossy, G., and M. F. Greaves. *Lymphocyte Activation. I. Response of "T" and "B" Lymphocytes to Phytomitogens.* (Submitted for publication.) 1971.
10. Douglas, S. D., R. M. Kamin, W. C. Davis, and H. H. Fudenberg. Third Annual Leucocyte Culture Conference, W. O. Rieke, Ed. New York: Appleton-Century-Crofts, Inc., 1969, p. 607.
11. Reisfeld, R. A., J. Börjeson, L. N. Chessin, and P. A. Small, Jr. *Proc. Nat. Acad. Sci. (U.S.)*, 58:2020, 1967.
12. Chessin, L. N., J. Borjeson, P. P. Welsh, S. T. Douglas, and H. L. Cooper. *J. Exptl. Med.*, 124:873, 1966.
13. Barker, B. E., M. A. Lutzner, and P. Farnes. Proceedings of the Third Annual Leucocyte Culture Conference, W. O. Rieke, Ed. New York: Appleton-Century-Crofts, Inc., 1969, p. 588.
14. Jandl, J. H., and R. L. Simmons. *Brit. J. Haemat.*, 3:19, 1957.
15. Caron, G. A., S. Poutala, and T. T. Provost. *Int. Arch. Allergy*, 22:76, 1970.
16. Marshall, W. H., F. T. Valentine, and H. S. Lawrence. *J. Exptl. Med.*, 130:327, 1969.
17. Penttinen, K., A. Vaheri, and G. Myllylä. *Clin. Exptl. Immunol.*, 8:389, 1971.
18. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. *J. Immunol.*, 101:262, 1968.
19. Twomey, J. J., O. Sharkey, Jr., J. A. Brown, A. H. Laughter, and P. H. Jordan, Jr. *J. Immunol.*, 104:845, 1970.
20. Harris, R., and J. D. Zervas. *Nature (Lond.)*, 221:1062, 1969.
21. Kourilsky, F. M., D. Silvestre, J. P. Levy, J. Dausset, M. G. Nicolai, and A. Senik. *J. Immunol.*, 106:454, 1971.
22. Shulman, N. R., J. V. Marder, M. C. Hiller, and E. M. Collier. *Progr. Hemat.*, 4:222, 1964.

23. Dausset, J., and F. T. Rapaport. Transplantation, 4:182, 1966.
24. Colombani, J., M. Colombani, A. Benajam, and J. Dausset. In Histocompatibility Testing, E. S. Curtini, P. L. Mattvez, and R. M. Tosi, Eds. Copenhagen: Munksgaard, 1967, p. 413.
25. Svejgaard, A., F. Kissmeyer-Nielsen, and E. Thorsby. In Histocompatibility Testing, P. I. Terasaki, Ed. Copenhagen: Munksgaard, 1970, p. 153.
26. Möller, G. Cellular Immunol., 1:573, 1970.
27. Bain, B., M. R. Vas, and L. Lowenstein. Blood, 23:108, 1964.
28. Main, R. K., K. C. Cochrum, M. J. Jones, and S. L. Kountz. Nature - New Biology, 229:89, 1971.
29. Wilson, D. B., and P. C. Nowell. J. Exptl. Med., 131:391, 1970.
30. Nisbet, N. W., M. Simonsen, and M. Zeleski. J. Exptl. Med., 129:459, 1969.
31. So, L. L., and I. J. Goldstein. Biochim. Biophys. Acta (Amst.), 165:398, 1968.

DEMONSTRATION AND PURIFICATION OF A SPECIFIC ANTIGEN IN MORRIS HEPATOMA 5123*

By

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The immunologic characteristics of cancer have been under intense investigation during recent years, and antigenic differences between normal and malignant tissue are considered to be key factors in the immunologic approach to cancer therapy. Tumor-specific transplantation antigens (TSTA) have been demonstrated in a number of experimentally-induced tumors,¹⁻⁴ as well as in spontaneous tumors in man.⁵⁻⁷ Previous studies using more indirect methods have pointed out the presence of a tumor-related antigen in the "minimal deviation" hepatoma (Morris, line 5123) and its absence from the normal hepatic tissue of origin.^{8,9} The "minimal deviation" hepatoma was selected because of its close morphologic and biochemical similarities to normal liver tissue, and in the hope that such a study might give some insight into the antigenic characteristics of the early stages of neoplasia. The present studies provide additional and more direct evidence of the presence of a hepatoma-related antigen, and its absence from the normal tissue in which the tumor originated.

A specific antigen related to a minimal deviation hepatoma (Morris, line 5123) has been demonstrated through immunodiffusion and immunoelectrophoretic studies.

MATERIALS AND METHODS

Morris hepatoma tumors of the line 5123 growing in adult male rats of the highly inbred Buffalo strain were removed, minced with scissors and passed through a stainless steel hand-operated homogenizer to remove most of the connective tissue. The homogenate was suspended in ice-cold saline-0.3 M sucrose (1:4 v/v), and cell disruption was carried out by "freezing-thawing" the homogenate in a bath of dry ice-acetone.

Cell membranes were isolated by the method of Mann, et al¹⁰ and were suspended in 0.01 M Tris-HCl - 0.15 M NaCl, pH 8.4, buffer to a concentration of approximately 30 mg of protein per ml of suspension. To this, papain (crude papain type II, Sigma Chemical Co., St. Louis, Missouri) was added in the amount of 1 mg of papain per each mg of membrane protein to be digested. The mixture, which had a final pH of 8.4, was incubated for one hour at 37°C with continuous gentle shaking. Digestion was interrupted by adding iodoacetamide to a final concentration of 0.05 M. The digested membranes were centrifuged in a Spinco preparatory ultracentrifuge, Model L-2 50, at 52,038 x g for one hour. The supernatant was collected and dialyzed against sodium phosphate-sucrose buffer (0.01 M - 0.3 M, pH 6.2) for 24 hours. It was then centrifuged for half an hour as indicated above and concentrated to a volume of 2 to 3 ml in a diaflo cell using a UM-10 filter. Stepwise preparatory fractionation of the final product was carried out on a CM - Sephadex C-25 column previously equilibrated with sodium phosphate buffer (0.01 M phosphate - 0.3 M sucrose, pH 6.2. Column dimensions: 0.9 x 30 cm). Further fractionation

* This report is taken from a paper that appeared in Laboratory Investigation, 26:178, 1972.

was carried out on a Sephadex G-200 column equilibrated with 0.01 M Tris - 0.15 M NaCl, pH 8.4 buffer. Fractions of 3 ml were collected over a period of 5 days.

The same method was used in preparing, solubilizing, and fractionating a normal liver cell membrane fraction from the same strain of rats.

Analytical polyacrylamide disc electrophoresis was carried out by the method of Davies¹¹ using a 4 per cent small pore gel (separating gel). Solutions were prepared as described by Reisfeld and Small,¹² and brought to volume by substituting water for urea.

After polymerization of spacer gel, the tubes were filled to the top with a third layer of gel of the same composition as the large pore gel. The sample was introduced at the interface between the spacer gel and the upper gel, and the upper gel was allowed to polymerize with light for 15 minutes. The entire gel-column measured 5 cm. Electrophoresis was performed at 25°C with a constant current of 2-1/2 ma per tube. Bromophenol blue was used as the tracking dye in all runs. When the tracking dye had reached the end of the lower gel, electrophoresis was discontinued and gels were fixed in 12-1/2 per cent trichloroacetic acid (TCA) for one hour. Fixed gels were stained with 0.05 per cent Coomassie brilliant blue in 12.5 per cent TCA for one hour.¹³ Destaining was completed in 10 per cent TCA over a period of 48 hours, and finally gels were stored in 10 per cent TCA. When better resolution of a particular band was desirable, electrophoresis was allowed to continue for 40 minutes and the current was increased to 5 ma per tube.

Bands were identified by their R_f values. They were estimated by assigning the $R_f = 1.0$ to the moving front of the bromophenol blue and the $R_f = 0.0$ to the beginning of the separating gel.

Unstained gels for further study were frozen immediately after the run and kept frozen until use.

Disc electrophoresis - immunodiffusion were combined in order to study the immunologic behavior of certain protein bands separated by disc electrophoresis. For this purpose, frozen or freshly prepared gels were sliced in half longitudinally. One-half was immediately fixed and stained as described above, while the other half was placed on the surface of a recently prepared agar gel slide with the flat surface against the agar gel. Immediately thereafter the slide was placed in a humid chamber and kept at room temperature overnight so that the protein bands could diffuse out of the acrylamide gel and into the agar gel. After this time, longitudinal troughs were cut on the agar gel and filled with antiserum. The slides were kept at room temperature for 72 to 96 hours for development of the antigen-antibody reaction.

After completion of the reaction, the unstained half of the acrylamide gel was replaced with its stained counterpart and the reaction for each band was observed and recorded.

Antiserum Production

Antiserum to the tumor as well as to the normal liver was produced in a special hybrid strain of rabbits as described by Wissler, et al.¹⁴

Absorption of Antiserum

Absorption of antiserum was performed as follows. Normal serum and homogenates of livers, spleens, lungs, and kidneys from 10 Buffalo rats were pooled separately. To 2 ml of antiserum, 0.75 ml of each pool was added at room temperature, and periodically stirred gently for about one hour. The mixture was kept overnight at 4°C. It was then centrifuged at 2,000 r.p.m.

for 30 minutes and the supernatant was removed. The collected supernatant was tested in gel immunodiffusion plates against normal tissue extracts. The plates were kept at room temperature for 72 to 96 hours under continuous inspection. Absorbed antisera that reacted with normal tissue extracts were absorbed for a second or even a third time. In most cases, however, the second absorption was adequate for complete elimination of any nonspecific reaction with normal tissues. Portions of the reacted antiserum were further treated with embryonic serum and homogenates of embryonic livers, spleens, lungs, and kidneys. The amount of the embryonic tissue homogenates and the absorption procedure were as described above. This antiserum will be referred to as "completely absorbed antiserum."

Immunodiffusion, Immuno-electrophoresis

Immunodiffusion studies were performed on glass microscope slides with wells cut out at a distance of 5 mm from each other. The agar for these studies was prepared by heating 1 per cent ionager No. 2 (Oxoid) in potassium phosphate buffer 0.15 M at pH 7.4 in a boiling water bath for approximately 20 minutes. The hot agar was then centrifuged at 2,000 r.p.m. for 3 minutes to precipitate any nondissolved agar particles, and the clear gel was stored at 4°C.

Immuno-electrophoresis was performed according to the micromethod of Scheidegger,¹⁵ using a micro-immuno-electrophoretic apparatus (National Instrument Laboratories, Inc., Rockville, Maryland).

All protein determinations were by the method of Lowry, et al.¹⁶

RESULTS

After papain digestion of the crude insoluble product, protein determination showed a 16 to 18 per cent recovery of the initial protein concentration. Traces of papain, hemochromogens, and other nonantigenic substances were removed by a stepwise preparatory fractionation of the solubilized product on a CM-Sephadex C-25 column.¹⁷ This resulted in the chromatogram shown in Figure 1. By using a sodium phosphate buffer of increasing ionic strength at a constant pH 6.2, it was possible to recover four distinct fractions, which were then studied by immuno-electrophoresis. It was found that all fractions but the one that emerged second with a buffer of 0.05 molarity, were antigenically active. The immuno-electrophoretograms were developed by using antiserum against the tumor before and after complete absorption. At this step, the immuno-electrophoretic patterns for the crude solubilized product and the various fractions obtained by preparatory fractionation were compared. Both the crude solubilized product and the fraction eluted first from the CM-Sephadex C-25 column which contained about 10 per cent of the starting protein concentration, gave a whole spectrum of precipitin lines with unabsorbed antitumor antiserum. The use of the same antiserum after complete absorption resulted in only one precipitin line (Figure 2).

The first fraction obtained from the preparatory column, which contained the specific precipitin line developed with the absorbed antiserum, was further fractionated on a Sephadex G-200 column. Substantial improvement of the resolution of the emerging fractions was achieved by a considerable reduction in the flow rate of the column. This resulted in the chromatogram shown in Figure 3. Fraction I, contained in tubes 51-61, appeared immediately after the void volume; fraction II emerged in tubes 90-100. Immunodiffusion studies of the collected fractions are shown in Figure 4. It was apparent that both fractions contained antigenically active components

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

when reacted to the tumor antiserum. Fraction I-antiserum gave two precipitin lines while fraction II-antiserum gave three precipitin lines. When the same fractions were developed with the completely absorbed antiserum, only fraction I gave a persistent precipitin line. Cross-reaction studies demonstrated that this precipitin line was due to a component that was present in both the crude and the partially purified materials (Figure 2).

The distribution of protein components after digestion and throughout the fractionation steps, as well as the efficiency of the resolution of the chromatograms, were followed by analytical polyacrylamide disc electrophoresis using a 4 per cent gel and a basic buffer. Papain digestion of the crude material resulted in 15 well-distinguished components (Figure 5). Preparatory fractionation reduced the number to about 12. Following Sephadex G-200 fractionation, fraction I was found to be composed of only two large slowly moving components having R_f values 0.27 - 0.28 and 0.33 - 0.35 respectively, while fraction II contained several smaller and larger components

Figure 2. Immunoelectrophoretic analysis of papain-solubilized tumor-cell membrane fraction. Upper wells: Papain-digested product before any fractionation; Lower wells: First fraction from preparatory fractionation on CM-Sephadex C-25. Troughs: Antiserum to the tumor (A) before absorption; the same antiserum (B) after complete absorption.

Figure 3. Sephadex G-200 fractionation of the first fraction eluted from CM-Sephadex C-25. Eluant 0.01 M Tris-HCl - 0.15 M NaCl, pH 8.4. Flow rate 3.5 ml per hour. Column dimensions 87 x 2.5 cm. Fraction I, tubes 51-61; fraction II, tubes 90-100.

Figure 4. Gel immunodiffusion test. Central wells: Antitumor antiserum (A) before absorption; and (B) after complete absorption. Peripheral wells: 1, fraction I from Sephadex G-200 fractionation; 2, fraction II from Sephadex G-200 fractionation.

Figure 5. Analytical polyacrylamide disc electrophoresis on 4 per cent gel. Patterns obtained following analysis of (A) papain-solubilized tumor cell membrane fraction before any fractionation; (B) after preparatory fractionation on CM-Sephadex C-25; (C) and (D) fractions I and II from Sephadex G-200 respectively. Antigenically-active component specific for the tumor (arrows).

of various mobilities. From Figure 4 it is apparent that both components in fraction I are antigenically active. In addition, only the one component reacted with antitumor antiserum which was exhaustively absorbed with extracts of adult and embryonic organs. No such reaction occurred upon testing fraction II under identical conditions (Figure 4).

The antigenic specificity of the protein components appearing in fraction I was further tested by combining analytical disc electrophoresis and immunodiffusion. Figure 6 clearly shows that only the more slowly moving component with an R_f value 0.27 - 0.28 reacted with the completely absorbed antiserum. This reaction further supports the tumor specificity of this component.

Figure 6. Disc electrophoresis-immunodiffusion. Upper trough: Completely absorbed antiserum to the tumor. Lower trough: Antiserum to the tumor before any absorption. Note the reaction of the more slowly moving component with the completely absorbed antiserum (arrows).

The homogeneity of this specific component was tested by studying fraction I in polyacrylamide gels of increasing concentration and in 6 to 8 per cent urea. Under these conditions the specific components gave only one well-defined band (Figure 7).

DISCUSSION

The method of isolating cell membranes used in the present study¹⁰ was initially introduced for the isolation of mouse H-2 isoantigens.^{18,19} It was successfully applied in the extrac-

Figure 7. Analytical polyacrylamide disc electrophoresis in 6 per cent urea. (A) Crude solubilized product; (B) Fraction I from Sephadex G-200 fractionation. After the tracking dye had reached the end of the gel, electrophoresis was continued for 40 minutes and the current was increased to 5 ma per tube. Note the specific component (more slowly moving band, arrow) moving as a distinct individual band.

tion of transplantation antigens from human lymphoid tissues^{10,20} and in studies of membrane-associated tumor-specific antigens from rat hepatomas.²¹

Our experience shows that the isolation procedure is of paramount importance in preserving the antigenic specificity of the final product. Any harsh treatment, particularly during cell disruption is detrimental to the antigenic sites of the cell membranes. It was for this reason that the "freezing-thawing" technique for cell disruption was used throughout the present study.

The use of papain for the solubilization of membrane proteins introduced by Shimada and Nathenson^{22,23} was found to be superior to sonic disruption and the use of various detergents. When temperature, pH, and time of exposure to papain were controlled the results were repro-

ducible. During the present study, however, a certain degree of variation in the position of fraction II during Sephadex G-200 fractionation was found to be dependent upon the flow rate. This was consequently adjusted to 3.5 ml per hour, which gave an excellent resolution.

The absolute tumor-specificity of the detected antigenic component is a crucial question. It would imply the complete absence of this antigen from normal adult tissues as well as from embryonic tissues. Both questions have been at least partly answered by the present study. The antigen does not seem to be an isoantigen present in the tissues or the blood. The exhaustive absorption of the tumor antiserum with extracts of various tissues of the experimental animals and their sera was designed to eliminate such an antigenic determinant. The same conclusion may be drawn as to the possible embryonic origin of the detected antigen. The antigen is not extractable with perchloric acid; consequently it seems to be different from the carcinoembryonic antigen described by Gold and Freedman.²⁴ Since it is not absorbed with extracts of embryonic organs it is unlikely that it is similar to the alpha fetal globulin antigen present in mice²⁵ and rats²⁶ until the fourth week of life.²⁷ It should be mentioned that whenever completely absorbed antiserum was used in the present study the specific precipitin line obtained was weaker than that obtained with the unabsorbed antiserum (Figure 4). This was interpreted as reflecting the high dilution of the completely absorbed antiserum rather than any identity between normal liver and tumor-specific antigen. This is further supported by cross-reaction studies using solubilized liver antigen as well as antiserum prepared against normal liver tissue. Repeated cross-reaction tests failed to show any evidence of cross-reactivity between tumor specific antigen and normal liver components (Figure 8).

One may still challenge the definition of tumor specificity on the grounds of quantitative rather than qualitative differences between tumor and normal tissues. At present this challenge cannot be rejected completely. Although the methods used in the present study are characterized by a high degree of sensitivity it should be remembered that there is a threshold of sensitivity for any method, beyond which detection of a component is impossible. An antigenic component that was present in undetectable amounts in normal tissues might still be found in unusually high levels in tumors. We investigated this possibility by further diluting the final purified product while concentrating to a high degree the extracts of various tissues used for the absorption of antitumor antiserum. In neither case were we able to eliminate the specific precipitin line. Furthermore, concentrated normal liver cell membrane fraction did not show any reaction with the completely absorbed antiserum (Figure 8). Here, the production of an antiserum that would react specifically with the purified product would have been particularly valuable in clearly defining the specificity of the antigenic component. Unfortunately, the yield of the purified material has been too low to permit such a study.

Another possibility is that the present antigen is a true embryonic antigen, which appeared very early in embryonic life and persisted only for a very short time. Even if this hypothesis were correct, the fact that the antigenic component under discussion was able to elicit delayed-type-hypersensitivity and inhibit the migration of sensitized peritoneal macrophages, as was previously shown,⁸ is of particular importance.

The homogeneity of the specifically active component was studied in gels of increasing concentration and/or in the presence of 6 to 8 per cent urea. Increasing concentrations of gel would be expected to delay larger fragments, while more mobile components would move much faster. In addition, electrophoresis in 6 M urea should disrupt any protein-protein interaction thus re-

Figure 8. A. Immunoelectrophoretic analysis of papain solubilized tumor-cell membrane fraction diluted 10 times (upper well), and normal liver-cell membrane fraction concentrated 10 times (lower well). Trough: Antiserum to the tumor after complete absorption. The trough was filled twice in the first 5 hours, and the reaction was allowed to proceed for 5 days. Agar slides were scrutinized every 24 hours by using a dark field illumination. After the completion of the reaction slides were stained and photographed. Double arrows point at the specific reaction of the "tumor-cell membrane fraction - completely absorbed antitumor antiserum." No reaction was observed in the system "solubilized concentrated normal liver-cell membrane fraction - completely absorbed antitumor antiserum." Single arrows point at staining artefacts.

B and C. Gel immunodiffusion test. Upper wells: Purified tumor antigen. Lower wells: Left, antitumor antiserum; right, antinormal liver antiserum. Twenty-four hours after the initiation of immunodiffusion (B), and 5 days later (C).

solving any heterogeneous complex into its individual components.²⁸ Under these specific experimental conditions the specific component remained as a distinct individual band (Figure 7).

The ability to liberate and highly purify this antigen in soluble form should facilitate its isolation and characterization. Work now in progress is aiming at its isolation.

LITERATURE CITED

1. Cryan, W. S., R. M. Hyde, and S. Garb. *Cancer Res.*, 26:1458, 1966.
2. Heppner, G. H., and G. Pierce. *Int. J. Cancer*, 4:212, 1969.
3. Isojima, S., Y. Yagi, and D. Pressman. *Cancer Res.*, 29:140, 1969.
4. Kahan, B. D., E. C. Holmes, R. A. Reisfeld, and D. L. Morton. *J. Immunol.*, 102:28, 1969.
5. Hughes, L. E., and B. Lytton. *Brit. Med. J.*, 1:209, 1964.
6. Itakura, K. *Gann*, 54:93, 1963.

7. McKenna, J. M., R. P. Sanderson, and W. S. Blakemore. *Science*, 135:370, 1962.
8. Kyriazis, A. P., R. W. Wissler, and K. Dzoga. *Proc. Soc. Exptl. Biol. Med.*, 136:75, 1971.
9. Wissler, R. W., K. Craft, D. Kesden, B. Polisky, and K. Dzoga. In *Advance in Transplantation*, J. Dausset, J. Hamburger, and G. Mathe, Eds. Baltimore: The Williams and Wilkins Co., 1968, p. 539.
10. Mann, L. D., G. N. Rogentine, and J. L. Fahren. *Nature (London)*, 217:1180, 1968.
11. Davies, B. J. *Ann. N. Y. Acad. Sci.*, 121:404, 1964.
12. Reisfeld, R. A., and P. A. Small, Jr. *Science*, 152:1253, 1966.
13. Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari. *Anal. Biochem.*, 20:150, 1967.
14. Wissler, R. W., R. A. Baxter, M. M. Flax, M. F. La Via, and D. W. Talmage. *Cancer Res.*, 16:761, 1956.
15. Scheidegger, J. J. *Int. Arch. Allergy*, 7:103, 1955.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. *J. Biol. Chem.*, 193:265, 1951.
17. Stejskal, R. Personal communications.
18. Haughton, G., and D. A. L. Davies. *Brit. J. Exptl. Path.*, 43:488, 1962.
19. Nathenson, S. G., and D. A. L. Davies. *Ann. N. Y. Acad. Sci.*, 129:6, 1966.
20. Sanderson, A. R., and J. R. Batchelor. *Nature (London)*, 219:184, 1968.
21. Baldwin, R. W., and M. Moore. *Nature (London)*, 220:287, 1968.
22. Nathenson, S. G., and A. Shimada. *Transplantation*, 6:662, 1968.
23. Shimada, A., and S. G. Nathenson. *Biochem. Biophys. Res. Commun.*, 29:826, 1967.
24. Gold, P., and S. O. Freedman. *J. Exptl. Med.*, 122:467, 1965.
25. Abelev, G. I., S. D. Perova, N. I. Khramkova, Z. A. Postnikova, and I. S. Irlin. *Transplantation*, 1:174, 1963.
26. Stanislawski-Birencwajg, M., C. Frayssinet, and P. Grabar. *Arch. Immunol. Ther. Exp.*, 14:730, 1966.
27. Zilber, L. A., and G. I. Abelev. *The Virology and Immunology of Cancer*. Long Island City, New York: Pergamon Press, 1968.
28. Potts, J. T., Jr., R. A. Reisfeld, P. F. Hirsch, A. B. Wasthed, E. F. Voelkel, and P. L. Munson. *Proc. Nat. Acad. Sci. (U.S.)*, 58:328, 1967.

BILE ACID SULFATES: FORMATION, METABOLISM, AND EXCRETION OF LITHOCHOLIC ACID SULFATES IN THE RAT*

By

R. H. Palmer

Lithocholic acid is an endogenous human bile acid that possesses extensive biological toxicity. The implications of this toxicity, which includes the capacity to produce fever and inflammation,^{1,2} liver damage^{1,3} and tissue injury^{1,3,4} for human disease have been discussed previously.^{1,5-7} The metabolism of lithocholates, therefore, has relevance to human biology and disease.

In previous studies, sulfation was shown to be an important aspect of lithocholate metabolism in man.⁸⁻¹⁰ The present series of experiments was undertaken to answer the following questions: (1) Does sulfation of lithocholate also occur in the rat, and if so, is intestinal absorption a requisite for sulfation? (2) Can sulfated bile salts be absorbed from the rat intestine in vivo? (3) Does sulfation alter the biochemical pathways of lithocholate metabolism in vivo? (4) Does sulfation affect the route and rate of lithocholate excretion? The results demonstrate that sulfation of lithocholates does occur in rats, with consequent alterations in their patterns of metabolism and excretion.

METHODS

Thin-layer chromatography (TLC) was performed with Silica Gel H (Brinkmann Instruments) and the following phase systems: Butanol 1:¹¹ *n*-butanol 50, acetic acid 5, water 5 (pH 1); Butanol 3:^{9,10} *n*-butanol 50, 0.01 M tris buffer 9.25, propionic acid 0.75 (pH 3.0); T40:^{8,12} trimethyl pentane 40, ethyl acetate 20, acetic acid 0.5; S15:¹² trimethyl pentane 25, ethyl acetate 25, acetic acid 0.25; S11:¹² trimethyl pentane 25, ethyl acetate 25, acetic acid 5.0.

Lithocholic acid-24-¹⁴C (7.58 mC/mM, New England Nuclear Corp.) was shown to be > 99 per cent pure by autoradiography following TLC with system T-40. The glycine conjugate was prepared¹³ and esterified with sulfuric acid as described previously,¹⁰ except that the reaction mixture was purified by preparative TLC, Butanol 3 system. Samples of urine, bile, and fecal extracts were counted in a Nuclear Chicago liquid scintillation counter; quenching was not appreciable, and corrections were not made. Autoradiography of chromatograms was performed with RP-54 X-Omat Medical X-ray Film (Eastman Kodak), and quantitated with a Zeiss PMQ II chromatogram scanner.¹⁴

* This report is taken from a paper that appeared in *J. Lipid Res.*, 12:680, 1971.

The following trivial names and abbreviations have been employed: lithocholic acid, 3 α -hydroxy-5 β -cholanolic acid; glycolithocholic acid (G.L.), 3 α -hydroxy-5 β -cholanoyl glycine; taurolithocholic acid (T.L.), 3 α -hydroxy-5 β -cholanoyl taurine; chenodeoxycholic acid, 3 α ,7 α -dihydroxy-5 β -cholanolic acid; deoxycholic acid, 3 α ,12 α -dihydroxy-5 β -cholanolic acid; cholic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholanolic acid; β -muricholic acid, 3 α ,6 β ,7 β -trihydroxy-5 β -cholanolic acid; GLS, glycolithocholic acid-C-3 sulfate; TLS, taurolithocholic acid-C-3 sulfate; LS, lithocholic acid-C-3 sulfate; "sulfate" refers to the 3-sulfate ester of the corresponding bile acid unless otherwise indicated.

Solvolysis of sulfates and strong alkaline hydrolysis of amino acid conjugated bile acids was performed as described previously.¹⁰

EXPERIMENTAL DESIGN

Three experimental approaches were utilized. In the first, the ability of bile fistula rats to form sulfated lithocholates was examined. In the second, bile fistula rats were used to determine whether a sulfated lithocholate could be absorbed from the intestine in vivo. In the third, the biological disposition of a sulfated bile salt in intact rats was studied.

A. Sulfation of lithocholic acid-24-¹⁴C in rats. Three bile fistula rats received lithocholic acid-24-¹⁴C; bile was collected in hourly fractions, the isotope content was determined, and the labeled derivatives were analyzed. Rat F received 10.8×10^6 cpm through a gastric tube, and bile was taken for analysis from an initial small peak during the second hour, and from a second large peak during the fiftieth hour. Rat G received lithocholate-24-¹⁴C intraperitoneally. Most of the isotope appeared in the bile within three hours, and bile from the first hour was analyzed. Rat H was provided with a cannula in the duodenum, so that bile could be removed or infused back into the duodenum at will. The rat was kept in a restraining cage with food and water ad lib. After 24 hours with an intact enterohepatic circulation, bile was diverted to a fraction collector and replaced with a constant infusion of saline at an equal rate. The rat then received intra-peritoneal injections of lithocholic acid-24-¹⁴C (approximately 1×10^6 cpm) at the beginning of biliary diversion (H-24), after 16 hours of diversion (H-40), and 24 hours after restoration of an intact enterohepatic biliary circulation (H-72). Bile from all six samples was plated directly on Silica Gel H and chromatographed with the Butanol 3 system. Quantitative autoradiography was used to determine the distribution of isotope among four major groups of compounds (see Table 1). Various groups were then eluted with chloroform:methanol 1:1 and rechromatographed on appropriate systems as indicated.

B. Intestinal absorption of glycolithocholic acid-24-¹⁴C-3-sulfate. Two bile fistula rats were prepared, and bile was collected and analyzed as under A. The first rat received approximately 7×10^4 cpm G.L.S. in 0.5 ml 50 per cent ethanol by injection into the duodenum. Because of some seepage through the injection site in the first few seconds, the second rat received approximately 11×10^6 cpm G.L.S. in 3.0 ml 30 per cent ethanol through a small polyethylene stomach tube. Bile, urine and feces from the second rat were collected separately and the total excretion of isotope in bile, urine, and 80 per cent ethanol extracts of feces was determined. The chemical form of the isotope recovered in bile was inferred from its chromatographic behavior on TLC with the Butanol 1 and Butanol 3 systems; that in fecal extracts with the Butanol 3 and S-11 systems.

C. Biological disposition of lithocholic acid-24-¹⁴C and glycolithocholic acid-24-¹⁴C-3-sulfate in intact rats. Each of a group of three female Sprague-Dawley rats received lithocholic acid-24-¹⁴C (approximately 2×10^6 cpm) orally by intubation in 1.5 ml 30 per cent ethanol. A second group of rats received similar amounts of glycolithocholic acid-24-¹⁴C-3-sulfate. The rats were kept in individual metabolism cages, with food and water ad lib, and urine and feces were collected separately. Feces were extracted by refluxing twice with 80 per cent ethanol. Excretion of radioactivity was essentially complete after 9 days; the total amount recovered in feces was used to calculate the biological half-life ($T_{1/2}$), assuming that excretion of isotope could be described by the equation $\log Y = -kt$, where Y = the fraction of total radioactivity re-

Table 1

BILIARY METABOLITES OF LITHOCHOLIC ACID-24-¹⁴C IN BILE FISTULA RATS

Rat and hour of collection Type of injection [§]	Distribution of radioactive metabolites in bile %					
	F-2 I.G.	F-50 I.G.	G-1 I.P.	H-24* I.P.	H-40† I.P.	H-72‡ I.P.
Group I compounds - conjugated and sulfated	9	2	7	2	3	3
Group II compounds - conjugated, not sulfated	87	93	93	98	97	97
β-muricholic acid	15.5	73.0	18.2	38.0	51.0	56.0
3α,6β-dihydroxy-5β-cholanic acid	9.6	14.5	21.1	47.6	26.7	25.6
Chenodeoxycholic acid	29.0	2.1	20.4	2.0	9.0	4.1
Unknown	-	3.4	-	-	-	-
Lithocholic acid	33.0	tr.	33.3	10.4	10.3	11.3
Group III compounds - not conjugated, sulfated (Lithocholic acid sulfate)	-	0.5	-	-	-	-
Group IV compounds - not conjugated, not sulfated (Free bile acids)	4.0	4.5	-	-	-	-

* Injected at hour 24; enterohepatic circulation intact for 24 hours after operation.

† Injected at hour 40; after 16 hours of biliary diversion.

‡ Injected at hour 72; enterohepatic circulation reconstituted 24 hours previously.

§ I.P., intra-peritoneal; I.G., intra-gastric instillation.

covered from the feces remaining in the body at any time (t).^{*} The chemical form of the isotope in the fecal extracts was identified by its chromatographic behavior, using autoradiography for qualitative and quantitative analysis of thin-layer chromatograms.

RESULTS

A. Sulfation of lithocholic acid-24-¹⁴C in rats. Following the administration of labeled lithocholic acid to bile fistula rats, labeled compounds having the chromatographic mobility of glyco- or tauro lithocholic acid sulfate were observed in all six samples of bile obtained from three rats. As shown in Table 1, the amounts were small, constituting less than 10 per cent of the labeled metabolites in all cases. Further confirmation of the presence of sulfated derivatives of lithocholic acid was provided by relatively specific sequential degradation procedures. Compounds in Group I (Table 1) were eluted and treated with cholyglycine hydrolase according to Nair.¹⁵ This resulted in conversion of a large fraction of the isotope to a compound with the unusual and distinctive chromatographic mobility (Butanol 3 system) of lithocholic acid sulfate.¹⁰

* Regression lines were calculated for the data from each animal, using the method of least squares and a computer program kindly supplied by Dr. John Skosey.

Following elution of this spot and solvolysis,¹⁰ 73 per cent of the radioactivity had the same mobility as reference lithocholic acid when chromatographed with system S 15.*

In another approach, the Group I compounds from sample F-50 were subjected first to solvolysis, followed by strong alkaline hydrolysis. TLC (system S 11) revealed several small peaks, as shown in Figure 1, but the major peak at the front appeared to be a single compound with the mobility of lithocholic acid when re-run with system T-40.

Figure 1. Sulfated metabolites of lithocholic acid-24-¹⁴C in rat bile. The conjugated sulfate fraction was subjected to solvolysis, strong hydrolysis and chromatography with system S 11. Radioactivity, determined by autoradiography is quantitated below. Hatched areas are reference bile acids: (C), cholic; (3 α ,6 α), hydrodeoxycholic; (3 α ,6 β), 3 α ,6 β -dihydroxycholic; (CD), chenodeoxycholic; (D), deoxycholic; (L), lithocholic.

Analysis of Group II compounds by elution, strong alkaline hydrolysis, and chromatography with system S-11 showed an unremarkable pattern of expected murine metabolites of lithocholic acid (Table 1). Quite clearly, the distribution of metabolites in the Group I compounds (sulfates) shown in Figure 1 differs from that of the Group II compounds, the former being primarily monohydroxy cholanic acids and the latter being primarily dihydroxy- and trihydroxy cholanic acids.

B. Intestinal absorption of glycolithocholic acid-24-¹⁴C-3-sulfate. When glycolithocholic acid-24-¹⁴C was placed in the intestinal tracts of bile fistula rats, approximately 70 per cent of the administered dose was recovered in the bile. The major portion of the isotope was excreted 10-20 hours following its administration (Figure 2). In the second rat, the total isotope recovered was approximately 80 per cent of the administered dose; of the recovered isotope, 88.9 per cent was from bile, 11.0 per cent from feces, and 0.1 per cent from urine. Chromatographic analysis

* The identity of the remaining isotope derived from the sulfated fraction was not established, but it should be noted that conditions for either complete enzymatic hydrolysis of amino acid conjugates or complete solvolysis have not been established; and we have been unable to obtain complete enzymatic hydrolysis of reference compounds.¹⁰ Therefore, failure to obtain complete conversion of Group I labeled compounds to lithocholic acid need not imply the presence of significant amounts of other compounds.

Figure 2. Absorption and excretion of sulfated bile acids in vivo. Glyco-lithocholic acid-24- ^{14}C -3 sulfate was administered to bile fistula rats by intra-duodenal injection (top) or intra-gastric instillation (bottom). Excretion of radioactivity with time: solid line. Distribution of labeled metabolites determined at points indicated: lithocholic acid sulfate, stippled area; glycolithocholic acid sulfate, white area; tauro lithocholic acid sulfate, hatched area.

of the chemical form of the isotope in bile showed that most of it was G.L.S. Isotope appearing later in bile contained an increasing fraction as L.S. or T.L.S. All the isotope collected from bile had the chromatographic mobility of sulfated monohydroxycholates; no isotope was found in the nonsulfated derivative areas and no polyhydroxylated derivatives were observed.

Approximately 29 per cent of the isotope in the feces of the second rat was found in the sulfated fraction. Analysis of the nonsulfated fraction showed that 10 per cent of the isotope had the mobility of monohydroxycholates, 10 per cent had the mobility of 3-ketocholates, 3 per cent ran slightly behind (more polar than) cholic acid, and 48 per cent ran slightly ahead of (less polar than) cholic acid. Mild alkaline hydrolysis shifted most of the latter (front) peak to the peak just behind cholic acid, consistent with its being an ethyl ester formed during the extraction process.

C. Biological disposition of lithocholic acid-24- ^{14}C and glycolithocholic acid-24- ^{14}C -3-sulfate in intact rats. The disposition of sulfated and nonsulfated lithocholates differed in both route and rate of excretion. Rats fed labeled lithocholate excreted an average of only 6 per cent (3.3 per cent, 10.5 per cent, 4.2 per cent) of the administered isotope in the urine, whereas those fed the sulfated glycine conjugate excreted 23.8 per cent (20.2 per cent, 24.8 per cent, 26.4 per cent) of the administered isotope in the urine.

Fecal excretion of isotope was more rapid in rats fed the sulfated glycine conjugate than in those fed the free lithocholate. In both groups, fecal excretion of radioactivity was satisfactorily log-linear during the time required for more than 90 per cent excretion; after this time, it was considered that the probability of extensive microbial transformations and the low levels of

radioactivity involved made further analysis unjustified. It is clear, however, that during the period when most of the isotope was being excreted, the rate of excretion was greater in the rats given G.L.S. than in those given lithocholate. The mean biological half-life ($T_{1/2}$) for G.L.S. was found to be .78 days (.66, .73, .94), while the mean half-life for lithocholate was 1.30 days (1.04, 1.56, 1.30). The difference in k values and half-lives of the two groups was statistically significant (Student's t -test: $t = 3.023$, $p < .05$). The results from representative animals in each group are shown in Figure 3.

Figure 3. Fecal excretion of a sulfated and a non-sulfated ^{14}C -labeled bile salt in two representative rats. The total isotope excreted in the feces was used to calculate the rate of excretion of lithocholate-24- ^{14}C (L-3) and glycolithocholate-3-sulfate-24- ^{14}C (G-1). $T_{1/2}$ for L-3 was 1.304 days; $T_{1/2}$ for G-1 was .657 days.

Finally, two rats from each group were arbitrarily chosen, and the isotope excreted on the first and fourth day was subjected to chromatographic analysis. During Day 1, one rat (G-2) fed the sulfate excreted 15 per cent of the label as L.S. and about 5 per cent as G.L.S., whereas the other rat (G-1) excreted only a trace of sulfated material, mainly L.S. In each case, the remainder of the radioactivity was in the form of free bile salts: mainly lithocholate, isolithocholate, 3-ketocholanate, with traces of more and less polar metabolites. By Day 4, most of the label was in the dihydroxy and trihydroxy areas on TLC.

Rats fed lithocholate excreted a considerably greater proportion of the label as dihydroxy and trihydroxy bile salts during Day 1. No sulfated compounds were observed. During Day 4, marked differences were seen between the two lithocholate rats. Rat L-1, with the shortest $T_{1/2}$ of the three, excreted mostly di- and trihydroxy metabolites and virtually no monohydroxy bile salts, whereas rat L-2, with the longest $T_{1/2}$, excreted lithocholate predominantly, with only traces of polyhydroxylated compounds. No bile salts less polar than lithocholate were observed in either rat.

DISCUSSION

The many toxic properties of lithocholic acid make it important to understand the metabolism, physiological disposition, and pharmacological properties of this compound and its metabolites. In the preceding communication, the role of sulfation in the metabolism of lithocholic acid in humans was described.¹⁰ The present studies (part A) demonstrate that sulfate esters of lithocholic acid can also be formed in the rat, and suggest that this experimental animal could be used for further investigations on the site or sites and mechanism of sulfation. The significance of this observation lies not in the extent of sulfation in this species, which is small, but in the fact that it occurs at all, so that the enzymatic mechanism must be present. This metabolic pathway is probably quantitatively less important in the rat than in the human because hydroxylated derivatives of lithocholic acid, which are the expected products of lithocholate metabolism in rat liver¹⁶ but are not formed in the human liver, do not seem to undergo sulfation to any great extent. Similarly, it is of interest that sulfated lithocholates do not appear to undergo hydroxylation either, since significant amounts of polyhydroxylated bile acids were not isolated from the sulfate fractions in any of the present experiments. Hence, hydroxylation and sulfation may be alternative mechanisms for processing the less water-soluble steroids.

Identification of sulfated derivatives in the bile of rats that had received labeled lithocholate by intra-peritoneal injections suggests that sulfation probably occurs in the liver, although other tissues, such as the intestinal mucosa, may also have this capability.

Current information on the physiological disposition (intestinal absorption and excretion) of lithocholic acid indicates that it can certainly be absorbed from the intestinal tract of man and experimental animals, but the quantitative aspects of the processes involved are poorly documented. Lithocholic acid formed endogenously is considerably less well absorbed than its dihydroxy counterpart, deoxycholic acid, but whether this is a result of the physical properties of these molecules or whether it reflects the further microbial transformation of lithocholate to even less soluble derivatives⁸ remains unclear. Any lithocholate, or its taurine or glycine conjugates, that reach the upper intestine by ingestion or via the enterohepatic circulation can presumably be absorbed by ionic or non-ionic monomeric diffusion,^{17,18} although their relative water insolubility may limit access to the mucosa. These same compounds can also probably be absorbed by the active transport system in the terminal ileum, although their water insolubility may also limit this pathway of absorption in vivo just as it has complicated attempts to study their affinity for the transport system in vitro. The relative contributions of these two pathways to the absorption of lithocholates has not been evaluated, although it is a matter of some importance in considering their biological role. For example, rapid absorption of lithocholates in the upper intestine would short-circuit the normal enterohepatic circulation and effectively increase the exposure of the liver to these compounds.

Sulfation of lithocholic acid and its conjugates alters their physical properties by greatly enhancing their water solubility.¹⁹ The sulfate group, constituting a second and fully ionized polar group, would be expected to abolish non-ionic absorption from the intestine, and might inhibit ionic diffusion because of its size and position. Conversely, the increased water solubility of sulfated lithocholates might facilitate access to the mucosa, thus enhancing absorption by ionic diffusion and delivery to the active ileal transport system. There, the rate of absorption might depend on the relative affinity of sulfated lithocholates for the transport system, and the extent to which other bile salts compete for the uptake sites.

The experiments described in Part B demonstrate that a representative sulfate, G.L.S., can be absorbed from the intestinal tract and excreted in the bile. However, the time lag of 8-12 hours prior to the major peak of isotope excretion in bile suggests that passive absorption in the upper intestine was minimal, and that the major route of absorption was by the active ileal transport mechanism. This hypothesis (active ileal transport but little or no passive jejunal absorption of sulfated lithocholates) has been strongly supported by the detailed studies of Lack on the in vivo and in vitro absorption of sulfated and non-sulfated lithocholates in the guinea pig.²⁰

The results in Part B also strongly suggest that the sulfated lithocholates were absorbed without disruption of the sulfate linkage, for had the sulfate moiety been removed, the results from Part A would indicate that less than 10 per cent of the labeled lithocholates could have been expected to be re-sulfated, whereas in fact all of the isotope appearing in bile was found in the sulfate fraction.

Conversely, there was a progressively increasing fraction of the excreted isotope that had lost the glycine moiety and was either re-conjugated with taurine and excreted as T.L.S., or excreted without amino acid conjugation as L.S. Thus sulfation does not prevent microbial deconjugation in vivo, although the possibility of altered enzyme kinetics remains. Clearly, sulfated taurine and glycine conjugates of lithocholic acid can serve as substrates for the cholyglycine hydrolase of Nair¹⁵ in vitro, as demonstrated in this and the preceding report,¹⁰ but no kinetic data are available for sulfated compounds.

The excretion studies described in Part C deserve some preliminary comments. Glycolithocholic acid sulfate was chosen arbitrarily to facilitate differentiation of administered isotope from in vivo metabolites recovered from urine and feces. However, as demonstrated in Part B, variable hydrolysis of the glycine moiety and variable re-conjugation with taurine must be anticipated. Similarly, lithocholic acid can be assumed to have been almost completely conjugated with taurine on its first passage through the liver. Thus the studies probably compare the excretion of sulfated glyco- and tauroolithocholate with non-sulfated tauroolithocholate. If there were any significant difference between the physiological disposition of isotope in the two groups attributable to the state of amino acid conjugation at the carboxyl group, the difference should have favored enhanced excretion of the taurine conjugate,^{17,18,20-24} whereas these studies demonstrated enhanced excretion of isotope in the G.L.S. group. This suggests that the observed differences were in fact due to the presence of the 3-sulfate group.

The renal excretion of sulfated lithocholates was larger than would be anticipated in the absence of jaundice, and was of such magnitude (24 per cent of the administered radioactivity) that it should be considered in any discussion of the physiological disposition of lithocholates in general. Bile salts that escape hepatic extraction from portal vein blood pass into the systemic circulation and, if not protein bound, through the renal glomeruli. They then appear to be actively reabsorbed in the proximal tubules, so that bile salts are not normally present in the urine in large amounts.²⁵ Whether the increased excretion of sulfated lithocholates observed in these experiments was due to (a) decreased protein-binding and consequent increased filtration, as seems probable on the basis of solubility studies, (b) to decreased reabsorption, as seems likely by analogy with intestinal reabsorption studies, (c) to some other unknown mechanism, or (d) to some combination of these remains an open question. Increased exposure of renal tissue to sulfated lithocholates could be of pathological importance, if studies on the pharmacological properties of these compounds demonstrate appreciable toxicity.

The reason for enhanced fecal excretion of sulfated lithocholates is not obvious, although several possibilities clearly exist. Irrespective of the mechanism, however, sulfation apparently acts to deplete the circulating pool of lithocholates by enhancing fecal excretion as well as urinary excretion. Sulfation may thus constitute a second explanation, in addition to the extensive microbial transformations previously described⁸ for the relative paucity of lithocholates in bile.

LITERATURE CITED

1. Palmer, R. H., P. B. Glickman, and A. Kappas. *J. Clin. Invest.*, 41:1573, 1962.
2. Palmer, R. H. *Proc. Soc. Exptl. Biol. Med.*, 119:108, 1965.
3. Holsti, P. *Nature (London)*, 186:250, 1960.
4. Palmer, R. H. *Nature (London)*, 201:1134, 1964.
5. Kappas, A., and R. H. Palmer. *Pharmacol. Rev.*, 15:123, 1963.
6. Palmer, R. H., and A. Kappas. *Med. Clin. N. Amer.*, 47:101, 1963.
7. Palmer, R. H., and Z. Hruban. *J. Clin. Invest.*, 45:1255, 1966.
8. Norman, A., and R. H. Palmer. *J. Lab. Clin. Med.*, 63:986, 1964.
9. Palmer, R. H. *Proc. Nat. Acad. Sci., U.S.*, 58:1047, 1967.
10. Palmer, R. H., and M. G. Bolt. *J. Lipid Res.*, 12:671, 1971.
11. Gänshirt, H., F. W. Koss, and K. Morianz. *Arzneimittel-Forsch.*, 10:943, 1960.
12. Eneroth, P. *J. Lipid Res.*, 4:11, 1963.
13. Norman, A. *Arkiv. Kemi*, 8:331, 1955.
14. Raderecht, H. J., and S. Binewies. *Clin. Chim. Acta*, 7:873, 1962.
15. Nair, P. P., M. Gordon, and J. Reback. *J. Biol. Chem.*, 242:7, 1967.
16. Thomas, P. J., S. L. Hsia, J. T. Matschiner, E. A. Doisey, Jr., W. E. Elliott, S. Thayer, and E. A. Doisey. *J. Biol. Chem.*, 239:102, 1964.
17. Dietschy, J. M., H. S. Salomon, and M. D. Siperstein. *J. Clin. Invest.*, 45:832, 1966.
18. Small, N. C., and J. M. Dietschy. *Gastroenterology*, 54:1272, 1968.
19. Small, D. M., and W. Admirand. *Nature (London)*, 221:265, 1969.
20. Low-Beer, T. S., M. P. Tyor, and L. Lack. *Gastroenterology*, 56:721, 1969.
21. Lack, L., and I. M. Weiner. *Am. J. Physiol.*, 210:1142, 1966.
22. Failey, R. B., Jr. *J. Lab. Clin. Med.*, 56:809, 1960.
23. Gordon, B. A., A. Kuksis, and J. M. R. Beveridge. *Canad. J. Biochem.*, 42:897, 1964.
24. Hislop, I. G., A. F. Hofmann, and L. J. Schoenfield. *J. Clin. Invest.*, 46:1070, 1967.
25. Weiner, I. M., J. E. Glasser, and L. Lack. *Am. J. Physiol.*, 207:964, 1964.

BILE SALT DEPLETION IN THE RUNTING SYNDROME*

By

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The runting syndrome in mice is a graft-versus-host reaction induced by the injection of immunologically competent parental cells into newborn F_1 hybrids.¹ Wasting and diarrhea are prominent features of the syndrome, and pathological changes have been described in the intestine.^{2,3} Although runted animals are capable of absorbing monosaccharides such as glucose, lactose malabsorption occurs due to a deficiency in mucosal lactase.⁴ It has been postulated that lactase deficiency contributes to the acid diarrhea and steatorrhea observed in these animals.

Since the pathological lesions may be severe in the ileum, the site of active bile salt absorption, we considered the possibility that a defect in bile salt absorption might also be present. In human beings, failure to absorb bile salts normally following resection or disease of the terminal ileum leads to a syndrome of cholerheic enteropathy.⁵ To determine whether malabsorption of bile salts might contribute to the runting syndrome, we measured the amount of bile salts in intestinal segments from runted mice and controls.

METHODS

Runting was produced in newborn (C57BL/6 x DBA/2) F_1 hybrid mice by the I.V. administration of 4×10^6 live spleen cells from 12 to 24 week old male C57BL/6 mice in 0.05 ml Hank's solution, as described previously.³ Litter-mate controls were injected with Hank's solution.

Tissue to be analyzed for bile salts was homogenized in 10 ml water, filtered, and the bile salt concentration of the filtrate was measured by the enzymatic method of Talalay, as described previously.⁶

For isotope studies, approximately 3.12×10^6 dpm of Na deoxycholate-24- ^{14}C (Nuclear Research Chemicals, Orlando, Florida, specific activity 3.6 mCi/mMole) was administered in 0.04 ml water to 9-day-old mice by intraperitoneal injection. The mice were sacrificed on day 10 and the small intestine was homogenized in 10 ml water. Protein was precipitated with ethanol, and the ethanol extract was taken to dryness. An appropriate aliquot was subjected to thin-layer chromatography on 2.5 mm thick plates of Silica gel G using a solvent system containing *n*-butanol, 50 ml; 0.01 M Tris buffer, 9.25 ml; and propionic acid, 0.75 ml.⁷ The fraction of unconjugated labeled metabolites was determined by quantitative autoradiography.⁸

Protein was determined by the method of Stadtman, Novelli, and Lipmann.⁹

RESULTS

In the first series of experiments, segments of proximal small intestine one-cm in length were removed from mice of 7- or 10-day old litters, and the bile salt content was measured. Table 1 shows that for each litter, the mean bile salt content of the runts was less than the mean of litter mate controls. Paired analysis of the means revealed that the reduction in bile salt con-

*This report is taken from a paper that appeared in *Transplantation*, 12:479, 1971.

Table 1
MEAN TOTAL BILE SALTS IN ONE-cm SEGMENTS
OF INTESTINE

Litter number	Age days	Controls nMoles (n)*	Runts nMoles (n)*
1	10	294 (2)	170 (3)
2	10	151 (2)	122 (3)
3	10	172 (2)	86 (2)
4	10	133 (2)	71 (5)
5	10	190 (2)	151 (4)
6	7	100 (2)	56 (4)
7	7	92 (2)	59 (4)
8	7	166 (2)	60 (6)

* Number of animals.

tent, approximately 40 per cent overall, was statistically highly significant; the mean difference of the means was 65.4 nMoles/cm (SD 33.6, $n = 8$), $t = 5.50$, $p < 0.001$.

Runts stop growing after about 7 days, and may even lose weight, whereas controls continue to grow. If body weight were a determinant of the bile salt pool size, differences in body size should be considered as possibly contributing to the difference in intestinal bile salts. Table 2 gives the body weights of the animals (A) and the bile salt content of the intestinal segments expressed per g body weight (B). There was a significant difference in body weight between the runts and controls in the 10-day old litters, and no significant difference in the bile salt content

Table 2
BODY WEIGHTS AND INTESTINAL BILE SALTS IN RUNTS AND
LITTER MATE CONTROLS

	Age days	Controls		Runts		Significance p
		Mean $\pm$ SD	(n)*	Mean $\pm$ SD	(n)*	
A. <u>Body weight (g)</u>						
Litters 1-5	10	6.78 $\pm$ 0.62	(10)	4.37 $\pm$ 0.95	(15)	< .001
Litters 6-8	7	4.72 $\pm$ 0.15	(6)	4.50 $\pm$ 0.22	(14)	N.S. [†]
B. <u>Bile salts (nMoles/cm/g body weight)</u>						
Litters 1-5	10	27.7 $\pm$ 10.9	(10)	25.9 $\pm$ 17.2	(15)	N.S. [†]
Litters 6-8	7	25.4 $\pm$ 8.3	(6)	13.1 $\pm$ 4.3	(14)	< .001
C. <u>Total intestinal bile salts (nMoles)</u>						
Litters 9-10	7	680 $\pm$ 20	(6)	570 $\pm$ 60	(9)	< .005

* Number of animals.

[†] Not significant.

expressed per g body weight. Conversely, in the 7-day old litters the runts weighed the same as the controls, but had a significant decrease in bile salt content. To confirm this difference and minimize any errors due to slight differences in lengths of intestinal segments, bile salts in the entire small intestine were measured in two 7-day litters, with comparable results (Table 2, C).

In order to relate the bile salt abnormalities to conventional measures of the severity of the graft-versus-host response, a "bile salt index" was calculated for each experimental animal in a manner analogous to the calculation of the "liver index" and "spleen index."¹ The bile salt content of each runt's intestinal segment was divided into the mean value for its litter mate controls, so that an experimental value less than the control mean would give an index greater than one. Figure 1 shows the result of plotting this bile salt index against the liver and spleen indices for each experimental animal. There is no correlation with the liver index, but with the exception of 5 animals, there is a reasonably good correlation between the bile salt and spleen indices ($r = 0.53$, $t = 2.98$, $p < 0.01$).

Figure 1. Bile salt depletion, expressed as a "bile salt index," compared with spleen or liver enlargement, expressed as the corresponding indices, in 7- and 10-day old runted mice.

In a second series of experiments, seven additional litters were studied to determine how early a decrease in the intestinal content of bile salts could be demonstrated. Bile salts in the entire small intestine were measured in 5-7 day old litters, and the results are shown in Table 3, A. A significant reduction in bile salts occurred on days 6 and 7, but was not apparent on day 5. In two final litters (16 and 17), the entire bile salt pool was measured by homogenizing the liver, gallbladder and small intestine (Table 3, B). By this method, a significant reduction in bile salt pool size could also be demonstrated by day 5.

Table 3

INTESTINAL BILE SALTS AND INTESTINAL WEIGHTS IN 5-7 DAY LITTERS

	Age days	Controls		Runts		Significance p
		Mean $\pm$ SD	(n) [*]	Mean $\pm$ SD	(n) [*]	
A. <u>Total small intestinal bile salts (nMoles)</u>						
Litters 9-10	7	680 $\pm$ 20	(6)	570 $\pm$ 60	(9)	< .005
Litters 11-12	6	704 $\pm$ 38	(7)	586 $\pm$ 59	(7)	< .001
Litters 13-15	5	397 $\pm$ 72	(9)	400 $\pm$ 56	(11)	N.S. [†]
B. <u>Total bile salt pool (μMole)</u>						
Litter 16	6	589 $\pm$ 38	(3)	483 $\pm$ 14	(3)	< .025
Litter 17	5	504 $\pm$ 21	(4)	444 $\pm$ 12	(4)	< .01
C. <u>Small intestinal weights (mg)</u>						
Litter 10	7	134 $\pm$ 4.5	(3)	209 $\pm$ 3.7	(5)	< .001
Litters 11-12	6	143 $\pm$ 11	(7)	182 $\pm$ 9	(7)	< .001
Litters 13-15	5	128 $\pm$ 14	(9)	144 $\pm$ 25	(11)	N.S. [†]

* Number of animals.

[†] Not significant.

In litters 10-15, the weights of the small intestine were recorded to see if there might be a relationship between intestinal mass and bile salt pool. Surprisingly (Table 3, C) the intestinal weights of the runts were significantly greater than those of the controls at days 7 and 6, but not at day 5. In litter 10 (7-day), the total protein in the intestinal homogenates was determined and shown to parallel the intestinal weights.

Finally, two 10-day old litters were studied to determine whether the decrease in intestinal bile salts could be related to abnormal intra-luminal deconjugation and absorption of bile salts. Analysis of labeled metabolites of ¹⁴C-labeled deoxycholate gave widely varying results and did not reveal any important or consistent increase in the unconjugated fraction (Table 4).

Table 4

UNCONJUGATED METABOLITES OF ¹⁴C-DEOXYCHOLATE IN THE
INTESTINES OF MICE FROM 10-DAY OLD LITTERS

	Controls % unconjugated	Runts % unconjugated
Litter 18	11.9, 24.9, 9.4, 32.8	26.0, 30.0
Litter 19	2.6, 6.2	6.0, 15.6

DISCUSSION

These studies demonstrate that the bile salt content of intestinal segments from runt mice is lower than in litter mate controls. The experiments in which the total intestinal contents were measured, or in which the total bile salt pool in the liver, gallbladder and small intestine were measured, indicate that this difference is not an artifact due to a change in the length, luminal diameter, or tone of the intestine, but represents a diminished total bile salt content.

The intestinal content of bile salts is determined by two main factors. The first is synthesis and delivery of bile salts into the intestine, which depends in turn on (a) the synthetic activity of the hepatocytes, (b) the capacity of the liver to discharge bile salts, either synthesized *de novo* or returned to the liver following intestinal absorption, into the canaliculus, and (c) those mechanisms affecting the discharge of bile from the gallbladder. The second main factor concerns absorption of bile salts from the intestine, which occurs by passive diffusion in the upper small bowel and colon, and by active transport in the terminal ileum. Increased absorption in the upper intestine, which can be considered to result primarily from an increase in the fraction of unconjugated bile salts, or decreased absorption in the terminal ileum would both decrease the intraluminal bile salt content. In the former case, however, absorption would be more complete, less bile salts would be lost into the colon and feces, and synthesis would be depressed, whereas in the latter case, more bile salts would be lost into the colon and feces, and synthesis would be increased. Measuring fecal loss would have been helpful in interpreting the present data, but unfortunately this was not technically feasible.

We believe that the results presented here are best explained on the basis of decreased absorption in the ileum, though other possibilities cannot be excluded. Liver lesions, which have been described in runt disease^{1,10-12} could signify a defect in bile salt synthesis or release. The initial changes appear to be proliferation of the Kupffer cells (with a consequent increase in liver weight), which occurs at about the time of arrested weight gain, and proliferation of mononuclear cells in portal tracts. In a few days, patches of liver necrosis appear. The hepatocytes remain remarkably normal in non-necrotic areas, with no alterations in sub-cellular structures such as mitochondria or Golgi. These changes would not be expected to seriously impair excretory function, and liver disease must be substantial to interfere with duodenal bile salt concentrations. Furthermore, bile salt abnormalities were noted prior to the arrest in weight gain, and did not correlate with the increase in liver size as measured by liver indices. Therefore, it seems probable that the observed changes were not the result of hepatic dysfunction.

Since a large part of the bile salt pool can be sequestered in the gallbladder, variations in gallbladder function could also explain differences in intestinal bile salt content. However, runt mice do not stop nursing, since their stomachs are usually full at the time of sacrifice, and they remain in positive nitrogen balance,¹³ so that physiological stimuli for gallbladder contraction would seem to be comparable. Furthermore, the experiments in which the bile salt content of the liver and gallbladder were also measured indicated that the difference was in the bile salt pool size rather than in the distribution of the pool. Hence the gallbladder is probably not responsible for the observed differences.

Finally, enhanced local absorption should be considered. Enhanced local absorption is usually expected when unconjugated bile salts are present, either as a result of failure of the liver to conjugate bile salts, or from deconjugation secondary to the overgrowth of intestinal micro-

organisms. The limited studies with labeled deoxycholate, however, gave no clear evidence for an increased fraction of unconjugated bile salts in the runted mice.

The results are therefore most consistent with the hypothesis that there is impaired absorption of bile salts in the ileum, leading to a decreased return to the liver and an increased delivery to the colon. This could be a direct result of the ileal lesions, or it could be secondary to other factors such as the osmotic effect of unabsorbed lactose. Meihoff and Kern¹⁴ have demonstrated increased excretion of bile salts in mannitol-induced diarrhea in humans, and Stanley and Nemchausk¹⁵ have shown an increased rate of excretion during viral enteritis. In either case, increased delivery of bile salts to the colon could contribute to the observed diarrhea through their effects on water and electrolyte excretion.^{5,16,17}

If bile salt wasting is severe, depletion of the bile salt pool leads to defective micelle formation and steatorrhea.^{5,18-20} Although the decreased intestinal content of bile salts was statistically significant, it is doubtful that it was sufficient (approximately 40 per cent at most) to cause steatorrhea in the presence of an otherwise normally functioning intestine, since half-normal bile salt concentration need not result in steatorrhea in humans.²¹ In the presence of intestinal disease, however, it could well be a contributing factor. Further information is necessary to completely delineate the mechanism of steatorrhea in the runting syndrome.

An unexpected finding was the increase in intestinal weight (and protein content) which was apparent by day 6. It is not clear whether this reflects increased intraluminal fluid and protein derived from serum proteins or desquamated epithelial cells,² or increased mucosal mass due to hypertrophy and/or hyperplasia, although the latter appears more likely. Reilly and Kirsner³ described an increase in dispersed RNA in the intestinal epithelial cells, suggesting increased protein synthesis, and preliminary data now indicate an active proliferation of the epithelium in the early stages of runt disease (Wall, Rosenberg and Reilly, unpublished observations). Further studies on these and other aspects of the runting syndrome are in progress.

LITERATURE CITED

1. Simonsen, M. *Progr. Allergy*, 6:349, 1962.
2. Cornelius, E. A. *Transplantation*, 9:247, 1970.
3. Reilly, R. W., and J. B. Kirsner. *Lab. Invest.*, 14:102, 1965.
4. Hedberg, C. A., S. Reiser, and R. W. Reilly. *Transplantation*, 6:104, 1968.
5. Hofmann, A. F. *Gastroenterology*, 52:752, 1967.
6. Palmer, R. H. In *Methods in Enzymology*, Vol. XV. Steroids and Terpenoids. B. Clayton, Ed. New York: Academic Press, 1969, p. 280.
7. Palmer, R. H. *Proc. Nat. Acad. Sci. (U.S.)*, 58:1047, 1967.
8. Norman, A., and R. H. Palmer. *J. Lab. Clin. Med.*, 63:986, 1964.
9. Stadtman, E. R., G. D. Novelli, and F. Lipmann. *J. Biol. Chem.*, 191:365, 1951.
10. Arakawa, K., A. M. Jezequel, S. I. Macvie, R. Johnston, Z. M. Perz., and J. W. Steiner. *Amer. J. Path.*, 49:257, 1966.
11. Bain, G. O. *Transplantation*, 9:383, 1970.

12. Schlesinger, M., and E. Essner. Amer. J. Path., 47:371, 1965.
13. Kretchmar, A. L., W. H. McArthur, and C. C. Congdon. J. Nutr., 87:261, 1965.
14. Meihoff, W. E., and F. Kern, Jr. J. Clin. Invest., 47:261, 1968.
15. Stanley, M. M., and B. Nemchausky. J. Lab. Clin. Med., 70:627, 1967.
16. Mekhjian, H. S., and S. F. Phillips. Gastroenterology, 59:120, 1970.
17. Mekhjian, H. S., S. F. Phillips, and A. F. Hofmann. Gastroenterology, 54:1256, 1968.
18. Austad, W. I., L. Lack, and M. P. Tyor. Gastroenterology, 52:638, 1967.
19. Hardison, W. G., and I. H. Rosenberg. Clin. Res., 13:536, 1965.
20. Hofmann, A. F., and S. M. Grundy. Clin. Res., 13:254, 1965.
21. Van Deest, B. W., J. S. Fordtran, S. G. Morawski, and J. D. Wilson. J. Clin. Invest., 47:1314, 1968.

HYBRIDIZATION OF MITOCHONDRIAL tRNA's WITH MITOCHONDRIAL AND NUCLEAR DNA OF GRANDE YEAST*

By

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It is now well established that the mitochondrion contains a unique species of DNA which in higher organisms is circular and about 5 μ in length. In yeast, the predominant form of isolated mitochondrial DNA (mit-DNA) appears to be linear,¹⁻³ although the release of 25 μ closed circular molecules has been demonstrated from osmotically shocked mitochondria.⁴ Mitochondria also contain ribosomes⁵⁻¹⁰ which, at least in higher organisms, are smaller than cytoplasmic ribosomes.¹⁰ Unique species of ribosomal RNA^{5,6,11-19} and transfer RNA²⁰⁻²³ are also present in mitochondria. Mitochondrial ribosomal RNA of yeast is smaller,^{9,11,13-16} has a different base composition from cytoplasmic ribosomal RNA,^{9,12} and has been shown to hybridize appreciably with mitochondrial DNA.^{16,24} Mitochondrial ribosomal RNA is thus apparently a genetic product of mitochondrial DNA. A variety of transfer RNA's have been localized in mitochondria of *Neurospora*,^{20,21} rat liver,^{22,23} and yeast.²⁵ Mitochondria contain amino-acid activating enzymes that specifically acylate mitochondrial transfer RNA.^{22,23,26} Although early hybridization studies were contradictory, it appears that at least some species of mitochondrial tRNA are specified by mitochondrial DNA. Wintersberger reported hybridization of ³²P-labeled 4S RNA with mitochondrial DNA in yeast, but it is not possible to eliminate completely the possibility that hybridization of degraded ribosomal or messenger RNA accounts for these results.¹⁶ Nass and Buck,^{27,28} using techniques essentially identical to those employed in the studies reported here, recently showed that some species of mitochondrial tRNA from rat liver hybridized with mitochondrial DNA.

Weiss, et al²⁹ and Scherberg and Weiss³⁰ have described a relatively specific hybridization procedure in which aminoacyl tRNA labeled in the aminoacyl group is hybridized with DNA. Hybridization is carried out at low temperatures in the presence of formamide at an acid pH to minimize deacylation of the tRNA. Using this technique we have found that several species of RNA hybridize with the mitochondrial DNA of a haploid strain of *Saccharomyces cerevisiae*.

This paper presents our results with mit-leucyl-tRNA and m-valyl tRNA. The use of hybridization of mitochondrial tRNA to define the genetic content of a variety of petite m-DNA's is described in another paper.³¹ Preliminary reports of this work have been presented.^{32,33}

METHODS

Strains and culture. The haploid strain of *Saccharomyces cerevisiae* D-234-2B-R1 (R1) employed is from the collection of Professor P. P. Slonimski, Centre National de Gntique Mole-

* This report is taken from a paper that appeared in *J. Mol. Biol.*, 63:431, 1972. The work was supported by grants from the U. S. Public Health Services and the American Cancer Society.

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culaire, Gif-sur-Yvette, France. R1 (a $P_{7ad_1} \rho^+$) is a "grande" (respiratory competent) a revertant with respect to respiratory deficiency of the chromosomal petite P_7 .³⁴ R1 was grown aerobically at 28° in a medium consisting of 1 per cent yeast extract, 1 per cent bacto-peptone and 2 per cent galactose, and collected in the mid or late exponential phase of growth.

Isolation of mitochondria. Yeast cell walls were digested with snail digestive juice (Glu-sulase, Endo Laboratories) by a previously described modification³⁵ of the single step method of Duell et al.³⁶ Protoplasts were washed four times in 1.2 M sorbitol to remove traces of glu-sulase, and each gram (wet weight) of protoplast was suspended in 6 ml of 0.9 M sorbitol, 1×10^{-3} M EDTA, 0.1 per cent bovine serum albumin, pH 7.2. The protoplasts were disrupted at high speed in a Waring blender for 30 sec. The nuclear and debris fraction was sedimented at 1,600 g for 10' at 0°C. The mitochondrial fraction was then sedimented at 27,000 g for 10'. Mitochondria used for DNA isolation were suspended in 0.9 M sorbitol, 0.01 M $MgCl_2$, 0.01 M Tris, pH 7.5 and digested with pancreatic DNase (Worthington) 50 μ g/ml for 1 hr at 30°C.

DNase was then removed by three centrifugation washes at 27,000 g for 10' in 0.9 M sorbitol, 2×10^{-3} M Tris, pH 7.5, and 2×10^{-3} M EDTA. Mitochondria used for transfer RNA isolation were freed from supernatant tRNA either by centrifugation through a step-gradient consisting of 1.2 M sorbitol and 0.9 M sorbitol, or by three washes in 0.9 M sorbitol at 27,000 g for 10'.

DNA and RNA isolation. DNA was extracted by a modification of the method described by Fauman.²⁴ The mitochondrial pellet, digested with DNase and washed as described above, was suspended in 1 per cent sodium dodecyl sulfate, 0.01 M EDTA, 0.01 M Tris, pH 7.5, incubated at 37°C for 15', adjusted to 1 M NaCl, extracted with an equal volume of chloroform-octanol 9:1, and purified by chromatography on hydroxylapatite. Nuclear DNA was extracted from the fraction that sedimented at 1,600 g and isolated by the procedure used for mitochondrial DNA. Further purification was achieved by gradient elution from hydroxylapatite columns.³⁷ The purity of the DNA was verified by CsCl isopycnic centrifugation in the analytic ultracentrifuge. The $A_{260} m\mu / A_{280} m\mu$ of the DNA was 1.85-2.0. DNA concentrations were calculated assuming $1 \mu g = 0.020 A_{260} m\mu$.

Aminoacyl tRNA was isolated from mitochondria obtained from 200 g wet weight yeast. The pellet was suspended in 50 ml of 2 per cent sodium dodecyl sulfate, 0.01 M Tris, pH 7.5, 0.1 per cent mackaloid and incubated at 30° for 30'. The suspension was deproteinized four times with an equal volume of phenol cresol³⁸ (500 g of reagent grade phenol, Mallinckrodt; 70 ml m-cresol twice distilled, 0.5 g 8-hydroxyquinoline 55 ml of H_2O). The residual phenol was removed from the aqueous phase by four ether extractions, and traces of ether were removed by bubbling N_2 through the aqueous solution. One-tenth volume of 20 per cent potassium acetate, pH 5.2 was added, and the RNA was precipitated with two volumes of cold ethanol. After standing at -20°C for several hours, the RNA was collected by centrifugation. The precipitate was dissolved in 15 ml of 1 M Tris, pH 8.8 and incubated at 37°C for 1 hr. The deacylated tRNA was precipitated with two volumes of cold ethanol at -20°C overnight. The tRNA was collected by centrifugation and the precipitate was dissolved in 10 ml of 0.1 x standard sodium citrate (SSC) (0.15 M NaCl, 0.015 M sodium citrate, pH 6.8). Ribosomal RNA was precipitated by the addition of an equal volume of 4 M LiCl.³⁹ The mixture was stirred for 1 hr at 4°C and the precipitate was discarded. Mitochondrial tRNA to be used for acylation with [3H]leucine or [3H]valine was dialyzed against 10^{-6} M Mg acetate overnight. Cytoplasmic and mitochondrial tRNA used for competition studies was prepared as described, except that deacylation and incubation at pH 8.8 were omitted and

the tRNA was purified by gel filtration through Sephadex G-100.²⁹

Isolation of amino acid-activating enzymes and conditions for acylation. Amino acid-activating enzymes were purified from whole cell extracts of strain R1. The yeast was broken in batches. Fifteen grams yeast and 30 g glass beads (0.45-0.55 mm diameter B. Braun) were suspended in 30 ml of 0.01 M Tris-HCl buffer, pH 7.5, 0.01 M MgCl₂ and 10 per cent glycerol and the yeast was disrupted at high speed in the Braun homogenizer for eight 15-sec periods. The disrupted cells were centrifuged at 198,000 g for 120'. The supernatant was purified on DEAE-cellulose according to the method of Muench and Berg.⁴⁰ The extract was applied to a DEAE-cellulose column equilibrated with 0.02 M phosphate, pH 7.5, 0.02 M 2-mercaptoethanol, 0.001 M MgCl₂ and 10 per cent glycerol. The peak of the 280 m μ absorbing material was concentrated 5- to 6-fold by dialysis against 60 volumes of buffer containing 0.001 M phosphate, pH 6.8, 0.02 M 2-mercaptoethanol, 10 per cent glycerol and 15 per cent polyethylene glycol, molecular weight 6,000, for 6-8 hr. The ratio A 280 m μ /A 260 m μ was 1.6-1.7.

Mitochondrial tRNA was acylated with [³H]leucine (40 c/mole, Schwartz Bio-Research, Orangeburg, N. Y.) or [³H]valine (16 c/mole) in the following reaction mixture: 0.05 M Tris-chloride, pH 7.5, 0.005 M MgCl₂, 0.001 M ATP, 0.005 M 2-mercaptoethanol, 1×10^{-4} M CTP, 0.005 M phosphoenolpyruvate, 1 mc [³H]leucine or [³H]valine, 15 enzyme units pyruvate kinase (Calbiochem), 0.6 mg deacylated mitochondrial tRNA (not purified on Sephadex G-100) and 500-800 μ g enzyme in a final volume of 2.5 ml. The mixture was incubated for 15' at 30° and the aminoacyl tRNA was isolated by phenol extraction.⁴¹ The phenol was removed by ether, and the acylated tRNA was precipitated with ethanol. The precipitate was dissolved in 1 ml of 0.05 M NH₄ acetate buffer, pH 5.2 and purified on a Sephadex G-100 column that had been equilibrated with the same buffer.²⁹ The tubes containing [³H]aminoacyl tRNA were combined, one-tenth volume of 1 M acetate buffer, pH 5.2 was added, and the tRNA was precipitated with two volumes of ethanol.

Hybridization. Hybridization was carried out using minor modifications of the procedure described by Weiss et al.²⁹ DNA was denatured in 0.2 N NaOH (pH 13.0), following which it was neutralized with HCl. The SSC concentration was adjusted to 6X and the DNA was slowly applied to membrane filters (Schleicher and Schull 25 mm) as described by Gillespie and Spiegelman.⁴² The filters were air-dried overnight, and then dried in a vacuum oven at 88° for 2 hr. The DNA was retained on the filters as indicated by absence of A 260 m μ material in the filtrate and, in selected experiments, by the retention after hybridization of more than 95 per cent of ¹⁴C-labeled grande or petite mit-DNA applied to the filters. Hybridization was carried out in 33 per cent or 50 per cent formamide, 2 x SSC final pH 5.0 (reading of pH meter) for 4.5 hr at 33°. The filters were washed by stirring in one liter of 2 x SSC pH 6.0 for 15'. The filters were then treated with 5 μ g/ml. T₁ RNase in 2 x SSC at 30° for 30' and again washed with one liter of 2 x SSC. The filters were dried and counted in 15 ml of a toluene-based scintillation mixture (0.5 per cent 2,5-diphenyl-oxazole, 0.05 per cent, 1,4-bis-2 [5-phenyloxazolyl] benzene), using a Packard liquid scintillation spectrometer.

E. coli tRNA was purchased from Nutritional Biochemical Laboratories.

RESULTS

Analytic isopyknic CsCl ultracentrifugation showed that mitochondrial and nuclear DNA preparations from the respiratory competent strain R1 were free from cross-contamination.

Conditions of hybridization of mitochondrial tRNA. Hybridization of R1 mitochondrial [^3H]leucyl-tRNA with R1 mitochondrial and nuclear DNA was carried out at 33° in 50 per cent formamide in early experiments and 33 per cent formamide in later experiments. Higher hybridization levels were obtained at the lower formamide concentration. Considering the high A-T content of mitochondrial DNA, the lower formamide concentrations conform to the conditions giving optimum specific hybridization suggested by McConaughy et al.⁴³ High blank values observed in early experiments were lowered to satisfactory levels, when amino acid-activating enzyme was purified on DEAE-cellulose and the [^3H]leucyl-tRNA was purified by gel filtration as described in the Methods section. Significant hybridization of [^3H]leucyl-tRNA was observed with mitochondrial DNA. Conditions for optimal hybridization were sought by altering pH, temperature and duration of hybridization. Hybridization at pH 5.0 yielded lower blanks than at higher pH and greater stability of the aminoacyl tRNA. Variations in temperature (30°, 33°, 36°) had little effect on hybridization, so the original temperature suggested by Weiss et al,²⁹ namely 33°, was used. Optimal hybridization was obtained in about 4-6 hr, and declined thereafter, probably due to deacylation. In 6 hr about 82 per cent of tRNA remained acylated, while only 62 per cent remained acylated in 24 hr. We chose 4-1/2 hr as a suitable hybridization time.

Hybridization of mit[^3H]leucyl or mit[^3H]valyl tRNA. Information about the relative fraction of R1 mitochondrial DNA coding for leucyl-tRNA may be derived from saturation studies. Apparent saturation was achieved with R1 mitochondrial DNA (5 μg) at about 20 μg mitochondrial tRNA, four times the amount of DNA fixed to the filters (Figure 1A). There was no detectable hybridization with nuclear DNA. Hybridization was also carried out between [^3H]mit-valyl tRNA and mitochondrial and nuclear DNA (10 μg) (Figure 1B). As with mit-leucyl tRNA, mit-valyl tRNA hybridized well with grande mit-DNA, saturation being reached at RNA concentrations of about 5 $\mu\text{g}/\text{ml}$. Again, no hybridization with yeast nuclear or with *E. coli* DNA was observed.

Specificity of hybridization. Competition experiments to confirm the specificity of the hybridization were carried out at saturating levels of [^3H]mit-leucyl-tRNA (Figure 2A) and [^3H]mit-valyl tRNA (Figure 2B). Increasing amounts of unlabeled mitochondrial tRNA, yeast cytoplasmic tRNA and *E. coli* tRNA were added to the hybridization reaction mixture. The addition of cytoplasmic tRNA and *E. coli* tRNA produced no significant reduction in hybridization (Figure 2A and 2B). In contrast, the addition of unlabeled mitochondrial tRNA led to a marked dilution effect, which was similar for hybridizations of both [^3H]mit-leucyl tRNA and [^3H]mit-valyl tRNA.

Melting curve of hybrid. The specificity of hybridization was also confirmed by melting curves of the hybrid. Filters containing [^3H]leucyl tRNA bound to 20 μg of mitochondrial DNA were incubated for 10' in 50 per cent formamide, 2 x SCC, 0.05 M NH_4 acetate, pH 5.2 at increasing temperatures. The radioactivity released at 5° increments was measured and the data used to plot the resulting denaturation curve (Figure 3). About 10 per cent of the radioactivity is released at low temperature, but most of the bound [^3H]tRNA is released between 31-45°. The calculated T_m was approximately 36°. This would correspond to a T_m of about 71° in 2 x SCC in the absence of formamide. The T_m for yeast mitochondrial DNA in 2 x SCC is approximately 75°.

Identification of nature of hybridized radioactivity. To confirm that the counts fixed to the

Figure 1. Hybridization of [³H]mit tRNA with mitochondrial and nuclear DNA.

A. Mit-leucyl tRNA hybridization: [³H]mit-leucyl tRNA (specific radioactivity 1.3×10^3 cts/min/µg RNA) was hybridized to filters containing 5 µg mitochondrial DNA o—o, or 5 µg nuclear DNA ▲---▲. Blank filters having no DNA fixed 0-35 cts/min which were subtracted from experimental values in this and in all subsequent experiments. A 50% formamide hybridization system was used in a reaction volume of 0.6 ml.

B. Mit-valyl tRNA hybridization: [³H]mit-valyl tRNA (specific radioactivity 10.1×10^3 cts/min/µg RNA) was hybridized with filters containing 10 µg mitochondrial DNA o—o; or 10 µg nuclear DNA ▲---▲. Blank filters containing no DNA fixed 5-12 cts/min. A 33% formamide hybridization system was used in reaction volume of 0.3 ml.

filters during hybridization really represented annealed aminoacyl tRNA, the [³H]mit-valyl-tRNA and the radioactive material fixed to the filters were characterized. [³H]valyl tRNA was eluted from the tRNA-DNA hybrids bound to filters by incubation in 80 per cent formamide, 20 per cent 0.01 M NH₄ acetate, pH 5.2 at 40° for 15'. The tRNA was passed through a Sephadex G-25 column (1 x 24 cm) equilibrated with 0.005 M ammonium acetate, pH 5.2, to remove the formamide. The [³H]valyl tRNA appeared in the void volume indicating that it had not been significantly degraded. The tRNA was then treated with pancreatic RNase according to the method of Marcker and Sanger.⁴⁴ The released [³H]valyl-adenosine was isolated and identified by high voltage electrophoresis (Figure 4). The mobility of the [³H]aminoacyl-adenosine derived from the digest of the hybrid was identical with that of [³H]valyl-adenosine obtained from RNase digest of input [³H]valyl tRNA. Yields of radioactive valyl-adenosine were between 80-90 per cent. It may therefore be concluded that the counts fixed to filters after annealing with DNA did represent [³H]valyl-tRNA. Similarly, [³H]valyl-tRNA and filters containing hybridized counts were

Figure 2. Competition of unlabeled yeast mitochondrial and supernatant, and *E. coli* tRNA's with the hybridization of $[^3\text{H}]$ mit-tRNA and mit-DNA.

A. Mit-leucyl tRNA hybridization: 20 μg $[^3\text{H}]$ mit-leucyl tRNA (specific radioactivity 2.0×10^3 cts/min/ μg RNA) was hybridized with 5 μg mit-DNA filters in a 0.6 ml 50% formamide hybridization mixture in the presence and absence of competitor. No-DNA blank filters bound 60-94 cts/min. 180 cts/min were fixed to the filters in the absence of competitor. Unlabeled yeast mit-tRNA o—o; unlabeled yeast supernatant tRNA ●; unlabeled *E. coli* tRNA Δ .

B. Mit-valyl-tRNA hybridization: 6.6 μg $[^3\text{H}]$ valyl mit-tRNA (specific radioactivity 5.2×10^3 cts/min/ μg DNA) was hybridized with 5 μg mit-DNA filters in a 0.3 ml 50% formamide hybridization mixture. 164 cts/min were fixed to filters in the absence of competitor. 37-41 cts/min bound to no-DNA blank filters have been subtracted from the data. Symbols are the same as in Figure 4A.

Figure 3. Melting curve of $[^3\text{H}]$ mit-leucyl tRNA-mit-DNA hybrid. 7.7 μg $[^3\text{H}]$ mit-leucyl tRNA (specific radioactivity 3.9×10^3 cts/min/ μg RNA) was hybridized to 20 μg mit-DNA in a 0.6 ml reaction mixture containing 50% formamide. 1090 cts/min were bound to filters containing mit-DNA. 41 cts/min were bound to blank filters containing no DNA. ●—●, cumulative % cts/min released; o---o, d cts/min/dt. All counts were recovered from the filters. Melting was carried out for 5 min at 5°C temperature intervals in 0.3 ml of 50% formamide, 2 x SSC, 0.05 M NH_4 acetate (pH 5.0).

Figure 4. High voltage electrophoresis of RNase and alkaline digests of the [^3H]mit-valyl tRNA hybrid, and of [^3H]mit-valyl tRNA. 54 μg of [^3H]mit-valyl tRNA (specific radioactivity 5.8×10^3 cts/min/ μg RNA) was hybridized to a filter containing 50 μg mit-DNA in a 0.3 ml 33% formamide reaction system. The [^3H]tRNA was released from the hybrid by incubation in 80% formamide, and the formamide removed as described in the text. Half of the released material was digested with RNase (Marcker et al, 1964) and half incubated at pH 12 for 30'. 1.2 μg [^3H]valyl-mit-tRNA was treated in the same way. Electrophoresis was carried out in 0.5 M acetic acid pH 3.5 containing 0.3% pyridine (75 min at 3000 V) as described by Marcker et al (1964). The paper was cut into 1 cm segments and counted in a liquid scintillation counter.

A. RNase digest of the hybrid; B. Alkaline digest of the hybrid; C. RNase digest of [^3H]mit-valyl-tRNA; D. Alkaline digest of [^3H]mit-valyl tRNA; E. [^3H]valine.

hydrolyzed at pH 12.0 for 30' at 37°. The [^3H]valine was identified by paper electrophoresis (Figure 4). The radioactive material had a mobility identical with authentic valine.

DISCUSSION

The genetic products of mitochondrial DNA are still not completely known. At present it is well established that mitochondrial ribosomal RNA is specified by mitochondrial DNA^{16,24,45,46} in all species where this has been examined. This paper provides evidence that at least two transfer RNA's are also coded for by mitochondrial DNA in yeast. Mitochondrial formyl methionyl

tRNA,⁴⁷ methionyl tRNA (Halbreich and Rabinowitz, unpublished data), isoleucyl, glycyl, alanyl, phenylalanyl and tyrosyl tRNA's (Cohen and Rabinowitz, unpublished) also hybridize with yeast mitochondrial DNA. Experiments are currently in progress to determine whether all, or only a few, tRNA species are coded for by yeast mitochondrial DNA.

Similar results and conclusions have been reported by Nass et al^{27,28} who find that 4 mitochondrial aminoacyl tRNA's hybridize with mitochondrial DNA. It is of interest that leucyl tRNA hybridizes with the heavy strand and tyrosyl tRNA with the light strand of liver mitochondrial DNA. Hybridization saturation curves with ³²P-labeled HeLa cell⁴⁶ and frog⁴⁸ mitochondrial tRNA suggest that only 11 or 12 tRNA's are specified by mit-DNA in these organisms.

The hybridization results reported here appear to represent specific annealing of transfer RNA molecules with homologous complementary regions. The conditions of hybridization are those which McConaughy et al⁴³ established as providing a high degree of specific hybridization. Furthermore, the melting profile of the hybrid is sharp and yields the T_m expected from the GC content of mitochondrial DNA. The radioactivity bound to the filters is associated with relatively intact tRNA molecules. tRNA eluted from the hybrid is of high molecular weight. RNase digests of the hybrid contained [³H]aminoacyl adenosine, showing that the filter-bound radioactivity was covalently linked in a tRNA molecule.

Although apparent saturation levels were obtained in the hybridization experiments with mitochondrial valyl and leucyl tRNA, it is still not possible to calculate accurately the number of cistrons coding for these tRNA's present in each mitochondrial DNA molecule. For such calculations to be valid, the hybridization conditions must reflect true saturation, aminoacylation must be complete, and the specific activity of the amino acid in the aminoacyl tRNA must be known. These criteria were not fulfilled in the study of tRNA-DNA hybridization after T₄ infection of *E. coli*,²⁹ nor do we know whether they are fulfilled in these studies. Calculations based on our data, however, yield 0.1-0.2 tRNA cistrons per 25 micron length of mitochondrial DNA. Although the results almost certainly reflect the errors involved in the calculation, it is possible that only a fraction of the mitochondrial DNA molecules in a cell contains cistrons for a mitochondrial tRNA.

It has been postulated that mitochondria evolved from bacteria that had entered a symbiotic relationship with the cells. This hypothesis is based upon the similarity in a number of features of the systems of protein synthesis in bacteria and mitochondria. Among these is the similarity in size of bacterial and mitochondrial ribosomal RNA, and ribosomes.⁴⁹⁻⁵¹ Furthermore, bacterial protein synthesis is initiated by formylmethionyl tRNA, whereas eukaryote cytoplasmic protein synthesis uses a nonformylated species of methionyl tRNA.⁵²⁻⁵⁴ Mitochondrial protein synthesis is probably also initiated by the formylated tRNA, as it is in bacteria. Formylmethionyl tRNA has been found in mitochondria of yeast, and rat liver,⁵⁵ but not in non-mitochondrial fractions. Methionyl tetrahydrofolic acid transformylase activity has also been shown to be present in yeast mitochondria.⁴⁷ Despite similarities in the mechanism of initiating protein synthesis in the two systems, no evidence for homology of mitochondrial and *E. coli* tRNA has been observed in this or in other studies.²⁷

As is described extensively by Cohen, et al,³¹ functional cistrons from mitochondrial tRNA may be retained in the mitochondrial DNA of some respiratory deficient cytoplasmic petite mutants derived from the yeast strain described here. In some petite strains these cistrons may be lost, whereas in others they are retained and perhaps tandemly duplicated. Hybridization of mito-

chondrial DNA from a series of petites with a set of mitochondrial tRNA markers may be a useful device for the characterization of the mitochondrial DNA molecule and its genetic information content.

LITERATURE CITED

1. Sinclair, J. R., B. J. Stevens, P. Sanghavi, and M. Rabinowitz. *Science*, 156:1234, 1967.
2. Guerineau, M., C. Grandchamp, Y. Yotsuyanagi, and P. Slonimski. *Comp. Rend.*, 266D:1884, 1968.
3. Shapiro, L., L. I. Gorssman, J. Marmur, and A. K. Kleinschmidt. *J. Mol. Biol.*, 33:907, 1968.
4. Hollenberg, C. P., P. Borst, R. W. J. Thuring, and E. F. J. VanBruggen. *Biochim. biophys. Acta*, 186:417, 1969.
5. Kuntzel, H., and H. Noll. *Nature*, 215:1340, 1967.
6. Rifkin, M. R., D. D. Wood, and D. J. L. Luck. *Proc. Nat. Acad. Sci., Wash.*, 58:1025, 1967.
7. Schmitt, H. *F.E.B.S. Letters*, 4:234, 1969.
8. Vignais, P. V., J. Huet, and J. Andre. *F.E.B.S. Letters*, 3:177, 1969.
9. Morimota, H., and H. O. Halverson. *Proc. Nat. Acad. Sci., Wash.*, 68:324, 1971.
10. O'Brien, T. W., and G. F. Kalf. *J. Biol. Chem.*, 242:2180, 1967.
11. Dure, L. S., J. L. Epler, and W. E. Barnett. *Proc. Nat. Acad. Sci., Wash.*, 58:1883, 1967.
12. Fauman, M., M. Rabinowitz, and G. S. Getz. *Biochim. biophys. Acta*, 182:355, 1969.
13. Leon, S. A., and H. R. Mahler. *Arch. Biochem. Biophys.*, 126:305, 1968.
14. Rogers, P. J., B. N. Preston, E. B. Titchener, and A. W. Linnane. *Biochem. Biophys. Res. Comm.*, 27:405, 1967.
15. Steinschneider, A. *Biochim. biophys. Acta*, 186:405, 1969.
16. Wintersberger, E. *Z. Physiol. Chem.*, 348:1701, 1967.
17. Suyama, Y. *Biochemistry*, 6:2829, 1967.
18. Dubin, D. T. *Biochem. Biophys. Res. Comm.*, 29:655, 1967.
19. Attardi, B., and G. Attardi. *J. Mol. Biol.*, 55:231, 1971.
20. Barnett, W. E., and D. H. Brown. *Proc. Nat. Acad. Sci., Wash.*, 57:452, 1967.
21. Epler, J. L. *Biochemistry*, 8:2285, 1969.
22. Buck, C. A., and M. M. K. Nass. *Proc. Nat. Acad. Sci., Wash.*, 60:1045, 1969.
23. Buck, C. A., and M. M. K. Nass. *J. Mol. Biol.*, 41:67, 1969.
24. Fauman, M. A. Ph.D. thesis, University of Chicago (paper in preparation), 1971.
25. Accuceberry, B., and A. Stahl. *Biochem. Biophys. Res. Comm.*, 42:1235, 1971.
26. Barnett, W. E., D. H. Brown, and J. L. Epler. *Proc. Nat. Acad. Sci., Wash.*, 57:1775, 1967.
27. Nass, M. M. K., and C. A. Buck. *Proc. Nat. Acad. Sci., Wash.*, 62:506, 1969.

28. Nass, M. M. K., and C. A. Buck. *J. Mol. Biol.*, 54:187, 1970.
29. Weiss, S. B., W.-T. Hsu, J. W. Foft, and N. H. Scherberg. *Proc. Nat. Acad. Sci., Wash.*, 61:114, 1968.
30. Scherberg, N., and S. Weiss. *Proc. Nat. Acad. Sci., Wash.*, 67:1164, 1970.
31. Cohen, M., J. Casey, M. Rabinowitz, and G. S. Getz. *J. Mol. Biol.*, 63:441, 1972.
32. Casey, J., H. Fukuhara, G. S. Getz., and M. Rabinowitz. *J. Cell Biol.*, 43:18A, 1969.
33. Cohen, M., and M. Rabinowitz. *J. Cell Biol.*, 47:37A, 1970.
34. Sherman, F., and P. P. Slonimski. *Biochim. biophys. Acta*, 90:1, 1964.
35. Rabinowitz, M., G. S. Getz, J. Casey, and H. Swift. *J. Mol. Biol.*, 41:381, 1969.
36. Duell, E. A., S. Inoue, and M. F. Utter. *J. Bacteriol.*, 88:1762, 1964.
37. Bernardi, G., M. Faures, G. Piperno, and P. Slonimski. *J. Mol. Biol.*, 48:23, 1970.
38. Kirby, K. S. *Biochem. J.*, 96:266, 1965.
39. Avital, S., and D. Elson. *Biochim. biophys. Acta*, 179:297, 1969.
40. Muench, K. H., and P. Berg. In Procedures in Nucleic Acid Research, G. L. Cantoni, and D. R. Davies, Eds. New York: Harper & Row, 1966, p. 375.
41. Holley, R. W., J. Apgar, G. A. Everett, J. T. Madison, S. H. Merrill, and A. Zamir. *Cold Spr. Harb. Symp. Quant. Biol.*, 28:117, 1963.
42. Gillespie, D., and S. Spiegelman. *J. Mol. Biol.*, 12:829, 1965.
43. McConaughy, B. L., C. D. Laird, and B. J. McCarthy. *Biochemistry*, 8:3289, 1969.
44. Marcker, K., and F. Sanger. *J. Mol. Biol.*, 8:835, 1964.
45. Wood, D. D., and D. J. L. Luck. *J. Mol. Biol.*, 41:211, 1969.
46. Aloni, Y., and G. Attardi. *J. Mol. Biol.*, 55:271, 1971.
47. Halbreich, A., and M. Rabinowitz. *Proc. Nat. Acad. Sci., Wash.*, 68:294, 1971.
48. Dawid, I. B. *Symp. Soc. Exptl. Biol.*, 24:227, 1969.
49. Borst, P., and A. M. Kroon. *Intern. Rev. Cytol.*, 26:108, 1969.
50. Nass, M. M. K. *Science*, 165:25, 1969.
51. Rabinowitz, M., and H. Swift. *Physiol. Rev.*, 50:376, 1970.
52. Rajbhandary, U. L., and H. P. Ghosh. *J. Biol. Chem.*, 244:1104, 1969.
53. Smith, A. E., and K. A. Marcker. *Nature*, 226:607, 1970.
54. Bhadrui, S., M. K. Chatterjee, K. K. Bose, and N. K. Gupta. *Biochem. Biophys. Res. Comm.*, 40:402, 1970.
55. Smith, A. E., and K. A. Marcker. *J. Mol. Biol.*, 38:241, 1968.

STUDIES ON THE HYBRIDIZATION OF MITOCHONDRIAL tRNA AND
MITOCHONDRIAL DNA IN PETITE MUTANTS OF YEAST*

By

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It is possible to isolate respiratory deficient petite strains of yeast which involve mutations in either the nuclear or mitochondrial (cytoplasmic) genetic systems. In chromosomal petites, the mitochondrial DNA is unchanged, whereas in cytoplasmic petites, there may be substantial alterations in base sequence,^{1,2} and in some cases, partial³ or complete^{3,4} deletion of mitochondrial DNA. Mounolou et al¹ observed that mitochondrial DNA of cytoplasmic petites may have an altered buoyant density, thereby showing that there may be substantial changes in average base composition of mit-DNA, which usually results in relative increase in A-T content.^{1,5}

It is also now apparent from genetic and RNA-DNA hybridization studies, that although different petite strains may be identical as far as respiratory deficiency and mitochondrial function are concerned, their mitochondrial DNA's may differ markedly from each other. Cytoplasmically-determined antibiotic resistance markers for chloramphenicol and erythromycin may be retained in some cytoplasmic petites and lost in others.^{6,7} Similarly, transcription of mitochondrial ribosomal RNA may be lost completely⁸ or partially retained.^{9,10} The degree of base sequence changes in mitochondrial ribosomal RNA may also vary in different petite strains.^{10,11}

In a preceding paper, we have shown that in wild-type (grande) yeast, mitochondrial valyl tRNA and mitochondrial leucyl tRNA hybridize with and are presumably transcribed from the mitochondrial DNA.¹² The present paper deals with the hybridization properties of mitochondrial tRNA isolated from grande and from cytoplasmic petite mutants, with grande and petite mitochondrial DNA. It is shown that cytoplasmic petite strains with substantial alterations in their mitochondrial DNA may retain cistrons for, and transcribe functional tRNA's. Petite strains vary in this capacity, illustrating the fact that mitochondrial DNA of cytoplasmic petites differ markedly even in a functional sense, from each other, although they have identical phenotypes with respect to respiratory activity. Furthermore, subclones isolated from the same spontaneously mutated parent petite strain may be heterogeneous with respect to hybridization with mitochondrial tRNA. Therefore, although they are all petites, they may consist of several cell types with respect to the mitochondrial genome. The use of several marker mitochondrial tRNA's in hybridization studies with different petite DNA's, especially if combined with genetic studies, offers an opportunity to investigate the structure of mitochondrial DNA in yeast.

* This report is taken from a paper that appeared in *J. Mol. Biol.*, 63:441, 1972. The work was supported by grants from the U. S. Public Health Services and the American Cancer Society.

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METHODS

Strains and culture. The haploid strains of *Saccharomyces cerevisiae* D-234-2B-R1 (R1) and D-243-2B-R1-6 (R1-6) were obtained from the collection of Professor P. P. Slonimski, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. R1(a, P₇, ρ^+) is a grande revertant with respect to respiratory deficiency of chromosomal petite p₇.¹³ R1-6 (a, ad₁, ρ^-) is a cytoplasmic petite spontaneously derived from the parent strain. The petite strain was isolated several years ago and has been subcultured many times. In the initial isolation of R1-6, subcloning was not done. Subsequently, we isolated ten random subclones of R1-6. These are referred to as R1-6/1, R1-6/2 and so on. R1 was grown aerobically at 28° in a medium consisting of 1 per cent yeast extract, 2 per cent bacto-peptone, 2 per cent galactose. R1-6 was grown in the same medium with the addition of 0.1 per cent glucose. The yeast was collected in the mid or late exponential phase of growth so as to facilitate digestion of the cell wall with snail digestive juice.

Isolation of mitochondria, DNA and tRNA. Mitochondria were isolated from yeast protoplasts as described previously.¹⁴ Mitochondrial DNA was extracted from both petite and grande mitochondria after DNase digestion.¹² The yield of mitochondrial DNA from grande R1 was 250-500 μ g/50 g-wet weight cells, and from petite strains 25-200 μ g/50 g wet weight. Transfer RNA was isolated from grande and petite mitochondria and from the postmitochondrial supernatant fluid. Aminoacylation and hybridization were also carried out as described previously,¹² except that in several experiments amino acid-activating enzyme was isolated from mitochondria¹⁵ instead of from whole cell extracts. More than 95 per cent of petite ¹⁴C-mit-DNA was retained on filters after hybridization.

RESULTS

Hybridization of grande mit-tRNA with petite mit-DNA. We have previously shown¹² that mit-valyl and mit-leucyl tRNA's isolated from respiratory competent grande cells hybridized specifically with mit-DNA obtained from grande cells. To determine whether cistrons for these tRNA's are retained or lost in different cytoplasmic petite strains, grande mit-tRNA's were hybridized with petite mit-DNA. The hybridization of grande (R1) [³H]mit-leucyl tRNA to grande (R1) and petite (R1-6) mit-DNA is shown in Figure 1A. Apparent saturation curves were obtained with each DNA. Not only was hybridization of grande mit-leucyl tRNA with petite DNA evident, but at saturation, the level of hybridization was twice as great with petite mit-DNA as with grande mit-DNA.

In contrast, grande [³H]mit-valyl tRNA hybridized to only a very small extent with petite mit-DNA (Figure 1B). A small but significant degree of hybridization of grande mit-valyl tRNA to petite mit-DNA was observed in most experiments. This result appeared to indicate the elimination, or gross alteration, of cistrons for mit-valyl tRNA in the R1-6 petite mit-DNA.

As we previously reported for the hybridization of mit-tRNA with grande mit-DNA,¹² only mitochondrial tRNA competed for the hybridization of grande mit-leucyl tRNA with petite mit-DNA (Figure 2). Yeast supernatant tRNA and *Escherichia coli* tRNA added in great excess did not compete in the hybridization.

Melting profile of mit-leucyl tRNA - petite mit-DNA hybrid. The specificity of the hybridization of grande mit-leucyl tRNA with petite mit-DNA was evaluated using melting curves of the

Figure 1. Hybridization of grande (R1) mit-tRNA with mit-DNA of petite (R1-6-0) and grande (R1) yeast.

A. Mit-leucyl tRNA hybridization: R1 [^3H]mit-leucyl RNA (specific radioactivity 1.3×10^3 cts/min/ μg RNA) was hybridized with filters containing 5 μg of petite (R1-6) $\bullet$ --- $\bullet$, grande (R1) $\circ$ — $\circ$ mit-DNA or nuclear DNA $\blacktriangle$ --- $\blacktriangle$. A 0.6 ml 50% formamide hybridization system was used. Blank filters containing no DNA bound 0 to 30 cts/min. In this, and in all experiments, blank values are subtracted from the hybridization data.

B. Mit-valyl tRNA hybridization: R1 [^3H]mit-valyl tRNA (specific radioactivity 10.1×10^3 cts/min/ μg RNA) was hybridized with filters containing 10 μg petite (R1-6) $\bullet$ --- $\bullet$ or grande (R1) mit-DNA $\circ$ — $\circ$. A 0.6 ml 33% formamide hybridization system was used. Blank filters containing no DNA bound 5 to 12 cts/min.

Figure 2. Competition by unlabeled yeast mitochondrial and supernatant tRNA's and *E. coli* tRNA with the hybridization of [3 H]mit-leucyl-tRNA (R1) and petite (R1-6) mit-DNA. 20 μ g [3 H]leucyl mit-tRNA (specific radioactivity 2.0×10^3 cts/min/ μ g DNA) was hybridized with filters charged with 5 μ g of R1-6-0 mit-DNA in the presence and absence of competitor. 725 cts/min were fixed to filters in the absence of competitor. 60-94 cts/min blanks have been subtracted from the data. Unlabeled yeast mit-tRNA o—o; unlabeled yeast supernatant tRNA ●, and unlabeled *E. coli* tRNA Δ .

hybrid (Figure 3). Filters containing [3 H]leucyl tRNA hybridized to petite mit-DNA were incubated for 10 min in 50 per cent formamide 2 x SSC and 0.05 M NH_4 acetate, pH 5.2, at increasing temperatures. The TCA precipitable radioactivity released at 2° increments was measured and the data were used to plot a denaturation curve. The results obtained with mit-DNA isolated from petite subclone (R1-6/8) are shown in Figure 3. The melting curve is quite sharp, with a T_m of about 36° corresponding to a T_m of 71° in 2 x SSC without formamide. The narrow range of temperatures at which [3 H]leucyl mit-tRNA is released from the hybrid attests to the specificity of the hybridization.

Hybridization of petite mit-tRNA with grande and petite mit-DNA. The hybridization of grande mit-leucyl-tRNA to petite mit-DNA indicated that the petite mitochondrial genome retains at least some homologous sequences of the leucyl tRNA cistron. We therefore sought to establish whether a functional tRNA is transcribed from the cistron in the petite. tRNA was isolated from petite (R1-6-0) mitochondria and aminoacylated with either [3 H]leucine or [3 H]valine. Hybridization of these labeled mitochondrial tRNA's with grande (R1) and petite (R1-6) DNA are shown in Figures 4A and 4B. Petite [3 H]mit-leucyl tRNA hybridized to mitochondrial DNA but not to yeast nuclear or *E. coli* DNA (Figure 4A). As with grande mit-leucyl tRNA, hybridization levels at apparent saturation concentrations of the tRNA were substantially greater with petite DNA than with the grande DNA.

Although grande mit-valyl tRNA hybridizes only to a small extent with petite mit-DNA, we were able to isolate a mit-valyl tRNA from this strain (R1-6) which could be acylated in vitro.

Figure 3. Melting curve of grande (R1) mit- $[^3\text{H}]$ leucyl tRNA-petite (R1-6/8) mit-DNA hybrid. $4.0 \mu\text{g}$ R1 $[^3\text{H}]$ leucyl mit-tRNA (specific radioactivity 1.6×10^3 cts/min/ μg RNA) was hybridized to $10 \mu\text{g}$ R1-6/8 mit-DNA in a 0.6 ml 33% formamide hybridization system. 903 cts/min were bound to the filter containing DNA. 88 cts/min were bound to filters containing no DNA. All counts were recovered from the filter. $\bullet\text{---}\bullet$, cumulative % cts/min released; $\circ\text{---}\circ$ $d(\text{cts/min released})/dt$. Melting was carried out for 5 min at 2°C temperature intervals in 0.3 ml of 50% formamide, $2 \times \text{SSC}$, 0.05 M NH_4 acetate ($\text{pH } 5.0$).

Figure 4. Hybridization of petite (R1-6) mit-tRNA with mitochondrial DNA of grande (R1) and petite (R1-6) yeast.

A. Mit-leucyl tRNA hybridization: Petite (R1-6) $[^3\text{H}]$ leucyl-mit-tRNA (specific radioactivity 4.0×10^3 cts/min/ μg RNA) was hybridized with filters containing $10 \mu\text{g}$ R1 $\circ\text{---}\circ$ or R1-6, $\bullet\text{---}\bullet$ mit-DNA.

B. Mit-valyl tRNA hybridization: Petite (R1-6) $[^3\text{H}]$ valyl-mit-tRNA (specific radioactivity 4.3×10^3 cts/min/ μg RNA) was hybridized with filters containing $10 \mu\text{g}$ of R1 $\circ\text{---}\circ$, or R1-6- $\bullet\text{---}\bullet$ mit-DNA.

In both A and B hybridization was carried out in 0.3 ml 50% formamide hybridization mixtures. Blank filters containing no-DNA bound 60 to 239 cts/min in A, and 7 - 23 cts/min in B.

This mit-valyl tRNA hybridizes well with grande mit-DNA, but surprisingly only very little to its own petite R1-6 mit-DNA (Figure 4B). The explanation for these results appears to be related to heterogeneity within the petite strain, evidence for which will be presented below. It is also worthy of notice that saturation of the mitochondrial DNA's with both leucyl and valyl petite mit-tRNA is obtained at 5-10 times the tRNA level required with grande mit-tRNA.

The competition studies shown in Figures 5A and 5B verify that the hybridizations of petite valyl and petite leucyl mit-tRNA with mitochondrial DNA do in fact represent hybridization

Figure 5. Competition by unlabeled grande (R1) mitochondrial and supernatant tRNA's with the hybridization of petite (R1-6) mit-tRNA and mit-DNA.

A. Mit-leucyl tRNA hybridization: 21.2 μg petite (R1-6) [^3H]mit-leucyl tRNA (specific radioactivity 4.0×10^3 cts/min/μg) was hybridized with filters containing 10 μg of grande (R1) and petite (R1-6) mit-DNA in a 0.6 ml 50% formamide mixture. 60 cts/min were bound to R1 mit-DNA and 154 cts/min to R1-6 mit-DNA in the absence of competitor after subtracting a no-DNA blank of 192 cts/min. Hybridization to R1 mit-DNA, open symbols; hybridization to R1-6 mit-DNA, closed symbols. unlabeled mit-tRNA (R1) o, ●; unlabeled supernatant tRNA (R1) □, ■; unlabeled E. coli tRNA Δ, ▲.

B. Valyl mitochondrial tRNA hybridization: 7.5 μg petite (R1-6) [^3H]valyl mit-tRNA (specific radioactivity 5.1×10^3 cts/min/μg RNA) was hybridized with filters containing 5 μg of grande (R1) mit-DNA in a 0.3 ml 50% formamide hybridization system. 129 cts/min were bound to mit-DNA in the absence of competitor. 6 to 9 cts/min were bound to no-DNA blank filters. Unlabeled mit-tRNA (R1) o; unlabeled supernatant tRNA (R1) □.

of a mitochondrial tRNA species. In each case, unlabeled mitochondrial tRNA competes with the labeled species in the hybridization, but yeast cytoplasmic tRNA does not.

In several of the hybridization experiments with petite mit-tRNA, high "no-DNA" blank val-

ues were encountered (in Figures 4A and 5A but not in Figures 4B and 5B). The results reported, however, were reproducible in many experiments.

Evidence for heterogeneity of petite strain R1-6. A possible explanation for the apparently contradictory observation that petite valyl mit-tRNA hybridizes well with grande mit-DNA but only very little with its own mit-DNA is that the strain is heterogeneous, being composed predominantly of cells having mitochondrial DNA lacking the cistron for valyl-tRNA, but also containing a small proportion of cells in which this cistron is retained. A small number of petite cells which transcribe a functional valyl tRNA would thus be present.

This hypothesis was tested by subcloning the petite R1-6 strain. Ten subclones R1-6/1, R1-6/2, etc. were isolated and mit-DNA was obtained from seven of them. Hybridization of grande mit-valyl tRNA to mit-DNA from the original strain (R1-6) and one of the seven subclones (R1-6/1) is shown in Figure 6. Also shown for reference is the hybridization of grande

Figure 6. Hybridization of grande (R1) mit-valyl tRNA with mit-DNA of original petite (R1-6) and a petite subclone (R1-6/1).

R1 [^3H]mit-valyl tRNA (specific radioactivity 10.1×10^3 cts/min/ μg RNA) was hybridized with filters charged with $10 \mu\text{g}$ of R1 $\circ$ — $\circ$; R1-6 $\bullet$ — $\bullet$; or R1-6/1 Δ — Δ mit-DNA. Hybridization was carried out in 0.3 ml 33% formamide systems. 5 to 12 cts/min were bound to no-DNA blank filters.

mit-DNA. Whereas hybridization to the original petite strain is barely above the blank level, substantial hybridization is obtained to the mit-DNA of the petite subclone R1-6/1. The very low level of hybridization observed with mit-DNA of the original petite strain R1-6 compared to that of the first subclone suggests that the original strain contains a high proportion of cells lacking cistrons for valyl tRNA.

Further evidence for heterogeneity within the original petite strain (R1-6) is obtained from hybridization of petite mit-tRNA isolated from this strain. R1-6 valyl mit-tRNA hybridizes very little with mit-DNA isolated from the same strain, but it hybridizes substantially with mit-DNA from a subclone (R1-6/1) of the original petite strain as well as with grande mit-DNA. Thus it

would appear that only a relatively few cells in the R1-6 strain continue to transcribe mit-valyl tRNA. This tRNA, however, cannot hybridize with the bulk of the mit-DNA of that strain.

Hybridization of mitochondrial leucyl tRNA to mitochondrial DNA of petite subclones. In an attempt to obtain data that may be useful in evaluating linkage of tRNA cistrons with mitochondrial genetic markers, grande mitochondrial valyl and leucyl tRNA's were hybridized with mit-DNA isolated from a series of subclones of the heterogeneous R1-6 strain. These subclones presumably originate either from mutations that occurred in a single parent cell, or from subsequent changes in the progeny. The results with leucyl tRNA are shown in Figure 7. Mit-DNA of

Figure 7. Hybridization of grande (R1) mit-leucyl tRNA with mit-DNA of subclones (R1-6/1 and R1-6/5 to R1-6/10) of the original petite (R1-6) strain.

In experiment A (ordinate scale on left). R1 [^3H]mit-leucyl tRNA (specific radioactivity 5.9×10^3 cts/min μg was hybridized to filters containing 10 μg mit-DNA following strains: R1 o—o, R1-6/1 Δ — Δ , R1-6/5 $\diamond$ — $\diamond$, R1-6/6 $\otimes$ — $\otimes$, R1-6/7 $\oplus$ — $\oplus$, R1-6/8 $\oplus$ — $\oplus$.

In experiment B (ordinate scale on right). R1 [^3H]mit-leucyl tRNA (specific radioactivity 31.5×10^3 cts/min/ μg RNA) was hybridized to filters containing 10 μg mit-DNA from the following strains: R1 $\bullet$ — $\bullet$, R1-6/9 $\blacklozenge$ — $\blacklozenge$, R1-6/10 $\blacksquare$ — $\blacksquare$. 0.6 ml. 38% formamide hybridization systems were used. No-DNA blanks were 32 to 60 cts/min in A and 27 to 148 cts/min in B. Ordinate scales have been normalized assuming equal levels of hybridization with R1 mit-DNA in the two experiments.

all petite subclones hybridized with grande mit-leucyl tRNA at least as well as did grande mit-DNA. The mit-DNA of the subclones appears to be composed of several groups. One group (R1-6/6) hybridizes at about the same level as grande mit-DNA. A second group (R1-6/1, R1-6/7) hybridizes approximately 2-3 fold greater than grande DNA and a third and perhaps fourth group (R1-6/5, R1-6/9; R1-6/8, R1-6/10) hybridize 4- or more-fold greater than does grande

mit-DNA. Thus if serial tandem duplication of the cistron for leucyl tRNA in the petite strains is the explanation for enhanced hybridization of mit-leucyl tRNA to petite mit-DNA, the process must vary quantitatively in the different petite subclones.

Hybridization of mitochondrial valyl tRNA to mitochondrial DNA of petite subclones. The hybridization of grande mit-valyl tRNA with mit-DNA from the seven petite subclones is shown in Figure 8. Again the subclones appear to be divided into three different classes. In one group

Figure 8. Hybridization of grande (R1) mit-valyl tRNA with mit-DNA of subclones of the original petite (R1-6) strain.

R1 mit-valyl tRNA (specific radioactivity 7.5×10^3 cts/min/ μ g RNA) was hybridized to filters containing 10 μ g mit-DNA from the following strains: R1 o—o, R1-6 ●—●, R1-6/1 Δ—Δ, R1-6/5 ◇—◇, R1-6/5 ⊗—⊗, R1-6/7 ⊞—⊞, R1-6/8 ⊕—⊕, R1-6/9 ◆—◆, R1-6/10 ■—■. A 0.6 ml 33% formamide hybridization system was used. 29 to 60 cts/min were bound to no-DNA blank filters.

the mit-DNA hybridizes very little if at all with mit-valyl tRNA and is thus similar to the original strain R1-6. Another group contains mit-DNA which hybridizes almost, but not quite as well as does grande mit-DNA, and the mit-DNA of a third group hybridizes to an intermediate level. The grouping of subclones obtained with mit-valyl tRNA hybridization only partially overlaps those obtained with mit-leucyl tRNA. For example, R1-6/8 and R1-6/10 have similar hybridization characteristics with both valyl and leucyl tRNA's and may represent genetically identical strains, but R1-6/6 and R1-6/7 differ considerably in their hybridization with mit-leucyl tRNA although they show similar patterns with mit-valyl tRNA.

DISCUSSION

In the cytoplasmic petite mutants of yeast, the base sequence of mitochondrial DNA is often

changed quite extensively. This is shown by an alteration of buoyant density T_m , and base comparison of the DNA,^{1,2,5} and by the presence of large segments of non-homologous sequences in petite and grande mit-DNA demonstrated by both RNA-DNA and DNA-DNA hybridization.^{9,10} The common defect in cytoplasmic petites appears to be the loss of capacity for mitochondrial protein synthesis.¹⁶ Since this loss can be brought about by a mutation that may affect only one or two mitochondrial tRNA's or by a massive alteration of the DNA, which may prevent the transcription of any meaningful mitochondrial RNA, it is apparent that mitochondrial DNA's from different petites may differ greatly from one another. Our observation that some petite strains continue to transcribe mitochondrial leucyl and valyl tRNA, shows that of the transcription of mitochondrial tRNA is apparently not dependent on mitochondrial protein synthesis.

Fauman has presented evidence that one of the petite strains (R1-6/1) used in this study has an overall change in the base sequence of mitochondrial DNA of approximately 50 per cent;¹⁰ similar changes are probably present in the other petite strains used. Despite the extensive alteration in sequence, however, the mitochondrial DNA still retained functional cistrons for leucyl tRNA and valyl-tRNA. The transcribed tRNA's were functional at least in the sense that they could still be enzymatically aminoacylated. In the case of leucyl tRNA, saturation hybridization levels were higher with petite DNA than with grande DNA. The latter result may be interpreted to indicate either a deletion of a substantial portion of the mitochondrial DNA with retention of the cistron for mit-leucyl tRNA, or serial tandem duplication of the cistrons for mit-leucyl tRNA. Cistrons for leucyl tRNA were retained in all the petite subclones studied, but enhanced hybridization saturation levels were observed only in some. The process appears to vary quantitatively in different strains.

The petite strain we originally studied appears to be heterogeneous with respect to mitochondrial DNA. Only a small proportion of the cells contain DNA that codes for mitochondrial valyl tRNA. Mit-valyl tRNA can be isolated from these cells, acylated in vitro and hybridized with mit-DNA. Other cells, however, appear to have lost their ability to transcribe a functional valyl tRNA and contain mit-DNA that is unable to hybridize with grande mit-valyl tRNA.

The hybridization system used in this study¹⁷ differs from other RNA-DNA hybridization systems in that only the aminoacyl groups of the tRNA are labeled. In this system a partial sequence homology between the tRNA and the DNA may not be reflected by a proportional partial hybridization. If base sequences are sufficiently homologous, complete hybridization may be observed, whereas with a somewhat greater degree of mismatching, no hybridization may be seen. It is possible, however, that an incomplete homology may be reflected in differing stabilities of hybrids. Furthermore, hybridization of grande tRNA and petite DNA does not necessarily indicate a functional cistron for the tRNA. In strains described here, positive hybridization is correlated with the production of a tRNA that can be acylated, and is in this sense functional. Nevertheless, it is quite possible that functional leucyl and valyl tRNA species derived from the petite strain do have some minor alterations in base sequence from those present in the grande parent.

Genetic analyses by Slonimski and colleagues¹⁸ of the various subclones used in this paper for tRNA-DNA hybridization show that all strains were chloramphenicol and erythromycin sensitive. The use of multiple tRNA markers, combined with genetic studies of antibiotic resistance markers should provide linkage data that may allow preliminary mapping of mitochondrial DNA.

LITERATURE CITED

1. Mounolou, J., H. Jakob, and P. Slonimski. *Biochem. Biophys. Res. Comm.*, 24:218, 1966.
2. Bernardi, G., M. Faures, G. Piperno, and P. Slonimski. *J. Mol. Biol.*, 48:23, 1970.
3. Goldring, E. S., L. Grossman, D. Krupnick, D. Cryer, and J. Marmur. *J. Mol. Biol.*, 52:323, 1970.
4. Nagley, P., and W. Linnane. *Biochem. Biophys. Res. Comm.*, 39:989, 1970.
5. Carnevali, F., G. Morpurgo, and G. Tecco. *Science*, 163:1331, 1969.
6. Coen, D., J. Deutsch, P. Netter, E. Petrochilo, and P. P. Slonimski. *Symp. Soc. Exptl. Biol.*, 24:449, 1970.
7. Gingold, E. B., G. W. Saunders, H. B. Lukins, and A. W. Linnane. *Genetics*, 62:735, 1969.
8. Wintersberger, E., and G. Viehhauser. *Nature*, 220:699, 1968.
9. Fukuhara, H. *Proc. Nat. Acad. Sci. (U.S.)*, 58:1065, 1967.
10. Fauman, M. A. Ph.D. thesis, University of Chicago (paper in preparation), 1971.
11. Fukuhara, H. Unpublished data.
12. Casey, J., M. Cohen, M. Rabinowitz, H. Fukuhara, and G. S. Getz. *J. Mol. Biol.*, 63:431, 1972.
13. Sherman, F., and P. P. Slonimski. *Biochim. biophys. Acta*, 90:1, 1964.
14. Rabinowitz, M., G. S. Getz, J. Casey, and H. Swift. *J. Mol. Biol.*, 41:381, 1969.
15. Halbreich, A., and M. Rabinowitz. *Proc. Nat. Acad. Sci. (U.S.)*, 68:294, 1971.
16. Plattner, H., M. M. Salpeter, J. Saitzgarber, and G. Schatz. *Proc. Nat. Acad. Sci. (U.S.)*, 66:1252, 1970.
17. Weiss, S. B., W.-T. Hsu, J. W. Foft, and N. H. Scherberg. *Proc. Nat. Acad. Sci. (U.S.)*, 61:114, 1968.
18. Slonimski, P. P. Unpublished data.

STAFF PUBLICATIONS

- Aschenbrenner, V., R. Zak, A. F. Cutilletta, and Murray Rabinowitz. Effect of Hypoxia on Degradation of Mitochondrial Components in Rat Cardiac Muscle. *Am. J. Physiol.*, 221(5):1418, 1971.
- Hamel, Ernest, Mohan Koka, and Tokumasa Nakamoto. Requirement of an *Escherichia coli* 50 S Ribosomal Protein Component for Effective Interaction of the Ribosome with T and G Factors and with Guanosine Triphosphate. *J. Biol. Chem.*, 247(3):805, 1972.
- Humphrey, David M., Frank W. Fitch, and Lionel W. Coppleston. Synergy of Antihist Antiserum with Donor Spleen Cells in the Graft-Versus-Host Reaction. *Transplantation*, 13(2):77, 1972.
- Lathrop, Katherine A., and P. V. Harper. Biologic Behavior of ^{99m}Tc from ^{99m}Tc -Pertechnetate Ion. In *Progress in Nuclear Medicine* 3, Vol. 1. Basel: Karger, 1972, p. 146.
- Lerch, Irving A. A Computer Analysis of the Role of Oxygen in the Modification of Radiosensitivity of Self-renewing Cell Systems. *Radiology*, 101(3):683, 1971.
- Lerch, I. A., and A. G. Haus. Development of a Diagnostic X-Ray Spectrometer with a Lithium-Drifted Silicon Detector for Intercomparison and Image Analysis. *Radiology*, 101(2):401, 1971.
- Palmer, Robert H. Bile Acid Sulfates. II. Formation, Metabolism, and Excretion of Lithocholic Acid Sulfates in the Rat. *J. Lipid Res.*, 12:680, 1971.
- Palmer, Robert H., and Merry G. Bolt. Bile Acid Sulfates. I. Synthesis of Lithocholic Acid Sulfates and Their Identification in Human Bile. *J. Lipid Res.*, 12:671, 1971.
- Palmer, Robert H., and Richard W. Reilly. Bile Salt Depletion in the Runting Syndrome. *Transplantation*, 12(6):479, 1971.
- Rabinowitz, Murray. Control of Metabolism and Synthesis of Macromolecules in Normal and Ischemic Heart. *J. Molec. Cellular Cardiol.*, 2:277, 1971.
- Rappaport, Henry, Stephen B. Strum, George Hutchison, and Lawrence W. Allen. Clinical and Biological Significance of Vascular Invasion in Hodgkin's Disease. *Cancer Res.*, 31:1794, 1971.
- Redding, Marshall E., Constantine E. Anagnostopoulos, and John E. Ultmann. The Possible Value of Mediastinoscopy in Staging Hodgkin's Disease. *Cancer Res.*, 31:1741, 1971.
- Rowley, Janet D. Loss of the Y Chromosome in Myelodysplasia: A Report of Three Cases Studied with Quinacrine Fluorescence. *Brit. J. Haematol.*, 21(6):717, 1971.
- Rowley, J. D., and W. F. Bodmer. Relationship of Centromeric Heterochromatin to Fluorescent Banding Patterns of Metaphase Chromosomes in the Mouse. *Nature*, 231(5304):503, 1971.
- Scanu, A. M., and C. Edelstein. Solubility in Aqueous Solutions of Ethanol of the Small Molecular Weight Peptides of the Serum Very Low Density and High Density Lipoproteins: Relevance to the Recovery Problem during Delipidation of Serum Lipoproteins. *Anal. Biochem.*, 44(2):576, 1971.
- Scanu, Angelo M., Celina Edelstein, and Chang T. Lim. Effect of Disulfide Cleavage on the Molecular Weight of One of the Major Polypeptides of Human Serum High Density Lipoprotein. *FEBS Letters*, 18(2):305, 1971.
- Strum, Stephen B., Lawrence W. Allen, and Henry Rappaport. Vascular Invasion in Hodgkin's Disease: Its Relationship to Involvement of the Spleen and Other Extranodal Sites. *Cancer*, 28(5):1329, 1971.

- Ultmann, John E. Hodgkin's Disease: Laparotomy or Not? 'Letters and Comments.' *Ann. Intern. Med.*, 76:330, 1972.
- Ultmann, J. E. The Management of Lymphoma. *Ca—A Cancer Journal for Clinicians*, 21(6):342, 1971.
- Vesselinovitch, S. D., E. L. Simmons, N. Mihailovich, K. V. N. Rao, and L. S. Lombard. The Effect of Age, Fractionation, and Dose on Radiation Carcinogenesis in Various Tissues of Mice. *Cancer Res.*, 31:2133, 1971.
- Weiss, Samuel B. Translational Control Mechanisms in Bacteriophage Development. In Nucleic Acid-Protein Interactions - Nucleic Acid Synthesis in Viral Infection. D. W. Ribbons, J. F. Woessner, and J. Schultz, Eds. Amsterdam: North-Holland Publishing Company, 1971, p. 237.
- Welander, Charles, Melvin L. Griem, Michael Newton, and James E. Marks. Staging and Treatment of Endometrial Carcinoma. *J. Reproductive Med.*, 8(1):41, 1972.