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950 EAST FIFTY-NINTH STREET • CHICAGO • ILLINOIS 60637

MASTER

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1971

ALEXANDER GOTTSCHALK, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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CORRELATION BETWEEN BRAIN TUMOR DETECTABILITY ON BRAIN SCAN AND PROTEIN LEVELS OF THE SPINAL FLUID*

By

S. M. Pinsky, P. B. Hoffer, R. Flom,[†] J. L. Quinn III,[†] and A. Gottschalk

The successful diagnosis of intracranial tumors by brain scanning has been reported as ranging from 65-90 per cent with 80 per cent accepted as the usual accuracy rate.^{1,2} Diagnostic accuracy is high for meningiomas, metastatic lesions, and rapidly growing gliomas,³ but more slowly growing astrocytomas and cystic tumors have a considerably lower probability of being visualized.

The exact mechanism whereby a nuclide localizes in a tumor is not known, and indeed, is probably not the same for all agents. It is assumed, however, that isotope localization within brain lesions is associated with interruption of the blood brain barrier. We have postulated that scanning agents such as pertechnetate-99m, which is loosely bound to serum protein,⁴ may localize in brain tumors by passive or active diffusion of protein into the lesion through an interrupted blood-brain barrier. Since the cerebrospinal fluid accurately reflects the contents of the extracellular fluid space of the brain,⁵ it follows that if protein leakage through an altered blood-brain barrier causes pertechnetate localization within a tumor, there is a high probability that protein levels of the cerebrospinal fluid may also be elevated in such patients. In other words, if about 80 per cent of all brain tumors are localized by pertechnetate brain scans; and if the localization is in large part due to diffusion of protein-bound pertechnetate into the extracellular space through an altered blood-brain barrier; then, granting that the extracellular space protein and CSF protein are comparable, approximately 80 per cent of all patients with brain tumors should also show an increase in CSF protein. The CSF protein levels, however, are elevated in only about 40-50 per cent⁶ of all patients with brain tumors. With this apparent paradox in mind, we undertook to determine if there was any correlation between cerebrospinal fluid protein levels and the detectability of brain tumors by brain scanning.

MATERIALS AND METHODS

Records of patients with proven primary brain tumors were reviewed from the University of Chicago Hospitals for the period January 1964 to December 1969 and the Chicago Wesley Memorial Hospital for the period January 1966-December 1969. The series consisted of 58 cases representing all tissue-proven brain tumors for whom both pertechnetate scans and spinal fluid protein determinations were available. A dose range of 10-20 mCi of pertechnetate-99m was utilized for scanning, and for approximately the second half of this series, doses of 0.4-1 g of perchlorate were used to premedicate the patients. Patients with meningiomas were eliminated from the study because of the high incidence of neovascularity in these lesions, which would introduce a second probable localization mechanism. Acoustic neuromas were also excluded from

* This report is taken from a paper that appeared in *Radiology*, 98:672, 1971.

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this study because of the frequent association of markedly elevated cerebrospinal protein with this tumor. In fact, our experience and that of others indicates that these tumors are highly detectable by brain scan.⁷

RESULTS

Protein levels were elevated in 25 (43 per cent) of the 58 patients. The normal value for spinal fluid protein levels at both institutions is considered to be below 45 mg per cent. Of the 25 patients with elevated spinal fluid protein levels, 22 had abnormal brain scans. Among the 33 patients with normal protein levels, only 17 had abnormal brain scans. Thus, if the protein level was elevated the brain scans were positive for tumor in 88 per cent of the patients, while if the protein levels were within normal ranges only 52 per cent were positive for tumor. These two groups of data are significantly different at the .005 level by Chi-Square test.

Two major histologic tumor types were present in large enough numbers for the cases to be considered individually. The largest group consisted of astrocytomas (34 cases). Of the 13 cases with elevated protein levels, 11 had abnormal scans; of the 21 patients with normal protein levels, only 12 had abnormal brain scans. For astrocytomas, therefore, 85 per cent were detected when protein levels were abnormally elevated, while only 57 per cent were detected with normal protein levels. The other histologic group of significant size consisted of glioblastomas (17 cases). Of the 9 patients with elevated spinal fluid protein levels, 8 had abnormal brain scans; of the 8 patients with normal protein levels, only 4 had abnormal brain scans. Other histologic types in the study included 4 medullablastomas and one case each of oligodendroglioma, chordoma, and craniopharyngioma. These results are summarized in Tables 1, 2, and 3.

Table 1

	All cases		Total
	CSF protein below 45 mg %	CSF protein over 45 mg %	
Abnormal brain scan	17	22	39
Normal brain scan	16	3	19
TOTAL	33	25	58

Table 2

	Astrocytomas		Total
	Normal CSF protein (below 45 mg %)	Elevated CSF protein (over 45 mg %)	
Abnormal brain scan	12	11	23
Normal brain scan	9	2	11
TOTAL	21	13	34

Table 3

	Glioblastomas		Total
	Normal CSF protein (below 45 mg %)	Elevated CSF protein (over 45 mg %)	
Abnormal brain scan	4	8	12
Normal brain scan	4	1	5
TOTAL	8	9	17

DISCUSSION

We have also analyzed the records of 10 patients from whose tumors cystic fluid was aspirated at the time of operation. All 10 of these cases had grossly positive brain scans. The protein levels of these cystic fluids were markedly elevated, ranging between 2700 and 6200 mg/per cent. Spinal fluid protein determinations were performed in only 4 of the 10 cases, and in all cases the protein was less than 16 mg/per cent. In these instances, a highly positive scan was correlated with regional protein accumulation, but because of the normal CSF protein the 4 cases were described in the overall results as being unassociated with increased protein levels. If we exclude these 4 cases, only 13 of 29 patients (45 per cent) with normal protein levels had abnormal brain scans.

The brain scan localized only 67 per cent of the tumors in the entire series. This low value is related to two factors. First, meningiomas and metastases were excluded from the study; second, the cases were highly selected, consisting exclusively of patients who were able to have a preoperative spinal tap. There was presumably less clinical evidence of markedly elevated intracranial pressure in the group, and therefore the tumors were likely to be smaller and more difficult to localize on the scan than those in an ordinary series of brain scan patients.

The series shows a definite correlation between the accuracy of detection of primary intracranial tumors on brain scans and the cerebrospinal fluid protein level. When the cerebrospinal fluid protein is elevated, there is a high probability of localization on brain scan (88 per cent). If, however, the cerebrospinal fluid level is less than 45 mg/per cent the probability of detecting the lesion is only 52 per cent. The difference is statistically significant. These data support the concept that tumor localization of pertechnetate-99m is often protein mediated.

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PANCREAS SCANNING - IS IT WORTH IT? *

By

S. Landman,[†] E. Polcyn,[‡] and A. Gottschalk

Ten years after its introduction into clinical medicine¹⁻³ pancreas scanning is still championed only by a few enthusiasts and distrusted by many who have either never used it or have abandoned the procedure after an early disappointing experience. Most practitioners of nuclear medicine either have not accumulated sufficient experience, or have not analyzed their experience well enough to know what may be expected from this procedure of limited, but definite value.

Unbiased by previous enthusiasm and sharing most of the general mistrust, we were still unwilling to abandon without a good clinical study one of the few radiologic procedures available in a field of great diagnostic difficulty. We therefore decided to re-analyze all our pancreatic scans with the object of establishing correlation between the appearance of the scan and the clinical outcome in order to assess the clinical value of the procedure.

MATERIAL AND METHODS

A total of 106 pancreas scans were performed at the University of Chicago from January 1966 until July 1, 1970. The examination did not include a special meal or any of the biologic and pharmacologic agents used by others in an attempt to increase concentration of the radio-pharmaceutical in the pancreas, but which had not proved to be clinically beneficial.^{2,4-26} We insisted, however, that our patients have breakfast, because starvation preferentially increases isotope uptake in the liver, since plasma protein synthesis has biologic priority over digestive enzyme synthesis in the pancreas.^{3,27}

After I.V. injection of 250 microcuries of ⁷⁵Se-selenomethionine,[§] the patient was placed supine under the Anger camera mounted with a 364 KeV parallel hole multiaperture collimator. The detector head was positioned over the mid-abdomen inclined 5-10 degrees toward the head in an attempt to "look under the liver."

Serial 10 minute anterior views were then taken, the patient being repositioned, if necessary, after the first view (Figure 1). The time of optimum visualization proved to be variable and different regions of the pancreas were best seen on different views. We firmly believe that this technique has been of essential value since it is the usual rather than occasional case in which the normal pancreas image is obtained by an amalgamation of the sequential views. The method, therefore, has a better chance of recording a diagnostic image than the one or two views that can be taken with the rectilinear scanner. The examination lasted for at least one hour. We analyzed these cases in an effort to establish optimum time of visualization, and found

* This report is taken from a paper that appeared in *Radiology*, 100:631, 1971.

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[§] Produced by Squibb by biosynthesis.

Figure 1. Serial 10-minute anterior views in patient with normal pancreas. In general, the image is evaluated by an amalgamation of the several views, for example, the mid portion of the body is seen intact only on the first image; whereas the head is best seen on the 20-30 minute view.

as will be seen in Table 1, that the best view was obtained most often in the first 40 minutes. Even when it occurred later, the early pictures would have been adequate for diagnosis. It is therefore considered unnecessary to prolong the examination beyond 50 minutes.

Table 1
ANALYSIS OF SEQUENTIAL VIEWS TECHNIQUE

Best view obtained	No. patients
10-20 minutes	9
20-30 minutes	17
30-40 minutes	12
40-50 minutes	4
50-60 minutes	3
Later than 60	3
Not visualized	<u>13</u>
Total	61

The normal variability of pancreatic shape has been the subject of much discussion.^{16,18,21,28,29} In our experience the decreased uptake at the junction of the head and body of the pancreas can cause more diagnostic difficulty. This is due to the underlying aorta, and when this organ complicates interpretation of the scan, we suggest a bolus nuclide injection to permit visualization of the aorta immediately following the last pancreas image. The patient should not be moved for this view so that the appearance of the aorta and pancreas can be correlated (see Figure 2).

Liver overlap has always caused the greatest difficulty in the interpretation of pancreas

Figure 2. Example of area of decreased uptake in the neck of the pancreas (left) shown to correspond to the abdominal aorta on nuclide angiogram (right).

scans because, although the uptake on a gram basis is less in the liver than in the pancreas, the mass of the liver is so great that it accumulates three times more selenomethionine than the pancreas.^{3,21,27,30-33} Numerous methods of overcoming this difficulty have been tried, from simple lead shields²⁰ to sophisticated electronic subtraction methods.^{18,35-38} We have also used a 1600 channel-analyzer in conjunction with the gamma camera to subtract the liver image obtained with ^{99m}Tc sulfur-colloid from the combined liver-pancreas image produced by ⁷⁵Se selenomethionine. An example is shown in Figure 3. Subsequent analysis of these cases (Table 2) permitted us to assess the value of the subtraction technique. In our hands subtraction, although useful at times is almost never essential, and we believe that the absence of subtraction equipment should never discourage a physician from scanning the pancreas.

RESULTS

The scans were interpreted by one of us (A.G.) without recourse to either clinical data or the initial report. Only three diagnostic categories were used (Figure 4): the normal pancreas; the not visualized pancreas; the partially or faintly visualized pancreas. Clinical correlation was obtained from autopsy, surgery, or clinical course. The series is shown in Table 3, and the results of this study are given in Table 4-6.

DISCUSSION

Eighty-eight per cent of our patients with a normal pancreas scan proved to have no detectable lesions in the organ. The remaining 12 per cent were false negatives with pathological conditions unrevealed in the normal scan. Mild chronic pancreatitis may be expected to leave the pancreas with sufficient enzyme-secreting capacity to produce a normal selenomethionine uptake, and the pancreas scan, therefore will commonly miss these cases. In the patient with carcinoma whose scan is shown in Figure 5 there was an adenocarcinoma of the duodenum which invaded most of the head of the pancreas in its posterior aspect, but only 1 cm of the anterior aspect, and left the ampulla and the pancreatic duct uninvolved, thus retaining enough function and uptake of radionuclide in the gland to produce a normal scan. The grossly demonstrable area involved by carcinoma was therefore below the resolution limit of the scanning procedure, which is about one inch.

Figure 3. Subtraction technique. Upper left: routine scintiphotograph showing the pancreas obscured by the lower edge of the liver. Upper right: digital matrix representation of the same image with the 1600 channel analyzer. Lower left: technetium-99m sulfur colloid image of liver and spleen with patient in the same position. Lower right: digital subtraction image of the technetium from the selenomethionine yielding a clearer view of the pancreas.

Table 2

ANALYSIS OF SUBTRACTION RESULTS

Essential	1 case
Corroborative	20 cases
Not contributory	26 cases
Total subtractions	47 cases

The vast majority of patients whose scans show either non-visualization or partial or faint visualization, will have significant impairment of the gland. Most of them will have either a carcinoma originating in or invading the pancreas, or chronic pancreatitis. The scan is unable to differentiate between these two lesions. Insulin-resistant diabetes has also been found to impair visualization of the pancreas,^{34,38} and this was substantiated in two of our cases. Our series contains fewer false positives than many series previously reported,^{18,40,41} probably because we use the technique of sequential imaging on the gamma camera.

Figure 4. Examples of diagnostic categories. Upper left: normal visualization. Upper right: non-visualization (carcinoma). Lower left: faint visualization (chronic pancreatitis). Lower right: partial visualization, the head is faint and deformed (carcinoma).

Table 3

SUMMARY OF SCAN DATA

Scan interpretation	No. cases	Lost to F/U	Adequate F/U
Normal visualization	52	4	48
Non-visualization	26	4	22
Abnormal or faint	<u>28</u>	<u>4</u>	<u>24</u>
Totals	106	12	94

Table 4

FINAL DIAGNOSIS IN PATIENTS WITH NORMAL VISUALIZATION OF THE PANCREAS

Diagnosis	Autopsy	Surgery	Clinical	Total
Normal pancreas	2	7	33	42 = 88%
Chronic pancreatitis	1	2	2	5 = 10%
Carcinoma	-	1	-	<u>1</u> = 2%
Total				48

Table 5

FINAL DIAGNOSIS IN PATIENTS WITH NON-VISUALIZATION IN THE PANCREAS

Diagnosis	Autopsy	Surgery	Clinical	Total
Carcinoma	2	5	2	9 = 41%
Chronic pancreatitis	1	4	4	9 = 41%
Diabetes	-	-	1	1 = 5%
Normal pancreas	1	2	-	3 = 13%
Total				22

Table 6

FINAL DIAGNOSIS IN PATIENTS WITH ABNORMAL AND FAINT VISUALIZATION

Diagnosis	Autopsy	Surgery	Clinical	Total
Chronic pancreatitis	-	4	9	13 = 54%
Carcinoma	1	5	-	6 = 25%
Diabetes	-	-	1	1 = 4%
Normal pancreas	-	-	4	4 = 17%
Total				24

Figure 5. Apparent normal visualization of the pancreas in patient with adenocarcinoma of the duodenum invading part of the head posteriorly and not involving the excretory ducts. This is the only case of carcinoma in this series with a normal scan.

Table 7 summarizes the appearance of the scan in patients examined for pancreatic disease by categorizing the scan readings according to the final diagnosis.

In conclusion, a normal pancreas scan has a high degree of reliability (88 per cent in this series). When the scan is abnormal, it is difficult or impossible to distinguish reliably between various pathologic entities, especially between pancreatitis and carcinoma. Severe diabetes, starvation,^{3,27} or postoperative states⁴¹ will also cause non-visualization of the pancreas. Since the patency of the excretory ducts seems to be a crucial factor in determining visualiza-

Table 7

APPEARANCE OF SCAN IN PATIENTS INVESTIGATED FOR PANCREATIC DISEASE

Final diagnosis	Normal scan	Non-visualization	Abnormal	Total
Normal pancreas	42 = 86%	3 = 6%	4 = 8%	49
Chronic pancreatitis	4 = 18%	9 = 33%	13 = 48%	27
Diabetes	-	1	1	2
Carcinoma	<u>1</u> = 6%	<u>9</u> = 56%	<u>6</u> = 37%	<u>16</u>
Totals	48	22	24	94

tion, small tumors not obstructing the duct system will therefore be missed when the lesion size is below the resolution limits of current imaging devices.

On the basis of these results, our own attitude to pancreas scanning has been revised. We believe that if the examination is used principally to establish normality or abnormality of the pancreas without attempting an etiologic diagnosis, the pancreas scan—done with sequential imaging—can be a useful diagnostic technique.

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SCANNING OF THE POSTERIOR FOSSA*

By

R. A. Moody,[†] J. O. Olsen,[†] A. Gottschalk, and P. B. Hoffer

It has been increasingly recognized by workers in nuclear medicine that brain scanning of the posterior fossa is nearly as successful as scanning of the supratentorial region.^{1,2} The neurological and neurosurgical literature, however, has generally reflected a more pessimistic view of the benefits of posterior fossa scanning. Furthermore, many of the published reports are small, consisting of less than 20 cases. We have been impressed with the consistency with which certain lesions in the posterior fossa have been detected, and the generally high detection rate of all lesions in this region. This has stimulated us to review the posterior fossa scans performed at this institution from January 1965 to June 1970, the results of which are reported here.

This report describes 37 cases in which the diagnosis was confirmed by direct biopsy or autopsy except for three cases of metastases, where the diagnosis was inferred by knowledge of the primary lesion, and location was confirmed by neurological examination and contrast studies. These cases were selected by first collecting (from the files of the neurosurgery department), all proven posterior fossa tumors that were diagnosed in the defined period. From these records were selected those cases for whom routine 4 view brain scans done on the ACRH scanner were available.

The Argonne Cancer Research Hospital brain scanner is a single organ scanner developed in the early 1960's³ which uses four collimated detectors arranged in two opposing pairs. The lateral views are done simultaneously as are the anterior and posterior views. The images are recorded as photoscans, utilizing a bell-shaped spot for data blending. No other contrast enhancement or background erase is employed. Low energy high-resolution collimators are used. The loss of sensitivity introduced by the high resolution collimators is offset by the use of multiple detectors.

The scans were performed using 10-12 mCi of pertechnetate-99m as introduced at this institution by Harper et al.⁴ Scanning is begun 10 minutes after intravenous injection of the isotope. An oral dose of 0.5 to 1.0 g of perchlorate is given 10 minutes prior to the injection of the tracer, to block the uptake of pertechnetate-99m in the choroid plexus and parotid glands. This improves visualization of the posterior fossa region, and in particular, of the area of the cerebello-pontine angles.

It is most important that the chin be well flexed on the anterior and posterior views. This "opens up" the posterior fossa, moving the venous sinuses upward so that the subtentorial region is not obscured. The average scanning time for all four views is 20 minutes, with the total examination taking about 40 minutes. The blended appearance of the scan, resulting from the use of the bell-shaped spot, is sometimes confusing to those who are unfamiliar with it, but normal structures such as the venous sinuses, bony sinus mucosa, and parotid glands are clearly seen.

*This report is taken from a paper that appeared in *J. Neurosurgery*, 35:181, 1971. The work was supported in part by the Junior Auxiliary of the University of Chicago Cancer Research Foundation.

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RESULTS

Table 1 summarizes the results according to lesion type and reviews the case material. It will be noted that 78 per cent of all posterior fossa lesions were definitely diagnosed, a figure that rises to 84 per cent if suspicious lesions are included. In general the scans did not present subtle diagnostic problems, and the diagnoses represent the collective opinion of all the authors obtained on retrospective reading of the studies.

Table 1
SCANNING BY TUMOR TYPE IN THE PRESENT SERIES

	Total	Positive	Suspicious	Negative
Acoustic neuromas	12	10	1	1
Metastases	7	6	0	1
Juvenile astrocytomas	7	6	0	1
Meningiomas	4	4	0	0
Medulloblastomas	2	0	1	1
Ependymomas	2	2	0	0
Hemangioblastomas	2	1	0	1
Cholesteatomas	<u>1</u>	<u>—</u>	<u>0</u>	<u>1</u>
Totals	37	29	2	6

Acoustic neuromas were detected in 83 per cent of cases. They are characteristically seen in lateral view in the anterior portion of the posterior fossa, near the floor, contiguous with the sigmoid sinus (see Figure 1). It is important not to confuse this lesion with asymmetry of the sigmoid sinus. This can be avoided by taking note of the globoid form of uptake, and by comparison with the posterior view, in which the degree of sinus asymmetry, if any, should be apparent.

Figure 1. Right and left lateral and posterior view of a right acoustic neuroma, arrows. The radiolucent vertical line is a scanner artifact where the two collimators meet.

On this view the area of uptake should appear in the paramedian position, often extending to the midline, just below the confluens sinuum.

Metastases were detected in 85 per cent of cases (see Figure 2). The one metastasis that was missed was a squamous cell carcinoma in the cerebello-pontine angle. In general, if the lesion is not midline, it will usually appear on one lateral view, and not on the other. The discrepancy in uptake between the two lateral views or the area beneath the transverse and sigmoid sinus is an important diagnostic clue to posterior fossa disease.

Figure 2. Right and left lateral views and posterior view of a left cerebellar metastasis from an oat cell carcinoma of the lung, arrows.

The juvenile astrocytomas were detected in 86 per cent or 6 of 7 cases. These astrocytomas are also seen in a midline or paramedian position on the posterior view (Figure 3). They are, however, higher, and tend to overlie the confluens sinuum. In lateral view they tend to be high, close to the transverse sinus. They may be seen either in the anterior portion of the fossa, close to the sinus, or posteriorly near the confluens sinuum.

Figure 3. Right and left lateral views and posterior view of a juvenile astrocytoma of the vermis, arrows.

All meningiomas were detected. In the case of those in the cerebello-pontine angle, there was little about their appearance or location to suggest a histological diagnosis that would distinguish them from the acoustic neuromas. As a general rule, meningiomas tended to have the greatest uptake of any of the lesions.

Neither of the two medulloblastomas was positively detected. Two other children with medulloblastomas, however, were examined with an Anger scintillation camera instead of the ACRH scanner because of their lack of cooperation, and are not included in this series. In both instances, the lesion was detected.

Both cases of ependymomas were detected, while only one of two hemangioblastomas were visualized, and the single cholesteatoma was missed.

DISCUSSION

It is difficult to compare the results of the present series with those recorded by others, for two reasons. First, the larger series were performed with a variety of agents—pertechnetate-99m, ^{197}Hg -chlormerodrin, ^{203}Hg -chlormerodrin, or ^{131}I -HSA—most of them using the latter 2 agents. Second, the types of scanning devices varied widely. As a result, the reported success rates have varied from 20 per cent to greater than 90 per cent.^{1,5-8} Many of the series consist of less than 10 cases, and it is difficult to draw significant conclusions from them. One author⁹ reports a detection rate of 87 per cent with ^{203}Hg -chlormerodrin and of 66 per cent with pertechnetate-99m, but although there are 38 mercury scans, only 6 pertechnetate scans were obtained, and it may be that a larger sample would have reduced this difference. In some series, the isotopes are either not specified, or are mixed. It seems to be generally agreed that the difficulties in posterior fossa scanning with many tracers are primarily attributable to the overlying venous sinuses, especially the confluence of sinuses, and the heavy investment of muscles with their rich vascular supply at the posterior fossa. These problems are compounded by poor patient positioning in the posterior view. Many scanning systems make adequate flexion difficult or impossible.

Despite the variation in scanning techniques and isotopes used, there seems to be general agreement that meningiomas are detected with great frequency.^{1,9-13} Metastases also are reported to be detectable in a large percentage of cases by some,^{9,12-15} although others^{1,10,16-18} report success in 50 per cent or fewer cases. Planiol¹⁹ was able to increase the percentage of detection considerably by the addition of special techniques such as contact counting and gamma-planigraphy using ^{131}I -HSA. Gates and Dore²⁰ report increasing success in detection by the use of delayed brain scanning with $^{99\text{m}}\text{Tc}$.

It is interesting that the literature on acoustic neuromas is equivocal. Although many authors report a high degree of success in detection of this lesion,^{1,12,14,16,17,19,21-24} others have found a detection rate well below 50 per cent.^{10,13} Although authors using similar isotopes report wide discrepancies in this case, there seems to be a tendency for greater success in detection of this tumor with pertechnetate-99m. We feel that adequate suppression of parotid activity is important in allowing visualization of cerebello-pontine angle tumors, and for this purpose, a dose of 1 g perchlorate is currently used routinely.

Astrocytomas, especially cystic astrocytomas, are also detected in a high proportion of cases,^{1,9,13,15,16,25-28} except by Planiol^{17,19} and Paillas¹² who used ^{131}I -HSA. The reason for this discrepancy in these large series remains to be elucidated.

Success with medulloblastomas has been mixed, ranging from nearly 100 per cent in some series to 50 per cent or less in others,^{1,16,18,19,28} while one author reports one positive, one equivocal, and one negative scan.¹⁴ As noted above, we detected neither of two positively with scanning, although one was suspicious. It should be noted that when the camera was used with the same isotope, we detected two of two. The difference, therefore, appears to be unrelated to isotope specificity.

Other lesions were reported in such small numbers as to defy meaningful comparison, but ependymomas^{1,9,10,14,17,19} may often be detected, and brain stem gliomas^{10,12,17,26} frequently so, although some authors rate this very low.^{1,27-29}

The location of the various tumors on the scan has been described by DeLand³⁰ and is the same as that noted in this series.

We believe that scanning of the posterior fossa should yield an accuracy close to that reported for the supratentorial region. Technetium-99m would appear to be a perfectly adequate agent if the parotid gland is well blocked with perchlorate. The proper positioning of the patient is essential for good results. When the scan is properly performed, one can expect to detect nearly 100 per cent of meningiomas, over 85 per cent of ependymomas, metastases, and juvenile astrocytomas, and over 80 per cent of acoustic neuromas. Our overall detection rate was 78 per cent.

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USE OF A DIGITAL COMPUTER FOR REDUCING RESPIRATORY ARTIFACT ON LIVER SCANS MADE WITH A CAMERA^{*}

By

B. E. Oppenheim

Respiratory motion is a major factor limiting the resolution obtainable on liver scans. It causes a disturbing "scalloped" appearance of the liver margins on rectilinear scans. This visual effect can be reduced by close spacing of the scan lines,¹ but this does not recover the information that has been lost due to motion.²

On liver scans made with a camera, respiratory motion degrades image quality by blurring margins. The better the intrinsic resolution of the camera, the more objectionable this blurring becomes. The blurring is increased if dyspnea is present, and frequently patients having liver scans are very ill and somewhat dyspneic.

An effective method is needed for reducing the respiratory motion artifact on liver scans made with a camera, and recovering the information that has been lost due to this motion. One method is to have the patient hold his breath while the camera is collecting counts.^{3,4} We use this method routinely, but it increases the time and difficulty of the study and unfortunately cannot be used for very ill, dyspneic, or uncooperative patients. A method of compensating for the motion of the liver during respiration is needed. Various ways of accomplishing this have been suggested,^{5,6} all of which require some kind of detector external to the camera for determining the position of the liver. Such methods present the technical problems of design, calibration, and maintenance of the required detector. Moreover, the detector must be properly applied to the patient, increasing the time and technical difficulty of the examination. To my knowledge none of these methods has yet been shown to be clinically useful on a routine basis.

A method is presented here for reducing the respiratory motion artifact which does not require a separate device for locating the liver, but derives the necessary positional information from the counts collected by the camera itself in the course of a routine study. The study is subdivided into a large number of time intervals which are so short that there is very little movement of the liver during any one of them, but enough counts can be collected during each interval to permit the position of the liver to be accurately determined. It then becomes possible to reposition the counts to compensate for the changing position of the liver.

MATERIALS AND METHODS

Studies were made with a liver phantom (Figure 1A) and a Picker thyroid phantom (Figure 1B). The liver phantom contained seven spheres of 11, 13, 17, 21, 27, 34, and 43 mm in diameter, which became "cold" areas when radioactivity was introduced into the phantom. The thyroid phantom filled with radionuclide contained three "cold" areas and one "hot" area of varying sizes. The radionuclide used in both phantoms was ^{99m}Tc-pertechnetate.

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Figure 1. Phantoms used in respiration studies. A. Liver phantom. B. Picker thyroid phantom.

The phantoms were scanned on a Nuclear Chicago Pho Gamma III camera, using a 4000 parallel-hole low energy collimator for the liver phantom studies and a pinhole collimator for the thyroid phantom studies. A count rate of approximately 25,000 counts/second was obtained in all studies. Studies were made of both phantoms with no motion as well as with motion of varying amplitudes. Motion was produced by placing the phantom on a specially constructed respiratory motion simulator,³ capable of moving backwards and forwards in a manner analogous to respiratory motion. The amplitude and rate of motion could be varied over a wide range. For all studies with movement a rate of 5 cycles/min was used.

Each study was divided into 16 intervals of 0.6 seconds duration, for a total collection time of 9.6 seconds and a total collection of about 250,000 counts. During any interval the phantom moved 1/20th of a cycle. Counts collected during each interval were stored in a 64 x 64 matrix in the core memory of a PDP-12 computer. At the end of each interval the contents of the matrix were dumped onto a magnetic type mounted on the PDP-12, and subsequently were transferred to a 7 track magnetic tape for processing by an IBM 360 Model 50 computer.

The manner in which a study was corrected for motion is illustrated in Figure 2. First, the IBM 360 identified the "median row" of each of the 64 x 64 matrices. If counts in a matrix are summed, row by row, the median row is the first row for which the sum of counts exceeds half the total number of counts contained in the matrix. Second, each of the matrices was shifted by the computer so that its median row coincided with the median row of the first matrix. Third, the counts in corresponding locations of each matrix were summed to produce a cumulative 64 x 64 matrix containing all the counts for the study corrected for motion. An additional cumulative matrix with no correction for motion was produced for each study by combining the 16 matrices without shifting them. The uncorrected and corrected cumulative matrices were transferred to a 7 track magnetic tape and subsequently to a PDP magnetic tape, and finally were displayed on the oscilloscope of the PDP-12 and photographed. The display utilized a 64 level gray scale with data blending.

Figure 2. Diagram of the method used to correct for respiratory motion. The input into the IBM 360 is counts from the camera, in the form of a series of matrices, one for each of the short time intervals into which the study is divided. The output is two cumulative matrices, each containing all the counts collected during the study, one with motion uncorrected, the other with motion corrected. See text for the definition of the "median row" (short arrows).

RESULTS

Figure 3 demonstrates the effectiveness of the method in correcting for motion on the studies made with the liver phantom. Figures 3A, 3C, and 3E were made without motion, and with back-and-forth motions of 25 mm and 50 mm respectively. Figures 3B, 3D, and 3F were made from the same data as Figures 3A, 3C, and 3E, but corrected for motion. A considerable improvement in image quality is apparent, with sharpening of the margins of the phantom, increased perceptibility of the "cold" areas, and elimination of the distortion caused by motion. Figure 4 similarly demonstrates the effect of the method on pinhole studies of the thyroid phantom. Figure 4A, 4C, and 4E were made without motion, and with back-and-forth motions of 12 mm and 25 mm respectively. Figures 4B, 4D, and 4F were made from the same data as Figures 4A, 4C, and 4E, but corrected for motion. Once again margins are seen to be much sharper, perceptibility of "cold" and "hot" areas is improved, and distortion due to motion is eliminated.

DISCUSSION

The method described uses only the counts collected by the camera to derive the positional information necessary to correct for motion. In clinical studies it would not require patient cooperation, nor any alteration in the technique of a liver scan, and would not be impaired by respiratory irregularities.

Figure 3. Liver phantom studies. A. No motion of the phantom, no correction for motion. B. Same data as in A, motion corrected. C. 25 mm motion, uncorrected. D. Same data as in C, motion corrected. E. 50 mm motion, uncorrected. F. Same data as in E, motion corrected.

Figure 4. Thyroid phantom studies. A. No motion of the phantom, no correction for motion. B. Same data as in A, motion corrected. C. 12 mm motion, uncorrected. D. Same data as in C, motion corrected. E. 25 mm motion, uncorrected. F. Same data as in E, motion corrected.

The theoretical aspects of this method have been investigated in detail and will be the subject of a subsequent publication. It can be shown that the "median row" is an accurate indicator of liver position. The effectiveness of the method is increased with decreased respiratory rate, increased depth of respiration, increased count rate, or increased detector resolution. Enlarging the computer matrix increases its spatial resolution, but since this can never exceed the resolution of the detector, beyond a certain point any increase in matrix size results in negligible gain in resolution, while entailing a considerable increase in the costs of equipment and processing. It has been shown⁷ that for the equipment presently available the optimum matrix size for recording static images is 128 x 128. Either lengthening or shortening the time interval tends to impair the accuracy with which the liver can be localized. Lengthening the interval increases liver motion during each interval, while shortening the interval decreases the number of counts collected during each interval, and thus increases the statistical error in determining liver position. The optimum time interval varies depending on the count rate and respiratory rate. On theoretical grounds it appears that 0.25 seconds would be a good compromise for clinical studies. For a respiratory rate of 12 breaths/min a 0.25-second interval represents 1/20th of a cycle, which is the fraction of a cycle covered by each interval in the phantom studies.

It can be demonstrated theoretically that with a count rate of 600,000 counts/min, storing counts for 0.25-second intervals in a 128 x 128 matrix, the method should produce about a five-fold reduction in respiratory artifact for normal patients breathing quietly, with greater reductions possible for dyspneic patients. With a count rate of 400,000 counts/min about a fourfold improvement should be obtained.

A comparison of the thyroid phantom studies with the liver phantom studies demonstrates the increased effectiveness of this method when detector resolution is improved. A greater improvement is noticeable between Figures 4C and 4D, made with only 12 mm movement of the thyroid phantom but with the high resolution pinhole collimator, than is noticeable between Figures 3C and 3D, made with 25 mm movement of the liver phantom but with a lower resolution multiple hole collimator.

Clinical application of this method will place much more stringent requirements on the interface than the phantom studies cited here. The interface should meet the following specifications:

1. It should utilize a 128 x 128 matrix for storing counts, though a 64 x 64 matrix might be used if a slight loss in resolution is accepted;
2. Either the dump time for a single matrix should be under 0.25 seconds, or the interface should contain a double core memory the size of two matrices, and be capable of alternately storing into and dumping from each region. Otherwise, since dump time is dead time with respect to count collection, clinical studies will be prolonged excessively;
3. The associated tape drive should be able to store at least 500 matrices on a single magnetic tape. With 0.25 second intervals, one minute of counting will generate 240 matrices, and the tape must be able to accommodate such vast amounts of data.

We are currently constructing an interface that will collect counts in a 128 x 128 matrix, will dump an entire matrix in 0.14 seconds, and will store about 1500 matrices on a single tape.

While initially all processing will be done on an IBM 360 computer, we are seeking a cheaper and more convenient device for doing this. Processing on a medium size computer appears feasible and will be attempted. Other computer configurations, such as task-specific computer

module assemblies, are worthy of consideration. In addition we are investigating analog techniques, which will permit the method to be applied very conveniently and inexpensively if they are successful.

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DEVELOPMENT OF A DIAGNOSTIC X-RAY SPECTROMETER WITH LITHIUM
DRIFTED SILICON DETECTOR FOR INTERCOMPARISON AND
IMAGE ANALYSIS*

By

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The body of information bearing on the production of radiographs in diagnostic radiology is chiefly the result of an empirical effort to relate image quality to operating tube characteristics for a variety of exposure techniques. The object of this effort has been the optimization of the imaging procedure in order to secure the highest quality image at the lowest possible patient dose by providing standard exposure parameters presumably valid for most diagnostic machines and patients. To attain this goal, the first concern was the development of an accurate but simple means of radiographic calibration so that standard operating characteristics could be assumed to be in operation from department to department and among the wide variety of devices. These efforts at standardization are undoubtedly far from complete, and it is also true that there is a formidable void in our knowledge of optimal imaging procedures and parameters.

It was our concern to develop an investigative tool with attendant techniques applicable in the analysis of diagnostic beams in order to investigate and catalogue systematically the impact of a given energy spectrum on the image quality and the patient dose. We have employed a lithium drifted silicon detector [Si(Li)] for measuring diagnostic energy spectra and we report on the first use of this spectrometer in resolving the imaging differences between two identically calibrated diagnostic units.

In recent years, investigators have exploited scintillation techniques in the analysis of x- and γ -ray spectra derived from diagnostic generators.^{1,2,3} Drexler and Perzl have examined the use of solid state detectors in the analysis of low energy x- and γ -rays in considerable detail.^{4,5,6} This work culminated in the publication of an atlas of a variety of spectra under varying conditions of x-ray tube potential and filtration by Drexler and Gossrau.⁷ These investigators, however, were concerned to characterize diagnostic beams in order to establish the relationship between the spectrum and the operating parameters of the facility (e.g., peak kilovoltage, tube current, filtration, and power supply characteristics). Other investigators have sought to develop radiographic calibration procedures for characterizing beam quality with half value layer determinations,⁸⁻¹² for determining the operating peak kilovoltage with K-shell fluorescence techniques¹³⁻¹⁶ and for measuring the impact on beam quality and exposure rates of differences in generator kilovoltage waveform and cable capacitance.^{14,17-19} A wide variety of factors affecting beam quality are summarized by the International Commission on Radiation Units and Measurements.²⁰ The present report will discuss the techniques employed in linking the photon energy spectrum of a diagnostic beam directly with its imaging characteristics in order to develop procedures and criteria for use in the analysis of the exposure operation in diagnostic image formation. Hence, the study deals with the effects of x-ray quality on images but not with the calibration of x-ray generators.

* This report is taken from a paper that appeared in *Radiology*, 101:401, 1971.

MATERIALS AND METHODS

A Si(Li) detector assembly which included a nitrogen dewar for cooling, a vacuum gauge, cold finger and pre-amplifier, was provided with a bias voltage and vacuum gauge supply for the purpose of conducting studies into the use of such detectors to characterize x- and γ -ray spectra. A pulse-shaping amplifier was procured which was employed to deliver the input to the 400 channel multi channel analyzer (MCA) that provided output in the form of a cathode ray tube (CRT) display and punched tape for further data processing by computer. In order to prevent electronic and thermal noise from degrading the operation of such a system, it is necessary to cool both the crystal detector and the pre-amplifier with liquid nitrogen and, further, to keep the crystal under vacuum to prevent physical degradation. Since the history and characteristics of the detector were unknown, it was necessary to determine the response function of the system in the diagnostic energy region and at the same time combine the crystal and electronics into a flexible but portable spectrometer facility for making measurements. The experimental set-up is illustrated in Figures 1a,b. The crystal-dewar-pre-amplifier assembly was modified for horizontal mounting on an optical bench with horizontal and vertical deflection controls to facilitate radiographic alignment. A lead shield was mounted on the optical bench proximal to the adjustable collimator found on most diagnostic x-ray tubes. This shield was designed to serve as a mount for a lead collimator that defined the focal spot of the tube being examined. Thus, upon measuring the focal spot with the ICRU recommended pinhole and camera technique,²¹ a defining collimator was fashioned from lead with a diameter approximately the same as the measured diameter of the focal spot for mounting into the shield. A second lead shield was mounted on the face of the crystal housing for the purpose of accommodating a pinhole over the center of the sensitive region on the detector. ICRU pinholes were employed to reduce the intensity of the incident beam at the detector in order to prevent too high a count-rate, which would result in spectral distortion ("flooding" of the detector). The entire experimental assembly was adjusted to provide a detector to target interval of 100 centimeters.

Calibration of the detector assembly was complicated by one preeminent factor: a very severe reduction in the charge collection efficiency as a function of increasing photon energy.⁴ A series of experiments was performed to determine the operating characteristics of the experimental array by the use of radioisotopes. For these experiments, the bias voltage was maintained at 250 volts, pinhole collimation at the detector face was not employed because of the relatively lower count rate of the sources selected, and the source-to-detector interval was varied depending upon the measurement required. An initial experiment demonstrated the energy scale to be linear within ± 190 eV in the interval of 0 to 140 keV. This analysis was performed using the characteristic radiations listed in Table 1.²² The efficiency of the detector system as a function of energy was examined by using the known relative efficiencies of characteristic lines from the sources listed in Table 2²² and fitting these data to an empirical expression for efficiency. Hence, by comparing the measured relative efficiencies with the reported values, a few points for the efficiency response function could be defined although the full function could not be resolved without a more extensive experimental effort. Since the counting efficiency of the detector system as a function of incident photon energy is dependent on undefined electronic characteristics as well as the physical crystal parameters, there was no analytic expression to which the experimentally derived data could be fitted. The following empirical formula for de-

Figure 1. Figure 1a is a diagrammatic representation of the experimental geometry depicted photographically in Figure 1b. The focal spot defining collimator assembly and shield is displayed in a position removed from the adjustable collimator housing in the photograph (1b) for better viewing.

tector efficiency, $\epsilon(E)$, was therefore derived, and is employed by the Center for Radiation Research of the National Bureau of Standards for characterizing Ge(Li) detectors.²³

$$\epsilon(E) = \sum_i a_i \left(\frac{1}{E}\right)^i \quad (1)$$

where E is photon energy, i is the index of summation for the expansion of the power series, and

Table 1

Nuclide	Mode of decay	Decay product	Characteristic line	Energy (keV)
^{99m}Tc			K_{α}	18.33
^{99m}Tc			K_{β}	20.66
^{241}Am	Alpha	^{237}Np	γ_{Np}	26.35
^{125}I	electron capture	^{125}Te	K_{α}	27.35
^{125}I	electron capture	^{125}Te	K_{β}	31.13
^{125}I	electron capture	^{125}Te	γ_{Te}	35.48
^{241}Am	alpha	^{237}Np	γ_{Np}	43.43
^{241}Am	alpha	^{237}Np	γ_{Np}	59.54
^{57}Co	electron capture	^{57}Fe	γ_{Fe}	121.97
^{57}Co	electron capture	^{57}Fe	γ_{Fe}	136.33

Table 2

Nuclide	Energy (keV)	Relative intensity
^{241}Am	11.89 Np L_I	2.2
	13.9 Np L_{α}	37.5
	17.8 Np L_{β}	51.2
	20.8 Np L_{γ}	13.8
	26.35	7.0
	59.5	100.0
^{57}Co	14.4	11.0
	122.0	100.0
	136.3	13.0

α_i are fitted coefficients for weighting the elements in the summation. The efficiency of Ge(Li) detectors in the diagnostic energy region is quite good, however, falling to 60 per cent at 140 keV for a detector with a 10 mm depth in the charge sensitive region.⁴ Thus, an expansion of the series given in expression 1 needs only be carried out to 3 or 4 terms in order to obtain an adequate fit for the data. The response of Si(Li) detectors is much more severely delimited, resulting in very rapidly declining counting efficiencies below 20 keV which would require an inordinate expansion of the above power series in order to achieve a representative characterization of the detector. We have selected an alternative technique which requires formulating an expression for a curve to approximate the efficiency function with enough parameters to assure an adequate fit of the experimental data. This was done by noting that below incident photon energies of approximately 18 keV, the efficiency of the Si(Li) detector is very nearly unity.⁴ Above 18 keV to about 70 keV, the detector efficiency falls off in a manner characteristic of a normal curve, after which it flattens into an almost exponential tail. The following expressions were thus employed,

$$\epsilon(E) = 1 \quad \text{for } E \leq \eta \quad (2)$$

$$\epsilon(E) = \epsilon_0 e^{-\left(\frac{E-\eta}{\sigma}\right)^2} + (1 - \epsilon_0) e^{-\left(\frac{E-\eta}{\gamma}\right)} \text{ for } \eta < E \leq 140 \text{ keV} \quad (3)$$

where $\epsilon(E)$ is the detector efficiency at the incident photon energy, E (in keV); ϵ_0 , σ , and γ , are the appropriate curve-fitting parameters and η is the energy offset (set at 18 keV in alignment with the above discussion) below which the detector efficiency is assumed to be unit. Figure 2 is the plot of the efficiency function defined by expressions 2 and 3 fitted to the data plotted

Figure 2. Approximate energy dependent counting efficiency function for the spectrometer assembly. The smooth curve represents the non-linear regression (least squares fit) of the given data points to expressions 2 and 3 given in the text. The dotted line enveloping the smooth curve is the estimate of the standard error derived from the fitting computation.

on the graph. The deterioration in detector efficiency with energy is marked. The estimated standard error for the fitted function is included as an envelope of dotted lines and may be seen to degrade rapidly with increasing energy, further reducing the useful range of this detector. The full width at half maximum (FWHM) was 2.2 keV at 59.54 keV, and rose to 4.8 keV at 136.33 keV.

Despite the limitations of the system, it was felt that sufficient data could be obtained to develop the procedures necessary for measurements and intercomparisons. Accordingly, an exam-

ination of the permissible counting rates was undertaken using the experimental configuration described previously for measuring x-ray spectra. Numerous spectra were recorded from a Super Rotalix tube supplied by a Philips Maximum DLX generator (6 rectifier 3 phase) with a tube focal spot of 1.2 mm^2 area and a focal spot defining collimator of 1 mm diameter. Figure 3 is a computer normalized plot of four spectra taken at 50 kVp and 40 mA tube current with 2 mm Al added filtration for 5 second exposures. The counting rate was reduced for the four

Figure 3. Computer normalized spectra (normalization to total count) for 4 five-second exposures taken at 50 kVp and 40 mA tube current with 2 mm Al added filtration. The four spectra indicate the effect on the raw data of employing $30 \mu\text{m}$, $75 \mu\text{m}$, $100 \mu\text{m}$, and $370 \mu\text{m}$ pinholes to attenuate the beam intensity at the detector. ΔN_E is the number of counts recorded at energy E (corrected for counter efficiency), and N is the total corrected number of counts.

spectra by using $30 \mu\text{m}$, $75 \mu\text{m}$, $100 \mu\text{m}$ and $370 \mu\text{m}$ pinholes. The last pinhole was a shop-fabricated insert made of lead. Aside from the very large statistical scatter inherent in the short counting time provided for these exposures, it appears that the $30 \mu\text{m}$, $75 \mu\text{m}$, and $100 \mu\text{m}$ defined spectra demonstrate little discernible deviation from each other whereas the large $370 \mu\text{m}$ pinhole produces a significant spectral shift. This is probably due to flooding of the detector. As a result, all subsequent work employed the $100 \mu\text{m}$ pinhole since it was considered that this was the largest available beam defining collimator that would adequately reduce beam intensity at the detector and thus prevent flooding, yet still provide acceptable counting statistics.

A large number of spectral measurements were made under varying conditions of peak kilovoltage, current loading and filtration with exposure times of from 1 to 10 seconds in order to approach the operating conditions prevalent in clinical use. Owing to the restriction such short exposures impose on the counting times, it was apparent that specialized data processing would be necessary in order to obtain a useful spectral characterization from highly statistically scattered and efficiency distorted data. Although for many applications prolonged or repetitive exposures could be employed, it was also true that characterization of transient spectral components or tubes operating under restrictive conditions would require a specialized data processing format. Accordingly, a computer package was developed which contained the following elements:

1. Translation of the spectral data from punch tape to IBM punch cards for higher order processing and permanent storage.

2. A curve smoothing program designed to extract the smoothed spectrum from the raw data by constructing the cubic spline which can be shown to minimize the estimate of the standard error for each data point about the smoothed function.²⁴

3. Efficiency demodulation (correcting for the energy response characteristics of the system).

4. Spectrum normalization to the total number of counts (corrected for the efficiency response).

5. Graphic and numeric output to include scaling and translation of the axes. Additional corrections as cited in ICRU Report 17²⁰ were not considered of sufficient importance to be undertaken for this pilot study. A brief summary of these factors and an estimate of their importance is given in Table 3. Resolution broadening of the measured spectrum most recently discussed by Unsworth and Greening²⁵ was also ignored since it was felt that the effect would be minimal with solid state detectors in conjunction with intercomparison studies.

Table 3

Experimental error	Comment
Attenuation in the entrance window of the detector	Due to the relatively thin entrance window (usually beryllium between .25 mm and .05 mm), the 100 cm air attenuation, 2 mm Al filtration and relatively high diagnostic energies examined, this was obviously of minimal importance.
Complete penetration of radiation through the detector	Implicit in the efficiency determinations discussed in the report
Escape of characteristic radiation	This factor was essentially ignored due to the low-energy cut-off selected. K shell fluorescence for both Si and Li are 1.84 keV and 55 keV respectively.
Escape of Compton and Rayleigh scattered radiation	Implicit in the efficiency determinations
Escape of secondary electrons	Of doubtful importance (ref. 20) but is implicit in the efficiency response
Characteristic radiation	The low energy lines were deleted from consideration by adjusting the counting threshold at low energy. This will pose a serious problem at higher energies where such components may have significant effects upon the image.
Target filtration	Not considered due to the use of photon energies above 10 keV

RESULTS AND DISCUSSION

Figure 4 is a data processed sequence of plots for spectra taken at eight console-indicated kVp settings (50, 60, 72, 85, 100, 112, 120 and 130 kVp). It will be noted that the noise components most prevalent in the lower keV channels have not been subtracted since our primary concern was the comparison of spectra and not absolute calibration. In each recorded plot in Figure

Figure 4. Normalized (to total efficiency corrected count), smoothed and efficiency demodulated plots of spectra with (F) and without (U) 2 mm Al added filtration and console kVp settings indicated. The exposure conditions were 75 mA tube current with exposure times of 2 seconds.

4, U denotes the unfiltered spectrum (inherent filtration) and F denotes 2 mm aluminum added filtration for purposes of comparison. For each plot the additional exposure conditions were 2 second exposure time and 75 mA tube current. The experimental setup and calibration procedure were as described in the preceding section. Two important features are represented in these data:

1. The apparent change in the low energy cut-off with changing filtration coupled with a trend of the converging high energy portions of the spectra toward the appropriate peak kV;
2. The recovery of certain spectral details (e.g., characteristic fluorescent lines from the tungsten target and other excitable impurities in the experimental array) due to the smoothing operation on the highly scattered raw data. (Note the introduction of plotting anomalies due to the failure of the smoothing operation in the region of very low signal to noise ratio in the high energy tail of spectra generated at 85 kVp and above.)

An experiment was performed at 85 kVp (console setting) with no added filtration, 2 mm aluminum and 3 mm aluminum + 1/2 mm copper added filtration under the exposure conditions indicated above. The processed spectra are given in Figure 5. Again, the progressive hardening of the spectrum is apparent although the high energy convergence of the spectra is obscured by the plotting anomalies inherent in this technique. Figure 5 thus serves to delineate the limits of interpretation imposed at the higher diagnostic energies.

Figure 5. 85 kVp spectra (console indicated with 75 mA tube current, 2 second exposure taken with no added filtration, 2 mm Al added, and 3 mm Al + 1/2 mm Cu added.

A comparison study was undertaken in an effort to characterize two similar diagnostic installations with divergent radiographic imaging qualities presumably calibrated in identical fashion. The difference in the imaging characteristics of the 2 units (to be denoted as Units A and

B) was brought to our attention by Dr. James R. Williams.* Unit A is the Philips unit described above and unit B is also a Super Rotalix tube but supplied by a Philips Super 100 generator (6 rectifier 3 phase) with falling load. This latter factor represents a significant difference in the operating characteristics of the two units. Both tubes have rated focal spots of 1.2 mm^2 area. The procedure currently used to standardize the operation of departmental machines is performed by measuring the voltage from the transformer secondary at the anode supply with a voltage divider coupled to a meter manufactured by Philips. Spectra were recorded from both units with 2 second exposures and 2 mm aluminum filtration + 1.5 mm aluminum inherent filtration for meter indicated peak kilovoltages of 50, 85, 100 and 120 kVp. The tube load for unit A was 75 mA (console reading) and variable for unit B (dependent upon the operating characteristics of the supply). These spectra are given in Figure 6. The spectral differences are quite apparent and form a consistent pattern. Unit B appears to be operating at a higher peak kilovoltage

Figure 6. Intercomparison of spectra taken from units A and B (see text) at the four indicated console kVp settings. The exposure conditions were as prescribed for the preceding figures and were taken with the normal 2 mm Al added filtration.

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although the complexity of the distributions above 85 kVp introduces additional obscuring elements. Although a careful determination of the statistical error inherent in the data processing techniques must be undertaken in order to measure the stability of the processed spectra, it is clear from Figure 6 that, indeed, a trend in the data does exist. To further illustrate the nature of these differences, radiographs of a test object were made with the same kVp settings as used for the energy spectra measurements. The test object consisted of 4 aluminum cylinders with 0.5, 1.0, 2.0 and 4.0 mm diameter and a 4.0 mm lucite cylinder placed approximately at the mid-line of a 20 cm thick lucite phantom. The images were made using Kodak RPL film with DuPont Par Speed screens. The focal spot to film distance was 104 cm with a 10:1 focused grid. Background densities of the radiographs were matched at a density of 1.60 within ± 0.03 . Figure 7 is a photograph of the radiographs obtained for visual comparison. Careful examination of these images verifies the trend apparent in Figure 6. At each energy setting, the image obtained from tube A exhibits higher radiographic contrast than that obtained from tube B (although the general trend of decreasing contrast at increasing energy is preserved). At 85 kVp (console setting), the

Figure 7. Test object radiographs (see text for phantom description) taken with units A and B for image intercomparisons. The exposure and technical details are given in the text. (Photographic reproduction mitigates the effect seen on the original radiographs.)

0.5 mm diameter aluminum cylinder is only marginally observed in the radiograph from tube B but is clearly visible when exposed with tube A. At 120 kVp (console setting), the 0.5 mm aluminum cylinder is still discernible in the radiograph from tube A but cannot be detected in the radiograph from tube B. These results are predictable from the spectroscopic data presented in Figure 6. The higher energy spectral components emitted by tube B at each kVp setting reduce the radiographic contrast of the recorded image. The clinical importance of this fact may be dramatized by noting that the aluminum cylinders employed in this phantom have x-ray attenuation characteristics similar to blood vessels containing the radioopaque dye used in angiography.

The image-spectrum analysis illustrated in Figures 6 and 7 represents a first step in determining the operating characteristics of two given units. Although certain general observations may be made with respect to the spectral differences observed, it is clear that no precise causal determinations can be made. Certainly the peak kilovoltage can be changed to bring the units into closer imaging alignment. In any event, the effect on the image is easily detected. This analytic technique determines the photon energy spectrum integrated over the time duration of the exposure and, hence, fails to provide information on transients or output waveforms such as those afforded by other techniques.^{14,18} A future concern will be the adaptation of the solid state detector to the measurement of short lived spectral transients so that, ultimately, a direct correlation may be made between the time varying beam quality and the exposure time integrated output of the x-ray generator. It is apparent from the intercomparison data reported in this investigation that peak kilovoltage calibration techniques of the type employed with units A and B are inadequate criteria in equating the performance of various diagnostic machines. Since image analysis requires a quantitative determination of imaging beam quality, it is desirable to further develop this spectroscopic procedure. This is the case since such techniques as oscilloscope coupled ionization chambers would not serve to provide direct information on the photon energy spectrum of an x-ray generator. An understanding of the imaging process requires a knowledge of the photon energy spectrum of the incident beam as well as such factors as the quantum efficiency and spatial frequency response of the image forming system (e.g., film and image intensifiers). Hence, how changes in exposure waveform and power supply transients affect changes in image characteristics over a variety of operating conditions will ultimately require a means for recording the spectral output of x-ray generators. The ultimate goal of such an effort will be the optimization of the exposure operation by prescribing imaging parameters for given diagnostic examinations which will produce diagnostic beams of lower patient exposure at the most reasonable level of image quality.

It is inadequate to specify operating conditions for given diagnostic procedures because there are large differences between facilities. Image enhancement will be obtained through careful analysis of a variety of parameters and their impact on the image formed. Quantitative x- and γ -ray spectrometry is essential if we are to establish an ordered view of what a diagnostic generator must do to provide the best image for the least risk to the patient. A corollary of this is the enhanced efficiency to be expected in the performance of the diagnostic radiologist in detecting and interpreting anomalous contours.

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EVIDENCE FOR THE EARLY SYNTHESIS OF T4 BACTERIOPHAGE-CODED TRANSFER RNA*

By

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The synthesis of viral-coded tRNAs after T-even phage infection of *Escherichia coli*¹⁻³ has now been reported from several laboratories. More recently, it has been found that bacteriophage T5 also codes for the synthesis of its own tRNAs.⁴

³⁵S-tRNA from pulse-labeled T4-infected cells show 3 peaks of radioactivity on methylated albumin kieselguhr (MAK) chromatography whereas only 2 major ³⁵S-tRNA peaks are seen with uninfected *E. coli*.⁵ The third ³⁵S-peak represents (in part) the *de novo* synthesis of T4-specific tRNA since 25 to 30 per cent of the labeled RNA is hybridizable to T4 DNA, and both leucine and proline T4-specific tRNAs are present in this same peak fraction.¹ This report describes results from studies with "third peak" T4 ³⁵S-labeled tRNA, as well as T4 (³H)leucyl-tRNA, which indicate that phage tRNA synthesis is an early function of the replicative process and suggests, in addition, that this synthesis is independent of viral-induced protein formation.

RESULTS AND DISCUSSION

Earlier observations on the timing of "peak 3" appearance after phage infection indicate that T4 ³⁵S-tRNA formation is probably an early viral function.⁵ This conclusion is more firmly supported by the following experiments. Nonradioactive RNA was prepared from cells infected with T4 phage in the presence of chloramphenicol (CAP) and incubated at 37° for various time intervals (0-60 min) after infection. The RNA extracted from CAP-blocked, T4-infected cells was tested for its ability to compete with T4 "peak 3" ³⁵S-tRNA for hybridization to T4 DNA. Figure 1 shows that despite pre-infection addition of CAP, RNA isolated as early as 2.5 minutes after infection competes with T4 ³⁵S-tRNA for hybridization sites on T4 DNA. With increasing times of infection the infected-cell RNA competes more effectively with the labeled T4 tRNA in the hybridization assay, suggesting the accumulation of RNA homologous to viral tRNA. On a weight basis, the rate of accumulation of viral tRNA competitor is slower in the presence of CAP than in its absence (compare the 20 minute infected RNA preparations in Figure 1). This difference may reflect a slower rate of viral RNA transcription in the presence of CAP or it may simply represent a lower percentage of viral tRNA competitor per µg of total RNA extracted, since, in the presence of CAP, host RNA synthesis is not shut off after phage infection.⁶⁻⁹

In agreement with the data presented above, Figure 2 shows that the tRNA isolated from *E. coli* cells infected with T4 phage in the presence of CAP also competes with T4 (³H)leucyl-tRNA for hybridization sites on T4 DNA. Once again, significant competition occurs with RNA

* This report is taken from a paper that appeared in *Biochem. Biophys. Res. Comm.*, 40:919, 1970.

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Figure 1. Hybridization of T4 ^{35}S -tRNA to T4 DNA in the presence of competing RNA isolated from CAP-blocked cells at different times after T4 infection.

The annealing mixture (0.60 ml) contained 60 μg of denatured T4 DNA fixed onto nitrocellulose filters, 0.66 μg of T4 "peak 3" ^{35}S -tRNA (1.1×10^4 cpm per μg), various amounts of competitor RNA as indicated, 0.3 M NaCl - 0.03 M citrate pH 5.5, and 0.30 ml of formamide. After annealing for 20 hours at 37° , the filters were washed and RNase treated as previously described,¹ and the radioactivity "fixed" onto the filters determined by scintillation counting. The preparation of T4 ^{35}S -tRNA (MAK peak 3) and the conditions for *E. coli* growth and T4 phage infection were as reported elsewhere.¹ Competitor RNA was extracted from *E. coli* B infected with T4 phage for 20 min in the absence of CAP, and for 0, 2.5, 5, 10, 20 and 60 min in the presence of CAP (50 μg per ml of culture) which was added to the culture medium 2 min prior to infection. The stopping of infection at the time intervals indicated, their collection and the isolation of RNA was as previously reported.⁵

isolated early after infection, and the capacity for the isolated RNA to compete with viral tRNA increases with longer periods of infection.

Additional evidence suggesting that viral tRNA is an early function of vegetative phage development was obtained from the hybridization characteristics of ^{35}S -labeled T4 tRNA to the separated *l* and *r* strands of T4 DNA.¹⁰ Table 1 shows that when annealing to the respective strands of T4 DNA was carried out, 25 per cent of the input counts hybridized to the *l* strand, whereas the radioactivity fixed to the *r* strand was at the background level. Since the early classes of T4 phage RNA are transcribed from the *l* strand,¹⁰ this result is consistent with the conclusion that phage tRNA transcription is an early event in the phage maturation process.

The presence of RNA molecules capable of competing with acylated phage tRNAs (Figure 2) in CAP-blocked infected cells, led us to enquire whether the competitor RNA itself was functional with respect to amino acid charging. Table 2 shows that T4 phage-specific tRNAs capable of accepting arginine, glycine, and leucine (proline and isoleucine were not tested) are

Figure 2. Time of RNA appearance in T4-infected CAP-blocked cells which competes with T4 (³H)leucyl-tRNA for hybridization to T4 DNA.

The annealing mixture was as described for Figure 1 except that the radioactive nucleic acid used was T4 (³H)leucyl-tRNA (12.8 μ g containing 108,800 cpm of (³H)leucine with a specific activity of 58 c/mM), filters contained 39 μ g of denatured T4 DNA, and 68 μ g of competitor RNA isolated from cells infected with T4 phage in the presence of CAP (50 μ g per ml of culture) for 0, 5, 10 and 20 minutes was individually added as indicated above. After annealing for 4 hours at 35° the extent of (³H)leucyl-tRNA hybridization to the T4 DNA-filters was determined as described for Figure 1.

Table 1
HYBRIDIZATION PATTERN OF T4 ³⁵S-tRNA WITH THE SEPARATED STRANDS OF T4 DNA*

DNA	Input radioactivity (c.p.m.) a	c.p.m. annealed	c.p.m. annealed corrected for blank filter b	% hybridized $\frac{b}{a} \times 100$	% l:r
T4 l strand	4436	1137	1003	25	100
T4 r strand	4436	131	0	0	0
λ dN DNA	4436	190	56	1.3	-
<i>E. coli</i> dN DNA	4436	198	64	1.4	-
Blank filter (-DNA)	4436	134	-	-	-

* T4 ³⁵S-tRNA collected from Peak 3 fraction of the MAK column was isolated from T4 infected *E. coli* B cells as described by Weiss et al.¹ Separation of the complementary T4 DNA strands with poly U,G was as described by Guha and Szybalski.¹⁰ The hybridization technique was essentially that of Nygaard and Hall.¹⁴

Table 2

THE ANNEALING OF CHARGED tRNAs ISOLATED FROM CHLORAMPHENICOL
T4-INFECTED CELLS, WITH T4 DNA*

Amino acid	Specific activity c/mM	Charged		DNA fixed on filter		Competitor <i>E. coli</i> tRNA	Radioactivity fixed on filter (c.p.m.)
		T4 μ g	tRNA (c.p.m.)	T4	T5		
Arg	45	24	(632,000)	+	-	-	551
		24	(632,000)	+	-	+	555
		24	(632,000)	-	+	-	186
Gly	20	24	(536,000)	+	-	-	1187
		24	(536,000)	+	-	+	1160
		24	(536,000)	-	+	-	94
Leu	58	24	(158,000)	+	-	-	330
		24	(158,000)	+	-	+	293
		24	(158,000)	-	+	-	55

* The conditions for annealing were as described for Figure 2. RNA was isolated from cells infected for 20 min with T4 phage in the presence of chloramphenicol (100 μ g per ml), purified and charged with the (3 H) amino acids shown as previously reported.¹ The amount of charged tRNA used is shown. The quantities of T4 and T5 DNA impregnated onto filters were 45 μ g and 35 μ g, respectively. Where shown, 150 μ g of *E. coli* tRNA was included in the annealing mixture. Hybridization of (3 H) aminoacyl-tRNA was determined as described for Figure 1. Five T4 tRNA species have been identified which include arg, gly, leu, ileu and pro.⁴

formed in CAP-blocked T4-infected cells. These results suggest the following possibilities:

(1) Phage tRNAs do not require chemical modification (i.e., methylation, thiolation, pseudouridine formation, etc.) for amino acid charging; (2) Phage tRNAs do require chemical modification for amino acid acceptor activity, but host-modifying enzymes, and not viral-induced enzymes, are employed for this function.

Several comments should be made in considering these two possibilities. Although the base composition of viral-tRNAs is unknown, several independent observations suggest that T4 tRNAs include methylated¹¹ and thiolated bases^{1,5} as well as pseudouridine.¹² Furthermore, the synthesis of phage-induced tRNA modifying enzymes in infected-cell preparations is indicated by the following: (1) Discrete changes in the relative activities of the different base-specific tRNA methylases¹³ as well as changes in rates of tRNA sulfuration⁵ have been observed after T-even phage infection of *E. coli*. (2) In the presence of chloramphenicol, T4 35 S-tRNA (MAK, peak 3) formation does not occur, suggesting that some viral-induced enzyme is responsible for T4 tRNA thiolation.⁵ These findings, including those reported in this paper, raise the possibility that when infection is carried out under conditions where protein synthesis is inhibited, T4 tRNAs are transcribed but not modified with respect to the formation of certain minor bases. If this interpretation is correct, then the fact that such tRNA molecules can be aminoacylated would indicate that these minor bases play no obvious role in the charging of viral tRNAs.

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TRANSLATION OF AN RNA VIRAL MESSAGE IN VITRO: ONE-STEP POLYPEPTIDE CHAIN ELONGATION*

By

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Translation of RNA viral messages in vitro occurs in three steps: initiation, elongation and termination. The components required for initiation differ from those for polypeptide chain propagation. Thus, whereas ribosomes, fMet-tRNA_f, GTP, and Mg⁺⁺ are required in the formation of the initiation complex, additional components, such as a high speed supernatant fraction of *E. coli* extract, ATP, all twenty amino acids and their cognate tRNAs are needed for the latter process.^{1,2}

In principle, therefore, if the initiation reaction is carried out under conditions that do not satisfy elongation, and the reaction mixture is diluted sufficiently to prevent further interaction between template RNA, initiator tRNA and ribosomes, only the formylmethionine present in the initiation complex formed prior to dilution should be converted into polypeptide chains upon introduction of the components required for elongation. Such an experiment using MS2 RNA as messenger RNA is reported in this communication, and is referred to as one-step elongation.

MATERIALS AND METHODS

Preparation of ribosomes and S165 supernatant fraction. Ribosomes and S165 were prepared as already described.³ Unwashed ribosomes were used throughout.

Preparation of charged tRNA. fMet-tRNA_f was prepared by charging *E. coli* tRNA_f (97 per cent pure: a gift of Dr. D. Novelli) with unlabeled or ³H-labeled methionine (3.3C/mmole) in the presence of leucovorin by the methods of Marcker and Sanger.⁴ ³H-aminoacyl-tRNA was prepared similarly by charging *E. coli* tRNA, in the absence of leucovorin, with a mixture of twenty amino acids containing the ³H-labeled amino acids desired. Specific activity of ³H-amino acids was 3C/mmole each and radioactivities of the isolated tRNA were, per μ g RNA, 1.0×10^4 CPM for tRNA mixedly charged with ³H-labeled alanine, glycine, isoleucine and tyrosine, 3.2×10^3 CPM for ³H-leucyl-tRNA, and 4.1×10^3 CPM for ³H-valyl-tRNA.

One-step experiment. The initiation complex was allowed to form under the following conditions: the binding reaction mixture (0.065 ml) contained 40 mM Tris (pH 7.5), 80 mM NH₄Cl, 5 mM Mg acetate, 4 mM β -mercaptoethanol, 2 mM GTP, 17 μ moles of fMet-tRNA_f (labeled or unlabeled, as indicated), 15 μ g of MS2 RNA, and 200 μ g of ribosomes (estimated from A260). Incubation was carried out for 15 minutes at 37°C. The initiation reaction was then interrupted by tenfold dilution with a medium containing Tris (pH 7.5), NH₄Cl, β Me, GTP, and Mg acetate (dilution medium) at the same concentration as in the binding mixture, and was equilibrated to 31°C. Polypeptide chain elongation was initiated by introduction of an equal volume of elongation mixture (0.65 ml) which contained Tris, NH₄Cl, GTP (each at the same concentration as in the binding mixture), 15 mM Mg acetate, 4 mM ATP, 4 mM PEP (Na⁺ salt), 5 μ g of PEP kinase, 750 μ g

* This report is taken from a paper that appeared in Biochem. Biophys. Res. Comm., 42:405, 1971.

of S165 (estimated according to Lowry⁵) and ³H-aminoacyl-tRNA; or alternatively, the mixture of *E. coli* B tRNA (250 μg) and twenty amino acids with or without ³H-labeled amino acid(s) as indicated. The temperature of incubation for elongation was 31°C.

Assay for the binding and ³H-amino acid incorporation. The binding of ³H-fMet-tRNA_f was determined by trapping the radioactivity on Millipore filters (HA) as described by Nirenberg and Leder.⁶ Incorporation of ³H-amino acids was followed by measuring the hot trichloroacetic acid-insoluble radioactivity as already described.³

³H-amino acids were purchased from Schwarz BioResearch, Orangeburg, New York, and aurointricarboxylic acid (ATA) from Aldrich Chemical Co., Milwaukee, Wisconsin.

RESULTS

The formation of initiation complex (MS2 RNA-ribosome-(³H)fMet-tRNA_f) is illustrated in Figure 1A: maximum binding was achieved in 10 minutes at 37°C. About half of ³H-fMet-tRNA_f was converted into initiation complex. Over 99 per cent of the bound radioactivity was rendered acid-insoluble upon heating, indicating absence of polypeptide synthesis.

Figure 1B shows that upon the introduction of chain elongation components to reaction mixtures where initiation complex formation has been completed and diluted tenfold, ³H-formyl-methionine in the initiation complex was rapidly converted into hot acid-insoluble polypeptide. Conversion was complete within 1.5 minutes, indicating an excellent synchrony of the process. The absence of any further increase in hot acid-insoluble radioactivity thereafter up to 15 minutes indicates that no significant reinitiation occurred and thus illustrates one-step elongation.

The one-step experiments were performed with various ³H-aminoacyl-tRNA and are illustrated in Figure 2A. ³H-amino acid incorporation terminates within 12 minutes after addition of the elongation mixture containing tRNA isolated after being charged with twenty amino acids, of which alanine, glycine, isoleucine and tyrosine were labeled. When ³H-leucyl-tRNA served as the label, incorporation stopped at around 9 minutes; when ³H-valyl-tRNA was used as the label, incorporation stopped as early as 4 minutes. Control experiments in which the prebinding reaction was omitted, indicated no significant incorporation of ³H-amino acids, thus reinforcing the previous conclusion that no significant reinitiation occurred under the conditions of one-step elongation experiments.

Similar results were obtained when free ³H-amino acids were used as the label (Figure 2B). Thus, with a combination of four ³H-amino acids, termination of their incorporation again occurred at 12 minutes; incorporation of ³H-leucine and ³H-valine stopped at around 9 and 4 minutes, respectively. Incorporation of ³H-histidine was very small.

The effect of ATA on polypeptide chain growth was examined by the one-step technique. Figure 3 shows the effect of ATA on the ³H-valine incorporation at two concentrations. It can be seen that ATA effectively depressed the rate of polypeptide chain growth. With prolonged incubation, the extent of ATA inhibition was reduced; thus, ATA at a concentration of 50 μM inhibited ³H-valine incorporation by 90 per cent at the end of 5 minutes, whereas the degree of inhibition was reduced to 31 per cent when incubation was prolonged to 30 minutes.

DISCUSSION

The data presented in this report indicate that polypeptide chain elongation can be accomplished in one step without the use of any external inhibitor, e.g., ATA. This is done by: (a) pre-

Figure 1. A: Binding of ^3H -fMet-tRNA_f on ribosomes in the presence of MS2 RNA. Conditions as in Methods. ^3H -fMet-tRNA_f served as label (specific activity, 2,200 CPM per μmole). At the time indicated, a 0.04 ml sample was withdrawn and the radioactivity bound to ribosomes was determined by trapping it on Millipore filters. B: One-step elongation measured by incorporation of ^3H -fMet. The prebinding reaction of ^3H -fMet-tRNA_f was carried out for 15 minutes at 37°C as in A. Dilution of the binding mixture and initiation of elongation were carried out at 31°C as described in Methods. The elongation mixture contained *E. coli* tRNA and 20 unlabeled amino acids. At the time indicated, a 0.10 ml sample was assayed for hot acid-insoluble radioactivity as described in Methods.

binding, (b) dilution, (c) elongation. The overall dilution is twenty-fold. Under these conditions, no significant synthesis of hot acid-insoluble polypeptide is detectable without prebinding (Figure 2A); therefore, the polypeptide chains that are synthesized originate solely from the initiation complex present prior to the dilution, and the elongation components. Hence, the time required for the incorporation of a labeled amino acid into a polypeptide to terminate in the one-step experiments represents the time required for a ribosome to travel from the initiation site to the last codon of the longest cistron coding for the amino acid employed as the label.

RNA phages carry three identified cistrons—coat protein, A protein and replicase cistrons.⁷⁻¹⁰ The coat protein of RNA phages contains no histidine and its incorporation, when an

Figure 2. Experimental and theoretical one-step elongation. **A:** The experimental plan is similar to that described in Figure 1B except that in the prebinding reaction a comparable amount of unlabeled fMet-tRNA_f was substituted for the label and that the elongation mixture (0.65 ml) contained 600 μ g of *E. coli* B tRNA precharged with 20 amino acids including the indicated H^3 -amino acid(s). Dotted lines represent corresponding control experiments in which the prebinding reaction was omitted. Samples of 0.10 ml were analyzed for hot acid-insoluble radioactivity. **B:** Experimental conditions were as above except that 20 amino acids containing H^3 -amino acid(s) as indicated and *E. coli* B tRNA were substituted for precharged tRNA in the elongation mixture, which was preincubated for 10 minutes at 31°C prior to introduction into binding mixture. Concentrations of labeled and unlabeled amino acids in the elongation mixture were 8×10^{-3} mM and 1.6×10^{-2} mM each, respectively. Specific activity of H^3 -amino acids was 3 C/mole when singly present and 1 C/mole when combined. The size of samples was 0.08 ml. Incorporations when the prebinding was omitted were around 110 CPM for H^3 -valine, 350 CPM for H^3 -leucine, 105 CPM for H^3 -histidine and 515 CPM for the mixture of four H^3 -amino acids, and were subtracted in the values shown. **C:** Theoretical incorporation curves for various amino acids expected from uniform rate of translation of the coat cistron were constructed by plotting cumulative number of amino acid residues against position from the NH_2 -terminal of the coat protein according to its amino acid sequence.¹⁸

RNA viral message serves as messenger RNA in a cell-free protein-synthesizing system, faithfully reflects the synthesis of non-coat proteins.^{11,12} Since no appreciable incorporation of H^3 -histidine occurs under the conditions of one-step translation (Figure 2B), the amount of non-coat proteins synthesized must be very small. Therefore, when a combination of four amino acids (alanine, glycine, isoleucine, and tyrosine) which are present abundantly in the COOH-terminal region of the coat protein¹³ is employed as the label, the time required for their incorporation to reach plateau should be the time required for a ribosome to translate

Figure 3. Effect of ATA on polypeptide chain propagation. Reaction conditions as described in Figure 2B in which ^3H -valine served as the label. ATA, when present, was added to the elongation mixture before it was introduced into the diluted prebinding mixture. At the times indicated, a sample of 0.05 ml was assayed for hot acid-insoluble radioactivity.

the coat cistron, assuming that complete protein molecules are synthesized. Since MS2 coat protein consists of 129 amino acid residues^{13,14} and the time required for the synthesis of this protein is 12 minutes (Figure 2A,2B), the rate of translation is approximately 0.18 amino acid per second at 31°C. On the other hand, when ^3H -valine is used as the label, its incorporation stops at around 4 minutes (Figure 2A,2B). Inspection of the theoretical incorporation curve for valine illustrated in Figure 3C, suggests that incorporation may be expected to continue until the last valine residue at the 105th position is reached; since, however, most valine residues (over 90 per cent) appear in the region preceding the valine residue at the 79th position, it is likely that termination of its incorporation signals chain propagation up to this point. If this is so, the rate of translation of this portion of the coat cistron is 0.33 amino acid per second, which is much larger than the value obtained from the use of four amino acids. This difference may indicate a decrease in the rate of translation as ribosomes move towards the COOH-terminal, possibly as a result of the change in topography of the coat cistron. When ^3H -leucine serves as label, its incorporation stops after 9-10 minutes of incubation. Since the last leucine residue is located at the 112th position,¹³ a rate of 0.20 amino acid per second results; this is in close agreement with the value obtained from the use of four labeled amino acids. The shape of the curve of incorporation, compared with that of theoretical incorporation expected from a uniform rate of translation (Figure 2C), seems to indicate again that translation of the early part of the

coat cistron is much faster than of the later part. The average rate of translation, 0.18 amino acid per second, is considerably smaller than that obtained by Webster and Zinder,¹⁵ who assigned the value of 0.4 to 0.5 amino acid per second at 32° or 33° C with a different technique. The reason for this discrepancy is not immediately clear.

Although not shown here, twenty-fold dilution still permits the binding reaction to proceed to a certain extent when monitored by the Millipore trapping of the initiation complex; the binding, however, is not detected as synthesis of acid-insoluble polypeptide chains. Forty-fold dilution is required for complete elimination of the binding reaction. One-step experiments employing this dilution yield essentially the same results.

It has been shown that ATA inhibits the binding of RNA viral messages to ribosomes.¹⁶ By the one-step experiments, ATA is demonstrated to depress the process of polypeptide chain growth, the rate in particular (Figure 3). This effect is in agreement with reports that ATA can affect protein synthesis at steps other than messenger binding to ribosomes,^{17,18} The effect is not due to the inhibition of aminoacylation of tRNA, however, since it is precharged in this experiment. The use of ATA as a specific inhibitor of initiation merits caution.

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THE PURIFICATION OF ERYTHROPOIETIN*

By

E. Goldwasser and C. K.-H. Kung

Erythropoietin is the primary inducer of red blood cell formation in vertebrates.¹ As such it is important both for the study of the molecular mechanisms involved in cell differentiation, and for possible therapeutic use in some types of refractory human anemias. Until recently the purification of erythropoietin had not reached the stage where its chemical and physical properties could be studied directly; nor could preparations be considered suitably pure for administration to patients. The present report describes the purification of erythropoietin derived from anemic sheep plasma to a state almost free of impurities.

Details of bioassay and of the first steps in the fractionation of sheep plasma have already been published.²⁻⁴ The starting material used in the work described in this preliminary communication was an ammonium sulfate fraction (Step IV) prepared as described earlier⁴ and generously supplied to us by Dr. L. O. Jacobson. It was dissolved in 3 M LiCl⁵ and the fraction that precipitated between alcohol concentrations of 50 per cent and 65 per cent (P7) was collected, de-salted, and chromatographed on sulfoethyl-sephadex. The fractions that were eluted with acetate buffers at the following concentrations were collected and pooled: 0.015 M, pH 5.0, 0.0175 M, pH 5.28 and 0.20 M, pH 5.6 (SE 4,5,6). This pool was de-salted and further purified by adsorption on, and elution from, calcium phosphate gel.⁶ The components that were eluted with 5×10^{-4} M phosphate buffer at pH 7.3 were collected (CaP eluate) and chromatographed on a methylated albumin-Kieselguhr column.⁷ The fraction eluted with 0.01 M phosphate at pH 7.2 (MAK-4) was found to have the highest potency. The results of the fractionation steps are summarized in Table 1.

Table 1

PURIFICATION OF SHEEP PLASMA ERYTHROPOIETIN

Fraction	Potency (units/mg protein)	Total units	Yield (%)	Purification factor
Anemic plasma	0.007	-	-	-
Step IV	26	36,000	100	3,700
P 7	250	27,500	76	35,700
SE 4,5,6	570	7,220	20	81,300
CaP eluate	1,670	2,380	6.6	238,000
MAK-4	7,450	690	2	1,050,000
MAK-7	8,250	-	-	1,180,000

* This report is taken from a paper that appeared in PNAS (U.S.), 68:697, 1971.

Figure 1. Gel electrophoresis of erythropoietin. Gel electrophoresis was done using 7.5% acrylamide and 0.2% methylene-bis-acrylamide to prepare the gels, which were 3 cm long and 0.2 cm diameter. After running for 90 min at 100V, the gels were removed from the tubes and cut into 32 slices which were counted in a crystal scintillation counter. The open circles in the top two frames show the total counts in the slice; the closed circles show the counts after fixing in 20% sulfosalicylic acid and washing. The bottom curve shows the biological activity recovered from slices of polyacrylamide gel. The total activity recovered from the polyacrylamide was greater than 80%.

When the MAK-4 fraction was examined by a micro adaptation of conventional polyacrylamide gel electrophoresis at pH 8.6, we found very much less protein after fixing with sulfosalicylic acid and staining with Coomassie blue⁸ than we expected from the known amount of protein put on the gel. A less pure preparation (6500 units per mg of protein) that had been iodinated with ¹²⁵I by Dr. P. M. Cotes of Mill Hill, London was examined by the same electrophoretic method and we found that more than 95 per cent of the labeled protein was lost in the fixation process. None of the alternative fixation processes that we tried yielded results that could be used for a test of heterogeneity and we were therefore forced to use the labeling method.

For studies of homogeneity we used a MAK column fraction similar to MAK-4 but derived from a larger experiment. This preparation (MAK-7) had a potency of 8500 units per mg of protein when repeatedly assayed by the fasted rat method² and by the polycythemic mouse method.³

When it was assayed in rat bone marrow cultures⁹ over the range 0.004 to 0.400 units per ml, there was no evidence of inhibition of either iron uptake or of hemoglobin synthesis and the log dose vs log response curve was linear except at the highest level. The MAK-7 material was labeled with carrier free ¹²⁵I¹⁰ and after removal of the greater part of the non-protein label by gel filtration the labeled erythropoietin was lyophilized and re-dissolved in a small amount of water. It was run by micro gel electrophoresis with the results shown in Figure 1. After the run was over, the gel was cut into 32 slices 0.91 mm thick and each slice was counted. The single major peak with a relative mobility of 0.52 has a small but definite shoulder on its slower side which contains about 7.5 per cent of the total radioactivity. There are also 2 minor peaks that run faster than the major one (less than 5 per cent of the total) and a small amount of label at the origin which is probably denatured protein. The labeled MAK-7 fraction was desialated by using a purified neuraminidase from Cholera vibrio, kindly supplied by Dr. S. Roseman and examined on a similar gel. The desialated erythropoietin had a relative mobility of 0.35 and the shoulder was no longer evident. We suggest that the shoulder was due to the presence of a small amount of partially or completely desialated erythropoietin. The figure also shows (closed circles) that the loss of labeled erythropoietin by fixation of the gel slices with sulfosalicylic acid is very much less when the hormone has been desialated.

Since desialation results in the complete loss of activity when erythropoietin is assayed by the in vivo methods we can infer that the active material in the MAK-7 preparation has a potency of 9200 units per mg of protein.

A less highly purified erythropoietin preparation was run on a gel that was sliced as described above. The erythropoietin activity was then eluted from the gel slices and assayed in vitro.⁹ The active material had a relative mobility of 0.54 and a small amount of activity at the position of the desialated erythropoietin. These data indicate that the MAK-7 peak was in fact, erythropoietin and that the major contaminant was desialated erythropoietin.

It is clear from the very low yield that we will have to find much improved methods of purification before we will be able to accumulate enough pure erythropoietin for detailed study of its chemical structure and before we can make tests of its clinical efficacy.

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ON THE MECHANISM OF ERYTHROPOIETIN-INDUCED DIFFERENTIATION
IX. INDUCED SYNTHESIS OF 9S RNA AND OF HEMOGLOBIN*

By

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The pivotal role of erythropoietin in the initiation of erythroid cell development is, by now, well established. We have shown in previous papers that primary cultures of rat bone marrow cells respond to erythropoietin by increasing the rate of hemoglobin synthesis.¹ This effect is preceded by increased uptake of iron² and by increased synthesis of a variety of RNA species, one of which has a sedimentation coefficient of about 9S.³ Similar RNA isolated from rabbit reticulocyte polysomes proved to contain messengers for the synthesis of the α and β chains of globin when tested in cell-free systems.⁴⁻⁶ Bone marrow cells in culture provide a system in which the relationship between the induced synthesis of 9S RNA and that of hemoglobin may be studied.

In the experiments reported here, we used marrow cells from normal rats and mice, as well as cells from animals made artificially polycythemic by the injection of red cells. Animals so treated show essentially no red cell formation, and the marrow cell population is virtually devoid of cells in the stages of development between the mature erythrocyte and the unknown, primitive precursor cell that is the primary target for erythropoietin action.⁷ Our data show that there is an interval of about 10 hours between the transcription of 9S RNA and its translation (hemoglobin synthesis), that there is 9S RNA synthesis even when hemoglobin synthesis is totally suppressed, and that erythropoietin appears to act on differentiated cells of the marrow as well as on undifferentiated cells.

METHODS AND MATERIALS

Preparation of polycythemic animals. Male, Sprague-Dawley rats (250 to 300 g) were made polycythemic by the intraperitoneal injection of 10 ml of packed, washed red cells from homologous donors on days 1, 2, and 6. Hematocrits and reticulocyte counts measured on day 7 were 65 to 80 and 0.02 to 0.10 per cent (normal 1.5 to 3.5 per cent), respectively. Bone marrow cells were harvested on day 8. Female CF 1 mice (15 to 20 g) were made polycythemic by the intraperitoneal injection of 1 ml of packed, washed red cells from homologous donors on days 1 and 3. Hematocrits were 65 to 75 on day 8, when the bone marrow cells were isolated. The mice were reticulocyte-free and had no cells in their marrow that were recognizably erythroid.⁷

Preparation of hyperplastic rats. Erythroid hyperplasia was induced in 250 to 300 g, male, Sprague-Dawley, rats by subcutaneous injection of 50 mg per kg of neutralized phenylhydrazine hydrochloride on 2 successive days. Bone marrow cells were isolated on the fourth day, when the hematocrit was about 30 and the peripheral blood showed 10 to 15 per cent reticulocytosis.

Cell culture. Rat marrow cells were cultured as previously described³ unless otherwise

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indicated. Bone marrow was obtained from the mice by flushing culture medium through the marrow cavity of the femora. These cells were cultured in the same manner as the rat marrow cells, with mouse serum replacing rat serum, unless otherwise indicated. We have found that it is important to maximize the yield of cells from the mouse femur by vigorous flushing of medium through the bone. If flushing is gentle and only the loosely attached cells are removed, the yield is about 6 to 8 million nucleated cells per femur, and little or no response to erythropoietin is seen. If the flushing is vigorous, the yield is about twice as great and the cells respond to erythropoietin. It is of interest that the concentration of colony-forming cells in the mouse femur is two to three times greater in the firmly attached cells than in those removed by gentle treatment.* All cell numbers are expressed as nucleated cells.

Measurement of RNA synthesis. RNA synthesis was determined by a 15-min pulse with 4 μ Ci per ml each of uridine- 3 H and cytidine- 3 H followed by RNA isolation using the Method II procedure previously described.³ Where indicated, cells were chased with actinomycin D (8 μ g per ml) and cold cytidine and uridine (5 mM final concentration) as described previously. In the experiment where rats were labeled in vivo, they were given an intravenous injection of 50 μ Ci each of uridine- 3 H and cytidine- 3 H. Marrow cells were collected and RNA was extracted by Method II 15 min later. The RNA was fractionated on sucrose density gradients using the SW 50 rotor in a Beckman model L-2 ultracentrifuge as previously described. The conditions of centrifugation are indicated in the figure legends. Absorbancy and radioactivity were determined as described earlier.³ Quantitative evaluation of gradient profiles was done by planimetric integration.

Determination of iron uptake and hemoglobin synthesis. Iron uptake was measured as the radioactivity in the marrow cell pellet after 3 washes with 5 ml of phosphate buffered saline (PBS).² Radioiron ($^{59}\text{Fe}^{3+}$) was added bound to rat serum for rat cells or to mouse serum for mouse cells. The amount of iron was enough for 50 to 80 per cent saturation of the transferrin in the serum. Radioiron was counted in a Nuclear Chicago model 4227 counter.

Hemoglobin synthesis was determined by one of three methods after labeling with ^{59}Fe , valine- ^{14}C , or glycine- ^{14}C . The first method was the heme isolation procedure of Fox and Thomson.⁸ The washed marrow cell pellet was mixed with 0.25 ml of PBS containing 10 μ l of packed rat red cells as carrier and then lysed by the addition of 0.03 ml of 2 per cent Nonidet-P40. After hemoglobin was precipitated by the addition of 4 volumes of acetone, heme was solubilized by the dropwise addition of a one-twentieth volume of 12 N hydrochloric acid with rapid mixing. After centrifugation for 5 min at 1,500 x g, the supernatant was combined with 5 ml of a 1:1 mixture of 1 N hydrochloric acid:methanol and 1 ml of chloroform. After mixing, the chloroform hypophase containing the extracted heme was washed twice with 5 ml of the acid methanol mixture, and then counted as an infinitely thin layer on aluminum planchets in a gas-flow, thin window counter.

The second method was the specific precipitation of rat or mouse hemoglobin with anti-serum prepared in guinea pigs against twice recrystallized rat hemoglobin. We found that rat hemoglobin, mouse hemoglobin, or a mixture of the two was quantitatively precipitated by the antiserum. To the washed marrow cell pellet, 0.25 μ l of packed rat red cells was added as carrier. The cells were then suspended in a total volume of 0.5 ml of PBS, and lysed by the addi-

* B. I. Lord, personal communication.

tion of 0.05 ml of 2 per cent Nonidet-P40. To the stroma-free lysate, 0.10 ml of antiserum was added, and the mixture was incubated at 37° for 30 min and then kept at 3° overnight. The precipitates were collected by centrifugation (5 min at 1,500 x g), washed three times with 0.50 ml of PBS, and then suspended in 0.50 ml of acetone. Heme was solubilized according to the method of Fox and Thomson, and the heme in the acid acetone supernatant was counted as an infinitely thin layer on aluminum planchets. The globin precipitate was dissolved in 0.50 ml of formic acid and similarly counted on planchets. If less than 0.10 ml of antiserum was added, the amount of hemoglobin remaining in the supernatant was inversely proportional to the volume of antiserum added. If, after precipitation of all of the hemoglobin, an additional amount of unlabeled hemoglobin and antiserum was added to the supernatant, no significant radioactivity was precipitated. Methemoglobin was not precipitated by the antiserum.

A third method was the procedure developed by Hrinda⁹ which depends upon the specificity of the binding of hemoglobin to haptoglobin. DEAE-cellulose was equilibrated with 0.5 M sodium acetate (pH 5.5) and then washed thoroughly with water. The stroma-free lysates from the marrow cells and 5 µl of packed rat red cells were dialyzed overnight against 0.005 M sodium acetate, pH 5.5 (acetate buffer), run through an 8 x 0.2 cm² DEAE cellulose column. Hemoglobin was washed through with the effluent using the same acetate buffer. Sufficient haptoglobin was added to the effluent to complex all the hemoglobin present. These samples were then applied to separate 8 x 0.2 cm² DEAE cellulose columns, to which the hemoglobin-haptoglobin binds at this pH and ionic strength. After the second column was washed with ten 1-ml volumes of acetate buffer, the hemoglobin-haptoglobin complex was eluted with 1 M sodium chloride, 0.10 M sodium phosphate (pH 7.2). The hemoglobin in the eluate was treated and counted as described for the antibody precipitation method, except that the heme was extracted with chloroform to remove the salt in the elution buffer.

We have found that all three of these methods agree in the determination of heme synthesis, and the antibody and haptoglobin methods agree in the determination of globin synthesis in rat marrow cell cultures maintained for as long as 42 days.¹⁰ In this system, measurement of heme synthesis is equivalent to measurement of hemoglobin synthesis.¹¹

Materials. NCTC-109 was purchased from Microbiological Associates, new-born calf serum from Colorado Serum Company, and culture dishes from Falcon Plastics. Uridine-5-³H (27.6 Ci/mMole), cytidine-5-³H (26.4 Ci/mMole), valine-1-¹⁴C (34.2 mCi/mMole), and ⁵⁹FeCl₃ (10 Ci/g) were bought from New England Nuclear Corporation. Glycine-2-¹⁴C (57 mCi/mMole) was bought from Nuclear Chicago. Actinomycine D was a commercial sample from Merck, Sharpe, and Dohme. Nonidet-P40 was a gift from Shell Chemicals. The anti-hemoglobin antiserum was generously provided by Dr. B. Hurn of the Wellcome Research Laboratories, Kent, England.

RESULTS

The time course of control and erythropoietin-induced iron uptake and hemoglobin synthesis by marrow cells from polycythemic rats is shown in Figure 1. In comparison with cells from normal rats, iron uptake was suppressed by about 75 per cent and hemoglobin synthesis by about 85 per cent. Stimulation of iron uptake by erythropoietin was evident about 7 hours after incubation was started, while stimulation of hemoglobin synthesis was not detected before 12 hours. Induced iron uptake (as shown by the difference curve) went through two maxima, at

Figure 1. Effect of erythropoietin on marrow cells from polycythemic rats. Quadruplicate cultures (1.0 ml) containing 10×10^6 cells per ml were incubated for increasing periods and pulsed with $1.0 \mu\text{Ci}$ of ^{59}Fe for 4 hours at the indicated times. The points are placed at the mid-points of the pulse periods. (●) control cells, (○) erythropoietin-treated cells (0.10 u per ml) and (△) the difference between control and stimulated. The upper portion of the figure shows iron uptake and the lower portion hemoglobin synthesis. Heme was isolated by the Fox and Thomson method.⁹ In comparison, parallel cultures with cells from normal rats under identical conditions at the first pulse time showed an iron uptake of 7250 cpm and heme synthesis equivalent to 2350 cpm. The vertical bars indicate the standard deviation of the means.

12 and at 48 hours, while induced hemoglobin synthesis rose steadily after the initial lag period, reaching a maximum at about 36 hours. The control rate of hemoglobin synthesis declined steadily from the start of the incubation, but the control rate of iron uptake showed an appreciable increase after about 18 hours of decline. We have shown previously¹² that this secondary

increase in rate of iron accumulation in the absence of erythropoietin, is due to the carrier iron added to the medium.

A similar study of the rate of hemoglobin synthesis by marrow cells from polycythemic mice showed that the control incorporation of iron into heme in these cultures was virtually zero throughout the 58 hours of incubation, as would be expected from observations on living mice. Erythropoietin caused an increase that was apparent between the 10th and 24th hours of incubation, and reached a maximum at about 33 hours. The stimulation of the rate of hemoglobin synthesis fell almost to zero by 58 hours (Figure 2). Nakao et al¹³ have reported similar findings in organ cultures of spleen from polycythemic mice.

Cells from normal rats and mice, in marked contrast to the suppressed cells, showed a small but significant effect of erythropoietin on incorporation of iron into hemoglobin as early as 4 hours. If the normal cells were pre-incubated for 24 hours before erythropoietin was added, there was no stimulation of hemoglobin synthesis until about 15 hours later (Table 1).

Figure 2. Effect of erythropoietin on hemoglobin synthesis by marrow cells from polycythemic mice. Triplicate cultures (1.0 ml) containing 3×10^6 cells per ml were incubated for increasing periods and pulsed with 1.0 μCi of ^{59}Fe for 4 hours at the indicated times. The points are placed at the midpoints of the pulse periods. (●) control cells, (○) erythropoietin-treated cells (0.2 u per ml), and (Δ) the difference between control and stimulated. In comparison, cells from normal mice synthesized 4 $\mu\mu\text{moles}$ of hemoglobin under identical conditions in the first 4-hour period.

Table 1

EARLY STIMULATION OF HEMOGLOBIN SYNTHESIS IN NORMAL MARROW CELLS*

Conditions	cpm ⁵⁹ Fe incorporated into hemoglobin			
	Rat cells		Mouse cells	
	Control	Erythropoietin (0.10 u/ml)	Control	Erythropoietin (0.20 u/ml)
All additions at zero, stopped at 4 hours	1900 (±130)	2280 (±180)	640 (±40)	740 (±25)
Erythropoietin and ⁵⁹ Fe added at 24 hours, stopped at 28 hours	870 (± 34)	720 (± 13)		
Erythropoietin added at 24 hours, ⁵⁹ Fe added at 39 hours, stopped at 43 hours	450 (± 36)	680 (± 17)		

* Rat marrow cell cultures contained 15×10^6 cells per ml, mouse cultures had 5×10^6 cells per ml. One μCi of ⁵⁹Fe was added to each plate at the indicated times. Rat cell cultures studied at 4 hours were in quintuplicate, others were quadruplicates, mouse cell cultures in triplicate. The difference between control and erythropoietin groups is significant [$0.001 < P < 0.01$] by t-test.

The data in Figure 3 show the effects of polycythemia and erythroid hyperplasia (induced by anemia due to phenylhydrazine) on the amount of 9S RNA from equivalent amounts of total marrow cell RNA. The suppression of red cell formation by polycythemia resulted in the disappearance of 9S RNA, while the acceleration of erythropoiesis in the anemic rats resulted in a doubling of the 9S RNA present.

Marrow cells from polycythemic rats in culture still synthesized some 9S RNA even though none could be detected in the absorbancy profile (Figure 4). When erythropoietin was added to the cultures 1 hour before pulsing for 15 min, there was an increase of about 40 per cent in the amount of labeled 9S RNA but still none was detectable by absorbancy measurement. With this short pulse the only difference between the meniscus and the 15S region of the gradient due to erythropoietin was in the 9S peak.

A chase experiment studying the fate of pulse-labeled 9S RNA indicated that all of it, in both control and stimulated cells, persisted for 3 hours (Figure 5). If the half-life of 9S RNA were 24 hours or greater, we would not have been able to detect any loss in three hours. Chasing for longer periods, however, is not possible since the cells do not survive long treatment with actinomycin. It is interesting to note that a considerable amount of RNA in the 4S region accumulated during the chase, possibly due to terminal labeling of t-RNA.

The unexpected synthesis of 9S RNA by cells from polycythemic rats might have been due to a small amount of erythropoietin in the calf serum used in the culture medium. In order to determine whether this was so, or whether that 9S RNA synthesis seen in cells not making hemoglobin might be unrelated to erythropoietin action, we did two experiments. In one of these (Figure 6), polycythemic, normal, and hyperplastic rats were pulsed in vivo with labeled uridine and cytidine and the RNA from marrow cells was examined on a sucrose gradient. Even

Figure 3. Presence of 9S RNA in rat marrow cells. RNA from hyperplastic rat marrow (o-o), normal marrow (●-●) and polycythemic rat marrow (-Δ-) was isolated by Method II. Sucrose gradients were run for 14 hours at 49,000 rpm.

under the *in vivo* condition of suppressed hemoglobin synthesis there was a small, but detectable, synthesis of 9S RNA amounting to about 60 per cent of that seen in normal rats. In the hyperplastic rats, as expected, 9S RNA synthesis was about 2.5 times as great as in normal animals. In the second experiment, we cultured marrow cells from polycythemic rats in media containing serum either from polycythemic animals (Figure 7A) or from normal rats (Figure 7B). Unstimulated cells in the medium containing "polycythemic" serum synthesized 9S RNA at a rate very similar to that of control cells in normal serum, indicating that at most 5 to 10 per cent of the 9S RNA synthesized by cells in normal serum could have been due to the presence of endogenous erythropoietin. In both cases, erythropoietin caused the same increase in 9S RNA synthesis. These data indicate that there is an erythropoietin-independent synthesis of 9S RNA in rat marrow cells.

Because the suppression of hemoglobin synthesis by artificial polycythemia in rats is not complete, we examined the synthesis of 9S RNA in cells from polycythemic mice, where red cell production is nil. The results (Figure 8) show that, as with rat cells, no 9S RNA is detectable by absorbancy measurement, and there is a similar synthesis of 9S RNA in controls, amounting to about 20 per cent of that seen in normal mouse cells. This level of synthesis was

Figure 4. Synthesis of 9S RNA by cultured cells from polycythemic rats. Triplicate cultures (2.5 ml) containing 20×10^6 cells per ml were incubated for 1 hour, then pulsed for 15 min with 4 μ Ci each of uridine-³H and cytidine-³H. Cells were pooled, RNA was isolated and run on sucrose gradients for 11 hours at 49,000 rpm. (●) controls, and (○) erythropoietin treated cells.

about the same when serum from polycythemic mice was substituted for calf serum in the medium. Addition of erythropoietin caused an increase of about 2-fold in 9S RNA synthesis when measured by a 15-min pulse after 4 hours of incubation.

The effect of erythropoietin on 9S RNA synthesis is limited to a relatively short period of time (Figure 9). When cells from polycythemic rats were pulsed with uridine and cytidine after 3 hours of incubation, the erythropoietin stimulation was about 2-fold, but when the cells were pulsed after 24 hours of incubation there was no erythropoietin effect; control 9S RNA synthesis

Figure 5. Chase of 9S RNA synthesized by cells from polycythemic rats in culture. Triplicate cultures (2.5 ml) containing 20×10^6 cells per ml were pulsed for 15 min after 3 hours of incubation. In (A) RNA was isolated immediately after pulsing, in (B) the cells were chased for 3 hours in the presence of actinomycin D ($8 \mu\text{g}$ per ml) and cold uridine and cytidine (5 mM). Sucrose gradients were run at 49,000 rpm for 14 hours (A) and 12 hours (B). (—●—) controls, (—○—) erythropoietin treated cells.

was reduced by about 65 per cent. During the 24-hour incubation in the presence of erythropoietin, however, the cells did accumulate a significant amount of 9S RNA as determined from the absorbancy profile. Cells that have been pre-incubated 24 hours in the absence of erythropoietin can respond to its addition at that time with an increase of about 40 per cent in total RNA synthesis. These data suggest that the lack of response to erythropoietin with regard to 9S RNA synthesis at 24 hours is not a generalized consequence of culture conditions, but represents a selective loss of a particular function.

Figure 6. Synthesis of 9S RNA in vivo. Marrow cell RNA from hyperplastic rats (-o-), normal rats (-Δ-) and polycythemic rats (-●-) was analyzed 15 min after the animals were given 50 μ Ci each of uridine- 3 H and cytidine- 3 H. Sucrose gradients were run for 12 hours at 49,000 rpm.

DISCUSSION

Our results indicate that at least two types of 9S RNA, one of which is the erythropoietin-dependent globin messenger, may be found in marrow cells. The synthesis of 9S RNA that occurs in marrow cells from polycythemic mice, however, clearly takes place in cells that do not form hemoglobin and, since this synthesis is independent of erythropoietin, it may represent an RNA having no role in globin synthesis. The same may be true for the 9S RNA synthesized by the cells 24 hours after incubation in the presence of erythropoietin but after its action has stopped. It is quite possible that marrow cells not concerned with red cell formation

Figure 7. 9S RNA synthesis by cells from polycythemic rats incubated in serum from polycythemic rats. Triplicate cultures (2.5 ml) containing 30×10^6 cells per ml were incubated in a medium containing 70% NCTC 109 and 30% serum from polycythemic rats (A) or serum from normal rats (B). The cultures were pulsed for 15 min at 4 hours and the pooled RNA samples run on sucrose gradients at 49,000 rpm for 12 hours (A) and 13 hours (B). (—●—) controls, (---○---) erythropoietin treated cells.

make 9S RNA that are messengers for proteins (other than globin) with a molecular weight of about 16,000 daltons. Another interpretation of these data, is that undifferentiated cells synthesize 9S RNA globin messengers that do not accumulate until some other aspect of erythroid differentiation (e.g., heme synthesis) is initiated.

The identification of that part of marrow cell 9S RNA formed in response to erythropoietin as globin messenger must await the demonstration that it can serve as a template for globin synthesis in a cell-free system. Since the unfractionated, normal marrow cell population con-

Figure 8. 9S RNA synthesis by cells from polycythemic mice. Duplicate cultures (2.5 ml) containing 15×10^6 cells per ml were pulsed at 4 hours for 15 min, the pooled RNA samples were run on sucrose gradients for 16 hours at 49,000 rpm. (●-) control cultures, (-o-) erythropoietin treated cells.

tains reticulocytes, it is certain to have some 9S RNA that is known to be globin messenger. The use of marrow from polycythemic animals would entail a massive experiment since so little 9S RNA is present. Such an experiment would best be done on fractionated marrow cells that are free of differentiated erythroid cells but are still responsive to erythropoietin. Until it is done, the suggestion that erythropoietin switches on the synthesis of globin messenger must be a presumptive one.

Initiation of 9S RNA synthesis by erythropoietin occurs in one hour in cells from polycythemic rats, and in four hours in cells from polycythemic mice. Induced hemoglobin synthesis, on

Figure 9. 9S RNA synthesis by rat marrow cells incubated for 24 hours in the presence of erythropoietin. Triplicate cultures (2.5 ml) containing 25×10^6 cells per ml were incubated for 3 hours with (-o-) and without (-●-) erythropoietin and for 24 hours with (-□-) and without (-■-) erythropoietin. All cultures were pulsed for 15 min at the end of the incubations. The pooled RNA samples were run on sucrose gradients for 13 hours at 49,000 rpm. In the lower part of the figure, (-Δ-) indicates marker RNA isolated from hyperplastic rat marrow cells.

the other hand, does not begin until about 12 hours. The delay in initiation of hemoglobin synthesis, both in vivo and in vitro, clearly must be due to a post-transcriptional regulatory process since the presumptive messenger (9S RNA) is synthesized several hours before its function is expressed. One aspect of this post-transcriptional regulation may be the transposition of RNA synthesized in the nucleus to the cytoplasm. We have found that the bulk of the RNA newly synthesized in erythropoietin-treated marrow cells is retained in the nucleus for at least three hours.¹⁰

The remaining interval between transcription of globin messenger RNA and hemoglobin synthesis may be required for those transcriptive and translational processes needed for the enzymes used for heme synthesis. We will present data elsewhere showing that there is a discrepancy between heme and globin synthesis in cells from polycythemic animals, and that globin synthesis can persist in the absence of heme synthesis.

Marrow cells that have been pre-incubated for 24 hours before erythropoietin is added, behave as do cells from polycythemic animals, in that erythropoietin has no effect on hemoglobin synthesis in the first four hours, as there is in normal cells. These findings suggest that erythropoietin can act on cells that are already at some stage in erythroid differentiation, since the difference between the populations is essentially the lack of recognizable erythroid cells in the "polycythemic" and pre-incubated cultures. We have shown elsewhere¹⁴ that the erythropoietin stimulation of hemoglobin synthesis by marrow cells from normal rats, occurring within the first four hours of incubation, is much less sensitive to inhibitors of DNA synthesis than is the response at 24 and 48 hours. Ortega and Dukes¹⁵ have made similar observations. In addition, Barcos and Goldwasser^{*} have found that the erythropoietin response of marrow cells from polycythemic rats is more radiosensitive than is the response of normal cells and that pre-incubated cells are more similar to cells from polycythemic animals than to normal cells in their response to ionizing radiation. These findings all suggest that the early erythropoietin stimulation of hemoglobin synthesis represents an action on cells already started on the pathway toward erythroid differentiation. There is evidence from studies in vivo which also suggests this type of action.^{16,17}

Although many aspects of this problem remain to be clarified, it is quite clear that marrow cells can synthesize 9S RNA and retain it in an unexpressed form for several hours, and that superimposed on the regulation of gene expression at the transcriptional level there are other regulatory mechanisms.

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MITOCHONDRIAL DEVELOPMENT IN LIVER OF FOETAL AND NEWBORN RATS*

By

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In the last few years considerable information about the synthesis and assembly of mitochondria has accumulated. Although our understanding is far from complete, it definitely appears that mitochondria have a degree of autonomy in the cell. They have their own DNA, ribosomes, tRNA and mechanism of protein synthesis,^{1,2} but little is known about how mitochondrial components, synthesized either inside or outside mitochondria, are assembled into a fully formed organelle. It is still not established whether the mitochondrion or a major unit of it is formed by the synchronous assembly of all or many of its elements or by the ordered sequential addition of its major components to pre-existing structures. The purpose of the present paper is to provide information on this problem by examining the development of different mitochondrial components in the foetal and postnatal period of the rat.

Several markers of mitochondrial structure and function have been followed. Activities of four respiratory enzymes of the inner mitochondrial membrane that belong to different parts of the electron-transport system have been evaluated during embryogenesis and postnatally. The concentration of the phospholipid cardiolipin, which has been shown to be localized exclusively in the mitochondria of rat and sheep liver^{3,4} and particularly in the inner mitochondrial membrane of guinea pig and rat liver,^{5,6} was also determined. The findings indicate that, in addition to a greater amount of inner mitochondrial membrane in adult liver, mitochondria from adult rats have an enhanced specific activity indicative of a qualitative difference between mitochondria from foetal and mature animals.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were used in all experiments. Total age was estimated from the time of conception and was accurate to within 12 h. Each animal was killed by decapitation. The liver was excised immediately and rinsed with ice-cold 0.3 M-sucrose containing 1 mM-EDTA, pH 7.4. All homogenates except for adult controls were prepared from pooled livers to obtain sufficient amounts of material for analysis.

Isolation of cell fractions. Except for the isolation of the purified nuclear fraction, which was carried out by the method of Lawford, Sadowski and Schlachter,⁷ 5 per cent (w/v) liver homogenates were prepared in 0.3 M-sucrose containing 1 mM-EDTA, pH 7.4, in a glass Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle at maximal speed with one up-and-down movement of the pestle for foetal liver and five for adult tissue. The homogenate was fil-

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tered through a layer of silk bolting cloth (110 mesh; Blaw-Knox Co., Chicago, Ill., U.S.A.) and centrifuged at 1100g for 10 min. The pellet was discarded and the supernatant was centrifuged at 7700g for 10 min. The mitochondrial pellet was washed by suspension in 0.3 M-sucrose containing 1 mM-EDTA, pH 7.4, and centrifugation at 5900g for 10 min. The washings were repeated five times in the experiment designed to evaluate mitochondrial localization of cardiolipin in foetal liver. In all other experiments mitochondria were washed three times. Microsomes were prepared by centrifuging the supernatant at 78500g in the Spinco ultracentrifuge for 1 h; the pellet was suspended in 0.3 M-sucrose containing 1 mM-EDTA, pH 7.4, and centrifuged at 12100g for 30 min. The pellet was discarded and the supernatant was then centrifuged 78500g for 1 h to yield the microsomal pellet. Samples were kept at 0°C throughout preparation.

Enzyme assays. Each sample was assayed in duplicate for the different enzyme activities and for protein content.⁸ Succinate-cytochrome c reductase activity was determined spectrophotometrically at 25°C by measuring the change in E_{550} in the presence of succinate.⁹ Antimycin-sensitive NADH-cytochrome c reductase activity was assayed after treating the mitochondria by a swelling and shrinking procedure described by Sottocasa, Kuylensstierna, Ernster and Bergstrand.¹⁰ The reduction of cytochrome c by NADH in the presence and absence of 1 µg of antimycin A per ml was measured in the same way as for the succinate-cytochrome c reductase and was calculated by subtracting the values obtained in the presence of antimycin from those obtained in its absence. Cytochrome c oxidase activity was assayed by measuring the rate of oxidation of cytochrome c reduced with dithionite in the Gilford automatic spectrophotometer by the modified method of Cooperstein and Lazarow¹¹ described by Rabinowitz, Getz, Casey and Swift.¹² Succinate dehydrogenase activity was measured with and without phenazine methosulphate as an intermediate electron carrier between reduced succinate dehydrogenase and tetrazolium. Formazans formed on reduction of tetrazolium salts were extracted with ethyl acetate and the E_{490} value was measured.¹³ Units of activity are expressed respectively as nmol of cytochrome c reduced/min per mg of protein for succinate-cytochrome c reductase and NADH-cytochrome c reductase, as mg of formazan produced/h per mg of protein for succinate dehydrogenase and as the first-order rate constant k/min per ml of reaction mixture per mg of protein for cytochrome c oxidase.

Phospholipid analysis. Phospholipids were extracted from each sample by the method of Letters.¹⁴ Lipid extracts containing approximately 20 µg of P were analyzed by two-dimensional t.l.c. as described by Getz, Jakovcic, Heywood, Frank and Rabinowitz.¹⁵ Phosphorus analysis was performed by the method of Barlett.¹⁶

Cytochrome determinations. Difference spectra (oxidized-reduced) of mitochondrial preparations solubilized with deoxycholate were obtained by the method of Williams¹⁷ with a Cary model 14 recording spectrophotometer. Cytochrome concentrations were calculated from these spectra.¹⁷

Electron microscopy. Small blocks of liver tissue were fixed for 1 h in 1 per cent OsO_4 in 0.1 M-phosphate buffer, pH 7.4. Small pellets of isolated mitochondria were fixed for 2-4 h in 4 per cent glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4, and 1.5 per cent sorbitol, and post-fixed in OsO_4 in phosphate buffer. Material was embedded in Epon 812; thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Results represent mean values for two to four separate experiments.

Enzyme patterns during the development of rat liver. Cytochrome oxidase, succinate-cytochrome c reductase, antimycin-sensitive NADH-cytochrome c reductase and succinate dehydrogenase activities in liver of foetal (15-21 days of gestation), postnatal (1-30 days post partum) and adult (3-month-old) rats were measured. Homogenate enzyme activities are presented in Figure 1. The mean specific activities $\pm$ 1 S.D. in adult rats were 48 ± 11 units for

Figure 1. Specific activities of respiratory enzymes of liver homogenates of foetal and neonatal rats. Activities are expressed as percentages of adult values: ▲, cytochrome oxidase; X, succinate dehydrogenase; ●, succinate-cytochrome c reductase; □, antimycin A-sensitive NADH-cytochrome c reductase. NB, Newborn. The mean $\pm$ 1 S.D. absolute specific activities for adult rat liver homogenates were 48 ± 11 units for cytochrome oxidase, 99 ± 18 units for succinate-cytochrome c reductase, 80 ± 17 units for antimycin-sensitive NADH-cytochrome c reductase and 21 ± 5 units for succinate dehydrogenase.

cytochrome c oxidase, 99 ± 18 units for succinate-cytochrome c reductase, 80 ± 17 units for antimycin-sensitive NADH-cytochrome c reductase and 21 ± 5 units for succinate dehydrogenase. Enzyme activities from liver of rats at 15-17 days of gestation were one-third to one-twentieth of those in adult liver. Except for cytochrome c oxidase, the activity of which is low and decreases further until birth, the activities of the other three respiratory enzymes start to rise during the last 3-4 days before delivery and continue to increase postnatally.

The specific activities of the respiratory enzymes of purified mitochondria (Figure 2) fol-

Figure 2. Specific activities of respiratory enzymes of purified liver mitochondria from foetal and neonatal rats. Activities are expressed as percentages of adult values: ▲, cytochrome oxidase; X, succinate dehydrogenase; ●, succinate-cytochrome c reductase; □, antimycin A-sensitive NADH-cytochrome c reductase. NB, Newborn. The mean absolute specific activities for adult rat liver mitochondria were 144 ± 17 units for cytochrome oxidase, 296 ± 17 units for succinate-cytochrome c reductase, 253 ± 18 units for antimycin sensitive NADH-cytochrome c reductase and 46 ± 14 units for succinate dehydrogenase.

lowed the same development pattern as that of the homogenate, but the increase was less marked. The values obtained 15-17 days prenatally were one-half to one-eighth of adult values. Mean adult specific activities ± 1 S.D. were 144 ± 17 units for cytochrome c oxidase, 296 ± 17 units for succinate-cytochrome c reductase, 253 ± 18 units for antimycin-sensitive NADH-cytochrome c reductase and 46 ± 14 units for succinate dehydrogenase. These results suggest that the increase in the specific activities of all respiratory enzymes is due both to the increased number of mitochondria postnatally and to the increased functional activity of individual mitochondrial units.

It also appears from Figures 1 and 2 that the rates of increase of respiratory-enzyme activities are not identical for the four enzymes studied. Succinate-cytochrome c reductase activity appears to increase more rapidly than do antimycin-sensitive NADH-cytochrome reductase and cytochrome c oxidase activities. Succinate dehydrogenase is the last to reach its maximal activity, at about 4 weeks after birth. A rather striking example of asynchrony is the decline of homogenate and mitochondrial cytochrome oxidase activities between days 18 and 21 of gestation, a period when the other three enzymes measured were showing marked increase in activity.

To evaluate the possibility that the difference in activity of foetal and adult cytochrome c oxidase is due to differences in enzyme activation during preparation and assay in vitro, enzyme

activities were measured in the presence of the detergent Lubrol (0.4 mg/mg of protein). It has been claimed that this pretreatment with Lubrol produces maximal activation of cytochrome c oxidase.¹⁸ Assays were carried out on liver homogenates and mitochondrial fractions in the foetal and postnatal rats (Figure 3). The activity increased by as much as 100 per cent in the homogenate and 80 per cent in the mitochondria in both foetal and adult samples. Irrespective of the degree of activation in vitro, the foetal activities remained one-third of adult values in the homogenate and one-half of adult values in the mitochondria.

Figure 3. Effects of addition of Lubrol (0.4 mg/mg of protein) on cytochrome c oxidase activities in liver mitochondria and homogenates of foetal and neonatal rats. ●—●, mitochondria; ●- -●, mitochondria + Lubrol; o—o, homogenates; o- -o, homogenates + Lubrol. NB, Newborn.

Cytochrome content. The lower specific activities of mitochondrial enzymes in foetal liver may be due to a smaller content of respiratory carriers or to their presence in less active form. To distinguish between these possibilities spectral analysis of purified mitochondria from foetal and adult rats was carried out. The mitochondrial fraction of 21-day-old foetal liver was compared with that of an adult control. No significant difference in the concentration of cytochrome a/mg of mitochondrial protein was found between the prenatal and adult liver mitochondria (Table 1).

Cardiolipin concentration of the foetal and adult liver. Cardiolipin was selected as a marker of the inner mitochondrial membrane. It was first necessary to establish that the mitochondrial membrane is the major, if not exclusive, site of cardiolipin in both foetal and adult hepato-

Table 1
CONCENTRATIONS OF CYTOCHROMES IN MITOCHONDRIA FROM
FOETAL AND ADULT RATS*

Cytochrome a		Cytochrome b		Cytochrome c ₁		Cytochrome c	
F	A	F	A	F	A	F	A
0.30	0.27	0.31	0.26	0.13	0.19	0.24	0.15

* Concentrations of cytochromes are given in nmol/mg of protein. F, foetal; A, adult.

cytes. Cardiolipin concentrations and the activities of mitochondrial markers, cytochrome c oxidase and succinate-cytochrome c reductase, were measured in the homogenate and in nuclear, mitochondrial and microsomal fractions. Results are presented in Table 2. The phospholipid composition of the adult liver cell fractions is similar to that previously reported with the use of different methods of phospholipid fractionation.⁴ Only a very small amount of cardiolipin was present in the nuclear and microsomal fractions. When corrections are made for mitochondrial contamination on the basis of marker enzyme activities, the calculated cardiolipin content in the nuclear fraction of adult rats is 0.31 per cent of nuclear phospholipids. Since pure nuclei contain a very small proportion of the total rat liver phospholipids this low content of cardiolipin is a negligible proportion of total hepatocyte cardiolipin.

Assuming that about 50 per cent of the homogenate protein is microsomal protein, as derived from the data of Dallner, Siekevitz and Palade,¹⁹ then the microsomes of foetal and adult livers do not contain much more than 10 per cent of the total homogenate cardiolipin (11.2 per cent and 11.9 per cent respectively). It is also apparent from Table 2 that cardiolipin is enriched in the mitochondria relative to the homogenate to about the same extent as are the enzymes cytochrome c oxidase and succinate-cytochrome c reductase. Thus, if the percentage of adult homogenate protein found in the mitochondria is calculated on the basis of the cyto-

Table 2
SUCCINATE-CYTOCHROME c REDUCTASE, CYTOCHROME c OXIDASE AND
CARDIOLIPIN CONTENT IN FRACTIONS OF FOETAL AND ADULT
RAT LIVER (F, foetal; A, adult)

	Homogenate		Nuclear	Mitochondrial		Microsomal	
	F	A	A	F	A	F	A
Succinate-cytochrome c reductase (nmol/min per mg of protein)	0	98.7	Trace	0	296.1	0	10.7
Cytochrome c oxidase (k/min per mg of protein)	8.1	48.5	0.49	41.9	144.9	5.5	5.7
Cardiolipin (μg/mg of protein)	0.1	0.21	0.0025	0.59	0.65	0.1	0.08
Cardiolipin (% of total phospholipids)	6.3	5.4	0.38	13.5	15.3	0.67	0.65

chrome c oxidase, succinate-cytochrome c reductase and cardiolipin contents, one arrives at the values 33.6 per cent, 33.4 per cent and 35.4 per cent respectively. Similar computations for the percentage of foetal liver homogenate protein found in the mitochondria on the basis of cytochrome c oxidase and cardiolipin contents yield values of 19.3 per cent and 16.9 per cent respectively. All these computations, employing conservative assumptions, suggest that 88-100 per cent of the cardiolipin in both foetal and adult rat liver is located in the mitochondria. Cardiolipin can therefore be used as phospholipid marker of the mitochondrial membrane in both foetal and adult rat liver. It is of note that the overall phospholipid compositions of foetal and adult liver mitochondria are similar.

The change in cardiolipin concentration during the development of rat liver was used as another parameter to elucidate the biogenesis of mitochondria. Figure 4 shows that the cardiolipin concentration/mg of liver homogenate protein in foetal animals (18-21 days of gestation)

Figure 4. Cardiolipin content of mitochondria and homogenates of foetal and neonatal rats. The broken horizontal lines represent ± 1 S.D. from the mean of the adult values. NB, Newborn.

was about twice that in adults. In contrast, similar cardiolipin concentrations were found in liver mitochondria from adult and foetal rats. A relatively elevated cardiolipin content in the 16-18 days of gestation was observed in both mitochondria and homogenates. From the ratios of homogenate to mitochondrial cardiolipin content (Figure 4) the mitochondria of foetal liver constitute about half the proportion of total homogenate protein compared to the situation in adult liver.

The ratio of respiratory-enzyme specific activities to cardiolipin concentration is lower in foetal liver and markedly increases during development. The same pattern of increased ratio during development is observed in the homogenate and the mitochondria. The changes in specific activities of respiratory enzymes expressed on the basis of cardiolipin are quite similar to those expressed per mg of mitochondrial protein. This confirms that the respiratory-enzyme specific activities increase much more significantly during liver maturation than does the cardiolipin concentration.

Figure 5 displays the changes in the concentration of mitochondrial phosphatidylethanolamine and phosphatidylcholine (lecithin) during the development of neonatal rat liver. Liver mitochondria from foetal and neonatal rats contain approximately the same amounts of phosphatidylethanolamine and more phosphatidylcholine than do liver mitochondria from adults.

Figure 5. Mitochondrial phosphatidylcholine and phosphatidylethanolamine concentrations during development. ●, phosphatidylcholine; ○, phosphatidylethanolamine.

Morphology of mitochondria of foetal, neonatal and adult liver. Electron micrographs of sections of rat liver (Figure 6) and of purified mitochondrial pellets (Figure 7) were obtained from 20-day-old embryos and from 4-day-old, 15-day-old and adult rats. Mitochondria of the embryo and newborn were wider than those of the 15-day and adult livers, but no consistent changes in quantity or development of cristae were observed. Mitochondrial pellets also showed no evidence for greater development of cristae in livers of mature rats. Contamination in the pellets was slight, consisting of occasional glycogen-particle microbodies, lysosomes and platelets. Mitochondria are almost exclusively of the large type ascribed to hepatocytes.

Figure 6. Sections of intact rat liver, showing regions of hepatocytes containing mitochondria. (A) 20-day embryo; (B) 4 days old; (C) 15 days old; (D) adult. Magnification approx. 12800 X.

Figure 7. Pellets of isolated rat liver mitochondria. (A) 20-day embryo; (B) 4 days old; (C) 15 days old; (D) adult. Magnification approx. 12800 X.

Table 3

PHOSPHOLIPID COMPOSITION IN FRACTIONS OF FOETAL AND ADULT LIVER (F, foetal; A, adult)

	Phospholipids (μg of P/mg of protein)								Phospholipids (% of total)					
	Homogenate		Nuclear	Mitochondrial		Microsomal		Homogenate	Nuclear	Mitochondrial	Microsomal			
	F	A*	A	F	A*	F	A	F	A*	A*	F	A*	F	A
Diphosphatidyl glycerol (cardiolipin)	0.10	0.21	0.0025	0.59	0.65	0.10	0.08	6.3	5.4	0.38	13.5	15.3	0.7	0.7
Phosphatidyl ethanolamine	0.38	0.88	0.13	1.88	1.43	1.53	2.72	23.8	21.7	20.2	42.7	33.6	21.9	23.0
Phosphatidyl choline	0.77	2.03	0.38	1.93	1.53	3.83	6.15	47.9	50.2	57.8	43.9	36.1	55.0	52.1
Phosphatidyl glycerol	n.d.	n.d.	n.d.	n.d.	0.07	n.d.	0.04	n.d.	n.d.	n.d.	n.d.	1.6	n.d.	0.4
Phosphatidic acid	n.d.	0.05	n.d.	n.d.	n.d.	0.01	n.d.	n.d.	1.3	n.d.	n.d.	n.d.	0.1	n.d.
Phosphatidyl serine	0.085	0.19	Trace	Trace	0.06	0.17	0.34	5.3	4.8	Trace	Trace	1.4	2.5	3.0
Lysophosphatidyl ethanolamine	n.d.	n.d.	0.015	n.d.	0.03	0.15	0.08	n.d.	n.d.	2.3	n.d.	0.6	2.2	0.7
Sphingomyelin	0.11	0.22	0.015	Trace	0.11	0.48	0.66	6.8	5.6	2.3	Trace	2.4	7.0	5.7
Phosphatidyl inositol	0.12	0.37	0.056	Trace	0.28	0.64	1.35	7.4	9.1	8.5	Trace	6.6	9.2	11.5
Lysophosphatidyl choline	0.04	0.08	0.051	Trace	0.05	0.08	0.25	2.5	1.9	7.8	Trace	1.3	1.2	2.2
Unidentified	n.d.	n.d.	0.005	n.d.	n.d.	n.d.	0.10	n.d.	n.d.	0.8	n.d.	n.d.	n.d.	0.8

* Means of five separate determinations; all other values are the results of single experiments. In the case of the foetal determinations, these were made on livers of 21-day foetuses. n.d., not detected.

DISCUSSION

In this study we have measured three parameters to follow the development of the inner mitochondrial membrane of the neonatal rat liver, namely; the activities of several respiratory enzymes in the homogenate and purified mitochondria, the spectrophotometrically determined contents of the cytochromes in the mitochondria, and the cardiolipin content in both the homogenate and mitochondria of the developing liver. The data confirm previous reports that the total activity of rat liver respiratory enzymes increases markedly after birth.^{20,21} Adult values are reached 2-4 weeks post partum. The increase in respiratory-enzyme activity begins 2-3 days before birth for all the enzymes studied, except for cytochrome oxidase, in which activity declines until the last day of gestation and begins to rise only after birth.

The increase in the respiratory enzyme activity of the homogenate could be the result of one or all of several changes. These include an increase in the total mass of mitochondria within the liver, an increase in the amount of respiratory element per mitochondrion, and/or an increase in the activity of each of the mitochondrial electron transfer units.

Evidence in favor of an increase in the mass of mitochondria present within the developing hepatocyte derives from two types of data. First, the cardiolipin content of the homogenate increases approximately twofold during this developmental period. These studies, as well as those conducted previously,³⁻⁶ have indicated that cardiolipin is a useful marker of mitochondrial mass, since most, if not all, of the hepatic cardiolipin is located within the mitochondrial membrane. Second, the ratio of homogenate to mitochondrial specific activities of respiratory enzymes suggests that during the course of development the proportion of hepatic protein made up of mitochondrial protein increases twofold. However, a twofold increase in mitochondrial mass is insufficient to account for the total increment in mitochondrial enzyme activity. The increasing specific activity of respiratory enzymes in mitochondria isolated from developing rat liver suggests qualitative changes in mitochondria at various stages of hepatic development. All four mitochondrial respiratory enzymes measured show lower specific activities in the purified foetal than in the purified adult mitochondria. Thus the relatively simple flavoprotein, succinate dehydrogenase and the more complex respiratory complexes, succinate and NADH antimycin-sensitive cytochrome c reductases and cytochrome oxidase all have lower activities in foetal mitochondria.

It is noteworthy that several investigators^{20,22} have suggested the existence of qualitative differences between the mitochondria of foetal and adult rat liver. This conclusion is based on the assumption that during the course of preparation of mitochondria from foetal and adult rat liver, there is no differential activation of respiratory enzymes occurring as a preparative artefact. The similar activation of respiratory-enzyme activity in both foetal and adult rat liver mitochondria and homogenates lends support to this assumption.

If the observed changes in the respiratory-enzyme specific activities of isolated rat liver mitochondria do indeed represent true changes in enzyme activity, they could be the result of variations in the amount of inner mitochondrial membrane as a proportion of total mitochondrial protein. That this is not the explanation for the changes in specific activity of respiratory enzymes is suggested by two factors. First, there is no apparent difference in the content of the cytochromes, determined spectrophotometrically per mg of mitochondrial protein. Second, the specific activities of the respiratory enzymes as expressed on the basis of cardiolipin, a pre-

sumed component of the inner mitochondrial membrane, reflects changes quite similar to those seen when these activities are expressed per mg of mitochondrial protein. Furthermore, the electron micrographs show no evidence of a change in the proportion of cristae between foetal and adult rat liver mitochondria. This is in contrast to the situation in developing rat brain²³ and in liver and skeletal muscle of hypothyroid animals treated with thyroid hormone^{24,25} (Gross & Swift, unpublished observations).

Aside from a definite increase in respiratory-enzyme activities in the postnatal period, there is a suggestion of an asynchronous pattern of elevation of the activities of each of these respiratory enzymes. The asynchronous development of mitochondrial components could conceivably reflect the changing cell population in the neonatal liver during development, if one assumes that a different type of mitochondrion is present in hemopoietic cells. It is extremely unlikely, however, that this is the sole explanation for the asynchrony of respiratory enzyme development, since hemopoietic cells, though constituting a large proportion of foetal hepatic mass, nevertheless contain relatively few mitochondria compared even with foetal hepatocytes. Thus, the respiratory activity and cardiolipin content probably reflect mainly the hepatocyte composition.

The asynchronous enzyme development observed during development does not necessarily conflict with studies measuring the turnover of various mitochondrial components which have led to the hypothesis that at least one major subunit of the inner mitochondrial membrane turns over as a unit.^{26,27} Simultaneous assembly and degradation of such a unit is assumed. The inner membrane cytochromes, c, b, and aa₃ all turn over with the same half-life in rat heart and liver.^{28,29} These studies were carried out under steady-state conditions and are valid only in the framework of the relatively long turnover times measured. They do not exclude the possibility of asynchronous assembly during such non-steady state conditions as are present in the rapidly growing foetal and newborn liver.

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CARDIOLIPIN CONTENT OF WILD TYPE AND MUTANT YEAST IN RELATION TO MITOCHONDRIAL FUNCTION AND DEVELOPMENT*

By

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The yeast, *Saccharomyces cerevisiae*, offers a unique opportunity for the study of mitochondrial biogenesis. It is a facultative anaerobe, being capable of anaerobic growth with glucose or related carbohydrate as carbon source, and of aerobic growth supported by a wide variety of carbon compounds, including lactate, glycerol or acetate. During anaerobic growth on a medium with a high glucose content, cytochromes are not formed and characteristic mitochondrial functions disappear, while during aerobic growth mitochondrial profiles and functions readily develop.^{1,2} During aerobic growth in the presence of large repressive concentrations of glucose, mitochondrial functions are incompletely developed.^{3,4} With glucose as carbon source, mitochondrial function is only fully expressed as the yeast culture passes into stationary phase, when all the medium glucose is exhausted. This situation clearly affords an excellent system in which to study the factors that regulate the emergence of typical mitochondrial activities. The availability in yeast of both cytoplasmic and chromosomal mutants,^{5,6} which affect mitochondrial function, furnishes yet a further advantage for the study of the mechanisms of mitochondrial formation in this organism. The mutants affecting mitochondrial function are readily identifiable by their inability to grow on a nonfermentable carbon source, e.g., glycerol. The lack of oxidative activities in anaerobic yeast is not associated with a complete absence of mitochondrial membrane profiles.^{7,8,9} This situation is reminiscent of that observed in the mitochondrial mutants, where recognizable (cytoplasmic mutants) and in some cases normal (chromosomal mutants) mitochondrial structures are seen without any evident terminal respiratory activity.² Thus both in anaerobically grown yeast and in certain mutant yeasts, microscopically identifiable mitochondrial membranes and characteristic mitochondrial function do not develop in parallel.

For the study of mitochondrial membrane biogenesis and its regulation, the availability of a membrane marker that is independent of respiratory function would be of considerable value. The characterization of, and analytical procedures for the structural or noncatalytic proteins are currently insufficiently refined for this purpose. We therefore turned our attention to the phospholipid components of these membranes, since the phospholipid, cardiolipin, is found to be particularly associated with mammalian mitochondrial membranes^{10,11} and especially with the inner mitochondrial membrane.^{12,13} We have embarked on an examination of the phospholipid composition of whole yeast and its subcellular fractions in order to evaluate whether in yeast also, cardiolipin is particularly associated with mitochondrial membranes. In this paper we also report on the phospholipid and cardiolipin content of several strains of yeast, diploid and haploid,

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normal with respect to respiratory status, and derived respiratory deficient mutants. With one exception (i.e., loss of auxotrophy for lysine in $R_1 P_7 \rho^+$ vs $scl_1 p_7 \rho^+$ —see Table 1), the respiratory mutants were isogenic with the wild type strains in all characters other than their respiratory status. We have analyzed the phospholipids of neutral and suppressive cytoplasmic mitochondrial mutants in view of the possibility that the basic defect in each of these two mutants might be different. This was considered especially important in the light of the observed differences in buoyant density of mitochondrial DNA in mutants of this type.¹⁴ The strains were cultured under growth conditions designed to induce variations in the complement of mitochondrial membranes.

MATERIALS AND METHODS

Strains of yeast. All strains of yeast employed were provided from the collection of Dr. P. P. Slonimski. Three sets of isogenic yeast strains were studied. The phospholipid composition of the first two of these sets was determined, while the third set was used for genetic testing. The characteristics of these strains are tabulated in Table 1. An explanation of the significance of the rho factor (ρ) was given elsewhere.¹⁵

The particular set of haploid strains was chosen because it contains a large amount of mitochondrial DNA in comparison to the diploid yeast 237.¹⁶ Several chromosomal loci controlling mitochondrial function have been identified.⁶ This set of strains varies in the locus designated P_7 (respiratory competent wild type) or p_7 (respiratory deficient mutant).

Culture media. Cultures were grown aerobically on enriched media as described by Getz et al.¹⁵ In some experiments 2 per cent galactose or 2 per cent Na lactate replaced glucose as the carbon source in the medium. For cell fractionation, diploid yeast, wild type ($YF\rho^+$) and "petite" ($YFA\rho^-$) were grown to mid-exponential phase in 2 per cent galactose. Strains of the haploid set ($scl_1 p_7 \rho^+$; $R_1 P_7 \rho^+$; $g p_7 \rho^-$; 116 $p_7 \rho^-$) were grown in the chemostat as described by Mounolou, Jakob and Slonimski.¹⁴ The medium used for chemostat growth (3-S) contained in 1 liter: 10 g yeast extract, 1 g KH_2PO_4 , 1.2 g $(NH_4)_2SO_4$ and 50 g glucose. Fifteen milliliters of antifoam A (Dow & Corning) 1 g streptomycin, and 5,000,000 U penicillin were added to 8 l of culture medium. Precultures were grown in 3S medium containing only 0.5 per cent glucose and collected in the exponential phase of growth in order to introduce a low glucose concentration at the beginning of the culture in the chemostat. The 500 ml chemostat flasks contained 88-136 ml of medium. The flow rate was about 18.0 ml/hr and generation times were between 4.2 and 5.4 hr. The collection of the first 48 hr was discarded. The optical density of the cultures was constant during the next 3 days of collection and was indicative of stationary growth phase. Glucose was not detectable in the medium during the steady state growth. Yeast cells were collected for phospholipid analyses on the third to sixth day. Cultures were tested daily for growth on different media to ensure that no respiratory sufficient revertants had appeared.

Strains of the haploid set were also grown in shaker flasks to the exponential phase of growth in 3S medium containing 5 per cent glucose. In some cases, the respiratory sufficient haploid strain $R_1 P_7 \rho^+$ was also grown to late stationary phase in 3S medium, and to late stationary and mid-exponential phases in enriched medium containing 2 per cent glucose.

Yeast collection and breakage. The yeast cells were harvested by centrifugation at appropriate stages of the growth cycle, washed twice with ice cold water and either broken or frozen immediately thereafter. When phospholipid analysis was to be of the whole yeast only, cell break-

Table 1
YEAST STRAINS

Strain	Cytoplasmic "petite"	Chromosomal "petite"	Mating type	Auxotrophy	Abbreviation used in text	Comment
<u>Diploid set</u>						
Yeast foam 237	-	-	-	-	YF ρ^+	
Yeast foam 237A	+	-	-	-	YFA ρ^-	
<u>Haploid set</u>						
D243-2B-Scl ₁	-	+	a	ad ₁ lys ₂ [*]	scl ₁ p ₇ ρ^+	reversion rate 1 in 10 ⁴ revertant with respect to respiratory defi- ciency
D243-2B-R ₁	-	-	a	ad ₁	R ₁ P ₇ ρ^+	
D243-2B-g	+(neutral)	+	a	ad ₁ lys ₂	g p ₇ ρ^-	
D243-2B-116	+(suppressive)	+	a	ad ₁ lys ₂	116 p ₇ ρ^-	17% suppressive [†] 92% suppressive [†]
<u>Test set (haploid)</u>						
C982-19D	-	-	δ	hi ₁ tr ⁻	19 d ρ^+	
C982-19dA	+	-	δ	hi ₁ tr ⁻	19 d A ρ^-	

* Note that in reversion with respect to respiratory deficiency, auxotrophy for lysine is lost.

† The degree of suppressiveness was obtained by measuring the percentage of diploid "petite" colonies obtained after crossing with "grande" test strain 19d ρ^+ and plating the zygotes on minimal medium with a low glucose content. The crosses were made with test set yeast according to standard procedures.^{33,34} The plates were stained using the tetrazolium overlay technique³⁵ to determine the number of respiratory sufficient colonies (which stain red).

age was performed at 0-5°C in the Ribi cell fractionator (Sorvall) as described by Getz et al.¹⁵ In most cases cells were broken within a week of collection. On the other hand, the cells of the haploid set ($R_1 P_7 \rho^+$ and mutant cells) grown in the chemostat, and log phase cultures of these same yeast strains grown in 3S medium, but not in the chemostat (grown in Gif-sur-Yvette) were stored as a yeast cake at -20°C for 6 months before mechanical breakage and lipid extraction. Small samples of the broken yeast cell suspension were saved for protein determination according to Lowry et al.¹⁷

Cell fractionation. Cell fractions used to study phospholipid distribution (Table 2) were isolated from protoplasts of diploid yeast. Protoplasts were prepared with glucanase (Endo Laboratories) as described by Rabinowitz et al.¹⁸ They were disrupted as previously described¹⁸ except that the concentration of EDTA in breaking solution was increased from 1×10^{-3} M to 5×10^{-3} M in the hope of minimizing phospholipolytic activity.

A portion of the broken protoplast homogenate was saved for lipid and protein analysis and for cytochrome oxidase assay. The remainder of the suspension was centrifuged in the Servall RC2B refrigerated centrifuge at 1500 g for 10 min. The pellet constituted the debris fraction. The supernatant was centrifuged again at 1500 g for 10 min and the pellet was discarded. This last step was repeated once more, to reduce contamination of mitochondria by whole protoplasts. The supernatant of the last 1500 g centrifugation was centrifuged at 17,300 g for 10 min. The pellet was resuspended in protoplast breaking solution and re-centrifuged at 17,300 g for 10 min. This last washing procedure was repeated twice more, the final three-times-washed pellet constituting the mitochondrial fraction. The supernatant from the sedimentation of the mitochondrial fraction was centrifuged at 48,000 g for 30 min. The pellet from this centrifugation is designated the intermediate fraction, and the supernatant was sedimented in the Spinco L2-65 centrifuge in rotor number 60 at 60,000 rpm for 60 min to yield the microsomal pellet and a supernatant that was discarded. The debris, mitochondrial, intermediate and microsomal fractions, were saved for lipid analysis, protein determination and cytochrome oxidase assay. The same fractionation procedure was employed for diploid petite yeast ($YFA\rho^-$) except that the mitochondrial fraction was washed at 27,000 g for 10 min. The mitochondrial fractions of both wild type ($YF\rho^+$) and petite ($YFA\rho^-$) yeast were examined by electron microscopy.

Electron microscopy. Mitochondrial pellets for electron microscopy were prepared from a small aliquot of the mitochondrial suspension removed prior to the final centrifugation for the third wash of the mitochondrial fraction. The entire pellet from this sample was fixed for electron microscopy in an attempt to ensure random sampling of the mitochondrial fraction. The mitochondrial pellets were fixed for 1 hr at 4°C in 4 per cent glutaraldehyde (Polysciences) in 0.1 M phosphate buffer and 1 per cent sorbitol. Shortly after introduction into the fixation medium, pellets were divided into pieces less than 1 mm across. These pieces were washed in phosphate-buffered sorbitol, postfixed in 1 per cent osmium tetroxide in phosphate-buffered sorbitol, and embedded in Epon. Thin sections were stained with uranyl acetate and Reynolds' lead citrate.

Cytochrome c oxidase assays. Cytochrome c oxidase was measured as described by Rabinowitz et al.¹⁸ In the case of the cell fractionation experiments, results are expressed as the first order rate constant K/min/mgm protein calculated as described by Cooperstein and Lazarow.¹⁹ In the case of yeast grown under various different conditions for lipid analysis of whole yeast, enzyme activities were determined on a mechanically broken yeast suspension and results are expressed as $m\mu$ moles cytochrome c oxidized/min/mg protein. Cytochrome c oxidase activity

Table 2

DISTRIBUTION OF PHOSPHOLIPIDS AND CARDIOLIPIN IN SUBCELLULAR
FRACTIONS OF WILD TYPE YEAST (YF ρ^+)

Fraction	Homogenate % PLP [†]	Debris % PLP	Mitochondria % PLP	Intermediate % PLP	Microsomes % PLP
DPG*	6.5	9.8	15.6	5.0	1.6
PE	14.1	17.2	22.0	16.0	12.4
Pc	28.6	31.6	32.5	29.4	40.5
DMPE	1.7	0.8	0.9	1.7	1.4
PG	2.7	1.4	2.0	1.5	
PA	1.5	2.6	1.0	2.6	0.7
PS	4.6	5.4	3.3	8.6	7.3
LPE	7.9	1.7	3.6	4.5	2.0
PI	12.7	22.1	10.7	16.6	27.8
LPC	10.8	2.5	4.2	5.7	2.7
?	1.8	0.5		0.7	
?	6.7	4.8	4.2	7.8	3.7
PLP $\mu\text{g/g}^{\ddagger}$ protein	3285	2050	8030	7920	5235
Cytochrome oxidase K/min/mg protein	21.9	29.9	160.9	37.0	6.5
DPG phosphorus $\mu\text{g/g}$ protein	214	201	1252	396	83.7
% of whole yeast DPG in fraction [§]	100	15.4	64.0	16.1	4.4
% of whole yeast DPG in fraction corrected for mitochondrial con- tamination		0		4.4	1.7
% of whole yeast PLP in fraction [§]	100	13.5	35.2	27.7	23.6
% of whole yeast pro- tein in fraction [§]	100	34.7	23.0	18.4	23.7

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

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

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

[§] The calculations of these percentages were all based upon setting the total cardiolipin, phospholipid or protein recovered in the sum of the fractions at 100%.

was largely inactivated by breakage of yeast in the Ribi cell fractionator at 40,000 pounds per square inch—the breakage method employed for whole yeast to be subjected to lipid analysis. For enzyme assays on whole yeast therefore, samples of yeast grown under similar conditions to the samples employed for lipid analysis were broken mechanically in the Braun shaker as

described by Henson, Weber and Mahler.²⁰ The homogenate was centrifuged at 1000 g for 10 min and the supernatant was used for oxidase assay. (We are grateful to Dr. B. Levin for help with these experiments.) Repeated assays for cytochrome c oxidase in "petite" yeast revealed no demonstrable activity.

Lipid extraction and chromatographic analysis. Procedures for the extraction, chromatographic quantitative determination of lipid and expression of results have been described.¹⁵ The only variation was that samples prepared in the cell fractionation experiments were initially extracted with 4 volumes of absolute ethanol (final concentration of ethanol 80 per cent), held for 15 min at 80°C, and shaken at room temperature overnight. This substituted for the overnight room temperature extraction with 19 volumes of ethanol (final concentration of ethanol 95 per cent) used with all other yeast samples. This variation was an attempt to limit the phospholipolytic activity in the fractions prior to analysis, as suggested by Letters.²¹ Evidence for the quantitative nature of this extraction procedure has been provided by Letters^{21,22} and Harrison and Trevelyan.²³ Although peroxides were not measured directly in the lipid extract, major deterioration of lipids did not appear to take place as judged by the very pale color of the extract and the absence of any significant material remaining at the origin of thin layer chromatograms. Sterol analysis was made by the Lieberman-Burchard reaction²⁴ performed on the whole purified lipid extracts. Ergosterol was employed as standard for these analyses.

RESULTS

Lipid composition of subcellular fractions of wild type diploid yeast (YF ρ^+). Only by the preliminary formation of protoplasts with glucylase can one expect to obtain a good yield of intact mitochondria. Mechanical breakage of whole yeast generally results in a low yield of mitochondria, probably due to the high rate of disruption. Disruption of mitochondria will mitigate against a satisfactory separation of mitochondrial membranes from other subcellular fractions. Since protoplasts are more readily formed from log phase than from stationary cells, galactose-grown cells, harvested in log phase were employed for yeast cell fractionation. Galactose was chosen as carbon source in order to obtain yeast with a large mitochondrial complement, and this carbon source could also be employed for petite (YFA ρ^-) yeast. Either because of the presence of lipolytic enzymes in snail juice or because of activation of endogenous yeast phospholipases during the preparation of yeast cell fractions, considerable difficulty was experienced in obtaining fractions that did not contain large quantities of lysophospholipids, such as lysophosphatidyl ethanolamine or lysophosphatidyl choline. That the extraction procedure per se was not responsible for the phospholipolysis, is suggested by the relatively low amounts of lysophospholipids found in extracts made according to the same procedure from yeast broken mechanically in the Ribi fractionator and extracted immediately. In attempts to minimize such artifactual degradation of phospholipids, the concentration of EDTA in the breakage solution was increased from 0.001 M to 0.005 M, and immediately after the fraction was obtained, extraction of lipids was initiated with ethanol (80 per cent) for 15 min at 80°C. Despite this, however, substantial amounts of lysophospholipids were found in the yeast cell fractions, the phospholipid composition of which is presented in Table 2. Continuing phospholipolysis during the course of fractionation also probably accounts for the fact that all fractions of the petite (YFA ρ^-) yeast contained less phosphatidyl inositol and more of an unknown phospholipid, probably lysophosphatidyl inositol than the starting homogenate (see Table 3). Comparison of the results for the homogenate,

Table 3

DISTRIBUTION OF PHOSPHOLIPIDS AND CARDIOLIPIN BETWEEN SUBCELLULAR
FRACTIONS OF PETITE YEAST (YFA ρ^-)

Fraction	Homogenate % PLP	Debris % PLP	Mitochondria % PLP	Intermediate % PLP	Microsomes % PLP
DPG*	4.9	5.7	9.1	2.7	1.2
PE	14.7	15.7	20.5	13.0	13.7
PC	37.6	41.6	35.8	36.1	38.1
DMPE	1.1	trace	trace	trace	0.8
PG	trace	0.5	0.8	0.6	0.6
PA	2.2	2.4	1.5	2.9	1.3
PS	9.7	9.2	7.4	5.7	3.9
LPE	trace	0.5	1.9	8.3	5.4
PI	23.9	19.9	16.7	8.9	7.2
LPC	1.6	trace	1.9	16.0	14.2
?	4.3	4.4	4.7	5.7	13.8
PLP $\mu\text{g/g}$ protein	3316	4500	14340	4640	3109
DPG phosphorus $\mu\text{g/g}$ protein	162.5	256.4	1224	125	37.3
% whole yeast DPG in fraction	100	40.8	45.0	9.4	4.9
% whole yeast PLP in fraction	100	36.6	25.2	17.7	20.7
% whole yeast protein in fraction	100	39.8	8.6	18.8	32.7

* For abbreviations and mode of expression and calculation of data see footnotes to Table 2.

shown in Table 2, with the results for galactose-grown cells disrupted mechanically prior to lipid analysis, suggests strongly that the presence of lysophospholipids in the glucosylase-broken homogenate of yeast is an artifact of the preparative procedure. The level of phospholipid per gram of yeast protein suggests that the lipolysis of phospholipid did not proceed beyond the stage of lysophospholipid. Most important, for the purposes of this paper, the lipolysis does not appear to have affected the cardiolipin.

Bearing in mind the limitations of this analysis, several features of the composition of cell fraction phospholipids are evident. The mitochondrial fraction is characterized by its high cytochrome c oxidase activity and its appearance on electron microscopic examination of a representative mitochondrial pellet (Figure 1). The distinctive feature of the mitochondrial phospholipid is the large proportion of cardiolipin (15.6 per cent) contained in this fraction. Mitochondria also contain a higher ratio of phosphatidyl ethanolamine to phosphatidyl choline than any other cell fraction. They contain a lower proportion of phosphatidyl serine and phosphatidyl inositol than is found in the other cell fractions, while the microsomal fraction contains large amounts of phosphatidyl choline and phosphatidyl inositol and relatively little cardiolipin and phosphatidyl ethanolamine. Similar characteristics were observed in the phospholipid composi-

Figure 1. Portion of mitochondrial pellet obtained from wild type ($YF\rho^+$) cells. Magnification 46,000X.

tion of a mitochondrial fraction and a microsomal fraction prepared in an equivalent fashion from glucose-grown aerobic stationary wild type diploid yeast ($YF\rho^+$). The relative cardiolipin content of a mitochondrial fraction of anaerobic wild type diploid yeast ($YF\rho^+$) was also twice that of the whole yeast. The mitochondrial fraction had the highest ratio of phospholipid to protein of all cell fractions. At least 64 per cent of the whole yeast cardiolipin was recovered in the mitochondrial fraction although it contained only 35.2 per cent of the whole yeast phospholipid and 23.0 per cent of the yeast protein. When corrections are made for the contamination of the debris, intermediate and microsomal fractions by mitochondrial membranes on the basis of their specific activity of cytochrome c oxidase, it is seen that all but a small proportion of the cardiolipin found in the intermediate and microsomal fractions (total ± 6.0 per cent) can be attributed to the contaminating mitochondria. In other words, in excess of 90 per cent of the whole yeast cardiolipin appears to be associated with mitochondrial membranes.

Lipid composition of subcellular fractions of respiratory-deficient yeast ($YFA\rho^-$). Respiratory-deficient diploid yeast grown to log phase in galactose was subjected to subcellular fractionation in the same fashion as was wild type yeast. However, to ensure a reasonable recovery of mitochondrial membranes, the mitochondrial fraction was sedimented at 27,000 g for 10 min rather than 17,300 g used for the sedimentation of the wild type mitochondrial fraction. The characterization of the mitochondrial fraction from the respiratory-deficient yeast rests upon the appearance of the electron micrograph from a representative pellet of the mitochondrial fraction which is shown in Figure 2. In general the distinction between phospholipid composition of the mitochondrial fraction and that of the other cell fractions was similar to that obtained for the wild type yeast cell fractions. The data are presented in detail in Table 3. The mitochondrial fraction had a high cardiolipin and relatively high phosphatidyl ethanolamine content. The mito-

Figure 2. Portion of mitochondrial pellet obtained from petite ($YFA\rho^-$) cells. Magnification 59,000X.

chondrial fraction had the highest ratio of phospholipid to protein of all the cell fractions. The cardiolipin content per gram of protein in the "petite" mitochondrial fraction was strikingly similar (1224 vs 1252) to that found in the corresponding mitochondrial fraction of the wild type yeast. Much of the whole cell cardiolipin (45 per cent) was recovered in the mitochondrial fraction, which contained 25.2 per cent of the yeast phospholipids and only 8.6 per cent of the yeast protein. A comparison of the subcellular distribution of cardiolipin with that of either cytochrome c or mitochondrial ATPase, both of which are present in petite mitochondria, would establish whether in the respiratory deficient yeast also, cardiolipin is an exclusive marker of mitochondrial membranes. Clearly however, "petite" mitochondria do contain large quantities of cardiolipin and it is likely that most or all of this phospholipid is also associated with mitochondrial membranes.

Effect of carbon source on phospholipid composition of exponential phase diploid yeast.

Since glucose is known to repress the formation of mitochondria, the effects of galactose and lactate replacement of glucose as the major carbon source in the enriched medium were investigated. The results of these experiments are presented in Table 4. The total content of phospholipid and the composition with respect to the major phospholipids were strikingly similar in both wild type ($YF\rho^+$) and petite ($YFA\rho^-$) log phase yeast irrespective of carbon source. The cardiolipin content however, varied significantly with carbon source and presumably with state of repression of mitochondrial function.

Effect of growth conditions on the cardiolipin content of diploid yeasts. The effects of variations of growth conditions on the cardiolipin content of wild type ($YF\rho^+$) and petite ($YFA\rho^-$) yeast are summarized in Table 5. The degree of repression of respiratory function, as represented by cytochrome c oxidase activity, and of cardiolipin formation were not similarly affected

Table 4

EFFECT OF CARBON SOURCE ON PHOSPHOLIPID COMPOSITION OF LOG PHASE
WILD TYPE ($YF\rho^+$) AND PETITE ($YFA\rho^-$) YEAST*

Yeast strain	$YF\rho^+$			$YFA\rho^-$	
Carbon source	Glucose(3) ^{§‡} % PLP [†]	Galactose(1) % PLP	Lactate(1) % PLP	Glucose(2) ^{§‡} % PLP	Galactose(1) % PLP
DPG [†]	3.3	7.1	6.6	1.3	3.8
PE	21.6	20.5	17.9	21.8	15.9
PC	41.6	39.6	47.3	39.8	43.4
DMPE	1.4	0.7		1.0	
PG	0.3	0.5	1.2	0.6	0.9
PA	0.8	0.5	2.1	1.8	2.3
PS	7.5	5.2	7.0	7.7	4.7
LPE	1.0	0.2		0.2	1.3
PI	20.3	24.6	16.0	21.8	26.1
LPC	0.3	1.3	3.0	2.2	1.5
?	0.4		1.4	0.7	
?	0.9			1.7	0.1
PLP μ g/g protein	2800		2990	2840	3200

* All cultures were grown in enriched medium in which glucose was replaced by the designated substrate at the 2% level. All samples of yeast were mechanically disrupted.

[†] For abbreviations and explanation of mode of expression of data see footnote to Table 2.

[‡] Numbers in parentheses refer to the numbers of independent cultures analyzed.

[§] The data in these columns are from Getz et al.¹⁵

in a quantitative sense by either glucose or anaerobiosis. Thus while respiratory activity of nonrepressed or incompletely repressed yeast was four- to seven-fold greater than glucose-repressed yeast and 16- to 30-fold greater than anaerobic yeast, the differences in cardiolipin content varied only over a 2- to 5-fold range. Cytoplasmic mutant yeast (ρ^-) had no demonstrable cytochrome oxidase activity, but contained one-third to two-thirds as much cardiolipin as wild type yeast (ρ^+) under the corresponding conditions of growth.

Effect of growth conditions and of mutations affecting mitochondrial function on cardiolipin content of haploid yeast. As in the case of diploid yeast, high glucose repressed the respiratory activity (in the respiratory competent $R_1 P_7 \rho^+$) and cardiolipin formation in both normal and mutant haploid yeast.

During the growth, harvesting, or storage of the haploid yeast and its derived mutants grown under chemostat conditions, there was activation of the phospholipolytic enzymes. This was evident from the relatively large amounts of lysolecithin, lysophosphatidyl ethanolamine, and lysophosphatidyl inositol found in extracts of these yeasts. Our major objective, however, was to ascertain whether the mutation was associated with a loss of mitochondrial lipids, especially cardiolipin, and whether there was a relationship between the cardiolipin content and the nature of the mutation affecting mitochondrial structure and function. This was why we chose

Table 5

LIPID AND CARDIOLIPIN CONTENT OF DIPLOID STRAINS UNDER
VARIOUS GROWTH CONDITIONS

Yeast	Gas phase	Carbon source	PLP μg [†] g protein	Cardiolipin (DPG) [†] % total PLP	Cardiolipin phosphorus $\mu\text{g/g}$ protein	Cytochrome oxidase $\text{m}\mu\text{moles/min}$ mg protein
A. Stationary Growth						
YF ρ^+	O ₂	glucose	2780(2) [*]	7.5	216.0	136
YFA ρ^-	O ₂	glucose	1830(3)	3.7	71.0	
YF ρ^+	N ₂	glucose	1050(2)	4.7	49.1	< 5
YFA ρ^-	N ₂	glucose	2260(1)	0.4	9.0	
B. Exponential Growth						
YF ρ^+	O ₂	glucose	2800(3)	3.3	92.4	20
YFA ρ^-	O ₂	glucose	2840(2)	1.3	36.9	
YF ρ^+	O ₂	galactose	3110(2)	6.8	211.5	152
YFA ρ^-	O ₂	galactose	3260(2)	4.4	143.5	
YF ρ^+	O ₂	lactate	2990(1)	6.6	197.7	82
YF ρ^+	N ₂	glucose	1790(2)	2.5	44.8	< 5
YFA ρ^-	N ₂	glucose	2210(2)	1.5	33.1	

* Figures in parentheses refer to the number of independent cultures analyzed.

† Expression of these results is as indicated in the footnote to Table 2.

mutants grown under chemostat conditions, i.e., conditions that would permit the maximal development of mitochondrial membranes. It can be seen from Table 6B that in each class of mutant the chemostat-grown cells contained more cardiolipin than the log phase cells. The chromosomal mutant contained a similar amount of cardiolipin to that of its corresponding wild type yeast. On the other hand, as in the diploid strains, the cytoplasmic "petites" had significantly less cardiolipin than the wild type $R_1 P_7 \rho^+$. In this respect there was little difference between the neutral and suppressive cytoplasmic "petites." In the case of log phase grown cells, respiratory competent and incompetent cells contained similar amounts of cardiolipin.

DISCUSSION

The phospholipid composition of the mitochondria of wild type diploid yeast (YF ρ^+) is strikingly similar to that reported for mitochondria of several mammalian tissues^{10,11} and more recently of yeast.^{22,25} The features of the phospholipid composition of mitochondria in all of these cell types is characterized by a high proportion of cardiolipin and a higher ratio of phosphatidyl ethanolamine to lecithin than is found in other cellular fractions. In few previous reports have the available data permitted an assessment of the proportion of total cellular cardiolipin that is located in the mitochondria. In this study, it is evident that more than 90 per cent of the total yeast cardiolipin is confined to the mitochondrial membranes, presumably the inner mitochondrial membrane. This is also true for fetal and adult rat liver²⁶ and probably for rat brain as well.²⁷

Table 6

LIPID AND CARDIOLIPIN (DPG) CONTENT OF HAPLOID STRAINS (D243-B) (NORMAL AND DIFFERENT MUTANTS)

Growth condition and medium	PLP [†] $\mu\text{g/g}$ protein	Cardiolipin [†] (DPG) % total phospholipids	Cardiolipin (DPG) phosphorus $\mu\text{g/g}$ protein	Cytochrome oxidase $\text{m}\mu\text{moles/min}$ mg protein	
A. Normal Cells ($R_1 P_7 \rho^+$) in Various Media					
Late stationary aerobic enriched 2% glucose	1410	13.8	195.0	92.0	
Late stationary aerobic chemostat medium	1990	13.6	271.1		
Late stationary anaerobic enriched 2% glucose	1230	3.3	40.5	<5.0	
Exponential aerobic chemostat medium	3080	1.7	51.1	19.0	
B. Normal and Mutant Cells Grown in Chemostat Medium. * Cells Grown in Chemostat.					
Yeast strain				Sterol mg/g protein	Log phase cells Cardiolipin (DPG) phosphorus $\mu\text{g/g}$ protein
$R_1 P_7 \rho^+ \dagger$	1870	9.9	185	20.2	68.0 [‡]
$\text{scl}_1 p_7 \rho^+ \S$ (chromosomal mutant)	1600	12.1	194	23.4	61.0 ^{**}
$g p_7 \rho^-$ (neutral cytoplasmic petite)	1800	5.9	106	27.1	41.4
116 $p_7 \rho^-$ (suppressive cytoplasmic petite)	1320	5.3	70	28.5	53.2

* All cultures referred to in this section of this table were grown in chemostat medium (3-S). The log phase cells were grown in this medium but not in the chemostat.

[†] Expression of these results is as indicated in the footnote to Table 2.

[‡] Sixteen per cent of this culture had become petite (ρ^-).

[§] On the last day of the collection from the chemostat, 29% of this strain had become double petite, i.e., $\text{scl}_1 p_7 \rho^-$. This was checked by crossing these cells with test yeast 19 d $A \rho^-$.

[‡] At harvesting of this culture 4.3% of cells were petite (ρ^-).

^{**} At harvesting of this culture, 8.5% of diploid colonies growing from a cross with test yeast 19 d $A \rho^-$ were petite, i.e., $\text{scl}_1 p_7 \rho^-$.

The information on the localization of cardiolipin to mitochondrial membranes suggests that this phospholipid may be used as a suitable structural marker of these membranes. Such a marker may be particularly useful in view of the recent findings by Fukuhara²⁸ that amounts of mitochondrial DNA do not fluctuate much with the physiological state of yeast. This situation has the particular advantage that it may be used to mark mitochondrial membranes, irrespective of their functional competence, as exemplified by the presence of significant concentrations of cardiolipin in anaerobic yeast, where it is concentrated in promitochondria,²⁵ and in respiratory-deficient yeast (this study). That mitochondrial membrane biogenesis may be regulated independent of the development of respiratory capacity is clearly demonstrated by the aerobic development of mitochondria in anaerobically grown yeast even in the presence of cyanide, an inhibitor of cytochrome oxidase.²⁹ Our own studies reported herein suggest that fluctuations in cardiolipin concentration (see Tables 5 and 6) reflect alterations in the development of mitochondrial membranes under a wide variety of growth conditions and genetic controls. Furthermore, they support the notion that the mechanism responsible for the regulation of mitochondrial membranes by glucose and anaerobiosis, as measured by variations in yeast cardiolipin content, is still operative even in yeast that is genetically unable to support terminal respiratory activity. Evidence in favor of such a relationship may be briefly summarized as follows:

(a) Mitochondrial development and respiratory capacity occur more prominently in the stationary phase of aerobic yeast growth in glucose.^{3,4} Cardiolipin content of stationary yeast was much higher than that of glucose-grown log phase cells of all strains examined, including the cytoplasmic mutants (see Table 5). For the wild type yeast, the cardiolipin content in various phases of growth was related to the cytochrome oxidase activity (see Tables 5 and 6A).

(b) The synthesis of respiratory assemblies and mitochondrial membrane formation is markedly suppressed under anaerobic conditions.^{1,2,30} Anaerobic yeast, at each phase of growth examined, invariably contains less cardiolipin than aerobic yeast, even in yeast with a comparable content of the major phospholipid lecithin. It is, however, noteworthy that cardiolipin was always present in measurable amounts in anaerobic yeast, even when grown in medium unsupplemented with Tween 80 and ergosterol (data not shown here). Assuming cardiolipin is exclusively localized in yeast mitochondrial inner membrane, as appears to be the case in liver,^{12,13} this may be taken to indicate that the mitochondrial membrane does not completely disappear from anaerobic yeast, even under conditions of minimal supplementation. Recent studies^{9,25,31} have demonstrated that mitochondrial membranes are present in anaerobic yeast and that cardiolipin is a component of such mitochondrial membranes. A similar situation apparently is encountered in the cytoplasmic mitochondrial mutants.

(c) Cytoplasmic mutants, both suppressive and neutral "petite," have relatively poorly developed mitochondrial profiles² and virtually no respiratory capacity. These mutants also contain less cardiolipin than the corresponding wild type cells (Tables 5 and 6).

(d) Chromosomal mutation affecting mitochondrial function is usually associated with relatively normal mitochondrial morphology when grown under conditions of maximal mitochondrial development² (i.e., stationary or chemostat growth) despite the absence of respiratory function. The cardiolipin content of such a mutant grown under nonrepressing conditions, was essentially normal (Table 6B).

(e) Lactate does not repress the development of mitochondrial membranes or of respiratory capacity at all and galactose does so less readily than glucose.^{30,32} Log phase yeast foam

"grande" ($YF\rho^+$) and cytoplasmic "petite" ($YFA\rho^-$) grown on lactate and/or galactose had a cardiolipin content at least as high as that found in late stationary yeast grown on glucose, i.e., in derepressed yeast. In the case of the diploid cytoplasmic petite ($YFA\rho^-$) yeast grown to log phase in galactose, the cardiolipin content was twice that found in similar cells grown to late stationary phase in glucose, presumably because the latter yeast, being unable to utilize the products of glycolysis for anabolic energy requirements, may enter a stage of cytoplasmic turnover earlier than wild type yeast.

Cardiolipin was present in all respiratory mutants studied. The relatively high concentration of cardiolipin in the diploid cytoplasmic petite yeast ($YFA\rho^-$) grown to log phase in galactose (Table 5) particularly suggests that there is no major defect in cardiolipin synthesis in this mutant. It is of interest that the chromosomal "petite," which as far as can be ascertained has qualitatively normal mitochondrial DNA,¹⁴ had a normal cardiolipin content when grown in the chemostat under maximally derepressed conditions. Thus, the genetic defect in this case probably does not diminish the mass of inner mitochondrial membrane under appropriate conditions.

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ISOLATION OF *Saccharomyces cerevisiae* MITOCHONDRIAL FORMYL
THFA[†]-Met tRNA TRANSFORMYLASE AND THE HYBRIDIZATION
OF MITOCHONDRIAL N-FORMYLMETHIONYL tRNA
WITH MITOCHONDRIAL DNA*

By

A. Halbreich and M. Rabinowitz

N-formylmethionyl tRNA has been detected in yeast and rat liver mitochondria¹ but not in the soluble cell fractions. Takeishi et al could demonstrate no formyl THFA-methionyl tRNA transformylase activity in yeast extracts, but were able to separate a methionyl tRNA species that could be formylated by an *E. coli* enzyme preparation.^{2,3} This tRNA species accounted for a large portion of the methionine acceptor activity of the whole cell tRNA and therefore was unlikely to be of mitochondrial origin. It has recently been identified, in the non-formylated form, as the initiator of extramitochondrial protein synthesis.⁴

Recently our laboratory has identified several yeast mitochondrial RNA species as gene products of mitochondrial DNA^{5,6,7} as part of a study of the roles of the mitochondrial and nuclear genomes in mitochondrial biogenesis.^{8,9} The known participation of N-formylmethionyl tRNA in initiating protein synthesis in bacteria combined with the similarity in the characteristics of the mitochondrial and the bacterial protein synthetic systems, prompted the examination of mitochondrial DNA as the source of the genetic information specifying the synthesis of this tRNA species.

We describe in these experiments the isolation of a fraction that contains transformylase activity from yeast mitochondria and evidence that the mitochondrial tRNA^{fMet} is a gene product of the mitochondrial DNA.

METHODS

A respiratory sufficient haploid yeast strain D243-2B-R1 (a , lys^- , ad_1^- , ρ^+) obtained from Prof. P. P. Slonimski was used in this study. Yeast was grown in a medium containing 1 per cent yeast extract, 2 per cent bactopectone, and 2 per cent galactose, and collected in the late exponential phase of growth. Mitochondria were isolated by digestion of the cell wall with glucanase (Endo Laboratories), homogenization and centrifugal separation.⁸ Mitochondrial DNA was isolated from DNase-treated mitochondria by lysis in 0.01 M Tris HCl, pH 7.5, containing 0.01 M EDTA and 1 per cent sodium dodecylsulfate, deproteinization by chloroform:n-octanol (9:1), and passage through a hydroxylapatite column.⁷ The DNA was tested for purity by isopycnic CsCl ultracentrifugation in the analytical ultracentrifuge.¹⁰

Yeast nuclear and *E. coli* K 12 DNA's were isolated by a similar method except that the

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† Abbreviations: THFA, tetrahydrofolic acid; β ME, β -mercaptoethanol; SSC, standard saline citrate (0.15 M NaCl, 0.015 M Na-citrate).

yeast nuclear DNA was further purified by hydroxylapatite chromatography according to Bernardi et al.¹¹ tRNA was isolated from washed mitochondria essentially according to Avital and Elson¹² by lysis with 0.01 M Tris HCl, pH 7.5, 2 per cent sodium dodecylsulfate and 0.1 per cent macaloid (National Lead Co.), deproteinization of the lysate with a phenol-cresol mixture,¹³ mild alkali deacylation and extraction with 2 M LiCl. The postmitochondrial supernatant was centrifuged for 2 hrs at 150,000 x g (Spinco ultracentrifuge, Model L2-65, rotor 50 Ti) and the ensuing supernatant was employed in the preparation of supernatant tRNA essentially by the procedure outlined above.

A mitochondrial fraction containing amino acyl tRNA synthetases and transformylase activity was prepared as follows: mitochondria from 100 g yeast cells were suspended in 20 ml 1 M KCl containing 0.02 M Tris-HCl, pH 7.5, 0.01 M β -mercaptoethanol (β ME) and 1 mM EDTA. The suspension was sonicated with a Branson sonicator in the cold for 10 sec at 3 mA, centrifuged first for 10 min at 48,000 x g (Sorvall RC2B centrifuge, rotor SS34) and then for 2 hrs at 150,000 x g. The final supernatant was dialyzed against 50 volumes of 0.01 M Tris HCl, pH 7.5, containing 0.02 M β ME, 0.01 M $MgCl_2$ and 10 per cent glycerol, applied to a DEAE cellulose column (3 x 11 cm)¹⁴ and eluted with a 0.25 M $K-PO_4$ buffer, pH 6.5, containing 0.02 M β ME, 0.01 M $MgCl_2$ and 10 per cent glycerol. A fraction, exhibiting both amino acid activation and transformylase activity, was eluted immediately after the void volume. An *E. coli* enzyme preparation was kindly furnished by Dr. P. Sigler.

Formation of [³H] methionyl (Met) and N-formylmethionyl (fMet) tRNA was tested according to Marcker and Sanger¹⁵ in a reaction mixture consisting of imidazole, pH 7.5, 0.06 M; $MgCl_2$, 0.01 M; KCl, 0.01 M; β ME, 0.05 M; ATP, 2 mM; [methyl ³H] L-methionine 3.3 c/mmole, 2.4 μ m (Schwarz BioResearch); 0.12 mM Ca-formyltetrahydrofolate (Leucovorin by Lederle); 15-50 μ g tRNA; and 20 μ l enzyme preparation in a final volume of 0.1 ml. The reaction proceeded for 10 min at 33°C, and was stopped by addition of 0.1 volume of 20 per cent K-acetate, pH 5.2, followed by 100 μ g *E. coli* tRNA, and a 20 times dilution with 2 per cent K-acetate, pH 5.2. The acylated tRNA was deproteinized with H₂O-saturated phenol and separated from free methionine by repeated alcohol precipitations. Finally Met and fMet adenosine were obtained by pancreatic RNase treatment of acylated tRNA and separated by high voltage electrophoresis in 0.5 M acetic acid, pH 3.5, containing 0.3 per cent pyridine (75 min at 3,000 volts).¹⁵ Two cm-wide strips of the paper were cut into 1 cm pieces, immersed in 0.2 ml H₂O in counting vials, 15 ml Bray's solution were added and the samples were counted in a Packard liquid scintillation spectrometer.

Tritiated mitochondrial fMet tRNA was prepared in the above mentioned reaction mixture in a final volume of 2.5 ml. Met tRNA was enzymatically deacylated by a second 10 minute incubation at 33°C in the presence of 30 mM $MgCl_2$, 15 mM AMP and 5 mM Na-pyrophosphate, pH 7.0.¹⁶ The reaction was stopped by addition of 0.1 volume 20 per cent K-acetate, pH 5.2, the mixture was diluted with an equal volume of 2 per cent K-acetate, pH 5.2, and extracted with H₂O saturated phenol. The aqueous phase was repeatedly extracted with ether to remove the phenol and N₂ was bubbled through it to remove the ether. RNA was precipitated by adding 2.5 volumes ethanol, redissolved in 50 mM NH₄-acetate, pH 5.2, and passed through a 3 x 50 cm Sephadex G-100 column with 50 mM NH₄-acetate, pH 5.2. The radioactivity and absorbance at 260 m μ of the eluate were followed, and the acylated tRNA was collected by alcohol precipitation from the fractions exhibiting the highest specific radioactivity. [³H] fMet tRNA represented more than 85 per cent of the labeled aminoacyl tRNA in these preparations as judged by electrophoresis

of RNase digests. Supernatant [^3H] fMet tRNA was prepared in the same manner except that an E. coli enzyme was employed for acylation and formylation.

DNA was immobilized on nitrocellulose filters according to Gillespie and Spiegelman¹⁷ following alkali denaturation, neutralization, and addition of 10 x SSC to a final concentration of 6 x SSC. DNA and blank (no DNA) filters were hybridized with [^3H] fMet tRNA according to Weiss et al¹⁸ in 0.3 ml of 33 per cent formamide containing 2 x SSC, 25 mM NH_4 acetate final pH 5.2, and varying amounts of [^3H] fMet tRNA. Following a 4 hr incubation at 33°C, the filters were removed, swirled for 15 min in 1 liter of 2 x SSC, pH 5.2, and washed on each side with 60 ml of this solution. Finally, the filters were dried and counted in a scintillation fluid consisting of 0.1 per cent PPO (2,5-diphenyloxazole) and 0.01 per cent Me_2POPOP (1,4-bis-2[4-methyl-5-phenyloxazolyl]benzene) in toluene.

fMet tRNA was eluted from filters containing the hybrid by three 20 minute incubations at 33°C in 0.3 ml portions of 95 per cent formamide in 50 mM ammonium-acetate pH 5.2. The filters were washed with 0.3 ml H_2O , the supernatants were combined, 100 μg E. coli tRNA was added and the mixture was passed through a 1 x 20 cm Sephadex G-25 column and eluted with 50 mM NH_4 -acetate, pH 5.2. The tRNA-containing fractions appeared in the void volume, were pooled, and evaporated to dryness. The residue was dissolved in H_2O , treated with pancreatic RNase, and electrophoresed as described above.

RESULTS

[^3H] fMet tRNA may be detected by high voltage electrophoresis of the RNase-treated reaction product.¹⁵ Formyl THFA-Met tRNA transformylase activity was thus demonstrated in a yeast mitochondrial fraction obtained by a 1 M KCl extraction of the mitochondria (Figure 1A). This enzyme preparation also exhibited Met tRNA synthetase activity. The presence of other synthetases was not tested. Transformylase was not released into the medium by a low-ionic strength extraction. No transformylase activity was detected in preparations of acylating enzymes obtained from the postmitochondrial supernatant.

Yeast cytoplasmic Met tRNA may be separated into two species,^{2,3} one of which may be formylated by E. coli enzyme. This is confirmed in Figure 1D. It may be seen in Figure 1C that although this tRNA is readily acylated by the mitochondrial enzyme, formylation is poor with the same enzyme preparation. The mitochondrial enzyme also formylates E. coli tRNA rather inefficiently (Figure 1E). Conversely, the E. coli enzyme exhibited an extremely low formylase activity towards mitochondrial tRNA (Figure 1B). The slight activity seen possibly represents contamination of the mitochondrial tRNA preparation by cytoplasmic tRNA.

Mitochondrial [^3H] fMet tRNA was prepared for hybridization experiments essentially free of labeled Met tRNA by an enzymatic deacylation of the latter in the presence of AMP and PPi ¹⁶ and Sephadex G-100 chromatography. Approximately 90 per cent of the radioactivity in the RNase digest of these preparations appeared as fMet adenosine upon electrophoretic analysis (Figure 3B). Mitochondrial fMet tRNA hybridized with mitochondrial DNA. Saturation was obtained by hybridizing 5 μg tRNA with 20 μg DNA (Figure 2). Unlabeled mitochondrial, but not supernatant, tRNA competed in this reaction (Table 1). The specificity of the reaction was further attested to by the fact that no detectable mitochondrial fMet tRNA was bound by either yeast nuclear or E. coli DNA. The results of a representative experiment are shown in Table 2. It should be pointed out, however, that the size of yeast nuclear and E. coli DNA, relative to that

Figure 1. Identification of products of the reactions between yeast mitochondrial and *E. coli* transformylases and mitochondrial, cytoplasmic and *E. coli* tRNA preparations. Samples were prepared as described in Methods and electrophoresed for 2 hrs at 1,500 V. The following reactions were tested: (a) mitochondrial enzyme with mitochondrial tRNA; (b) *E. coli* enzyme with mitochondrial tRNA; (c) mitochondrial enzyme with supernatant tRNA; (d) *E. coli* enzyme with supernatant tRNA; (e) mitochondrial enzyme with *E. coli* tRNA. The peaks were identified, using as markers [^3H] methionine and [^3H] labeled *E. coli* fMet and Met tRNA, as: M-methionine, FMA fMet-adenosine, MA-Met adenosine, M₂A-an isomeric form of Met adenosine.¹⁵

of the yeast mitochondrial DNA, may make it difficult to detect structural homology with tRNA at the specific radioactivity employed. Yeast supernatant [^3H] fMet tRNA prepared with the *E. coli* enzyme and [^3H] fMet tRNA (*E. coli*) did not hybridize with mitochondrial DNA (Table 3).

tRNA was eluted from the Met tRNA - Mit DNA hybrid with 95 per cent formamide, treated with pancreatic RNase and examined by high-voltage electrophoresis. It may be seen in Figure 3A that most of the radioactivity was recovered as fMet adenosine, proving that mitochondrial fMet tRNA had actually hybridized with the mitochondrial DNA.

Finally, it should be mentioned that the annealing mixture may be reused with fresh DNA filters and demonstrates an equal efficiency for hybrid formation. In fact this use results in lower blank values, which indicates the non-specific nature of the blank radioactivity associated with the blank filters. Similarly, inclusion of several DNA filters in the reaction mixture results in equal binding of tRNA to each of the filters with a consequent increase in the fraction of the

Figure 2. Saturation of mitochondrial DNA with mitochondrial [^3H] fMet tRNA. Varying amounts of [^3H] fMet tRNA, 390 cpm/ μg RNA, were hybridized with 20 μg DNA as described in Methods. The blanks consisted of filters without DNA that were incubated concurrently in the reaction mixture.

Table 1
COMPETITION BY MITOCHONDRIAL tRNA IN THE HYBRIDIZATION OF
MITOCHONDRIAL N-FORMYLMETHIONYL tRNA WITH
MITOCHONDRIAL DNA

Additions	Radioactivity bound to filters, cpm	
	Blank filter	DNA filter
None	17	71
6.75 μg Mitochondrial tRNA	14	37
115 μg Supernatant tRNA	20	84

20 μg of mitochondrial DNA filters were hybridized with 5 μg [^3H] N-formylmethionyl tRNA 390 cpm/ μg as described in Methods.

input tRNA utilized. These features should be expected in view of the very large excess of RNA required in this reaction.

DISCUSSION

A formyltetrahydrofolic acid-Met tRNA transformylase activity has been found in extracts of yeast mitochondria. No such activity was present in yeast post mitochondrial supernatant. The enzyme was most active with mitochondrial Met-tRNA but showed a low-level activity with yeast supernatant, and *E. coli* tRNA's. These findings explain observations of Smith and Marcker¹ that fMet tRNA is localized exclusively in the mitochondria in yeast and rat liver. The yeast supernatant fraction is known to contain two species of Met-tRNA, one of which may be formylated by the *E. coli* transformylase. This tRNA, in its non-formylated form, has recently been shown to be an initiator of extramitochondrial protein synthesis in mouse liver⁴ and rabbit re-

Table 2

HYBRIDIZATION OF MITOCHONDRIAL N-FORMYLMETHIONYL
tRNA WITH YEAST MITOCHONDRIAL AND NUCLEAR
DNA, AND WITH *E. coli* DNA

Type of DNA	μg DNA	Radioactivity bound to filters cpm
None	-	23
Yeast mitochondrial	10	58
Yeast mitochondrial	20	87
Yeast nuclear	100	20
<i>E. coli</i>	20	26

The annealing mixture contained 10 μg [^3H] N-formylmethionyl tRNA 665 cpm/ μg . Results are representative of six similar experiments.

Table 3

HYBRIDIZATION OF YEAST CYTOPLASMIC AND *E. coli* fMet tRNA
WITH YEAST MITOCHONDRIAL DNA

Source of [^3H] fMet tRNA	Radioactivity bound to filters, cpm	
	Blank filter	DNA filter
<i>E. coli</i>	21	17
Yeast cytoplasm	22	26
Yeast mitochondria	17	128

13.5 μg *E. coli* fMet tRNA 2870 cpm/ μg , 5.5 μg yeast cytoplasmic fMet tRNA 160 cpm/ μg , and 5 μg mitochondrial fMet tRNA 390 cpm/ μg were each incubated with 50 μg mitochondrial DNA filters and blank filters as described in Methods. An *E. coli* enzyme was employed in the preparation of *E. coli* and yeast cytoplasmic fMet tRNA. When 13.5 μg to 101 μg *E. coli* fMet tRNA 2870 cpm/ μg were annealed with 100 μg *E. coli* DNA, 16-85 cpm were bound to the filters.

ticulocytes¹⁹ as well as in yeast.^{3,4} It is likely that yeast mitochondrial fMet tRNA serves in the same capacity for mitochondrial protein synthesis.

We have also shown that the mitochondrial fMet tRNA hybridizes with and is probably specified by mitochondrial DNA. tRNA acylated in vitro with labeled amino acid was used in the hybridization studies, which were carried out in formamide, at low temperature and at acid pH to minimize deacylation. This annealing system, which is based on the procedure devised by Weiss et al,¹⁸ allows the specific detection of tRNA hybrids without interference from other RNA species. Mild alkaline hydrolysis of the aminoacyl tRNA completely removes the radioactivity bound to the DNA filters, showing that this binding is dependent on the ester bond between the amino acid and the tRNA molecule. The specificity is confirmed by electrophoretic identification of [^3H] fMet-adenosine in RNase digests of the tRNA hybridized to mitochondrial DNA. The fact that the tRNA, eluted from the hybrid, appears in the void volume of the Sephadex G-25 filtrate indicates that a high molecular weight tRNA was hybridized to mitochondrial

Figure 3. Analysis of tRNA eluted from the hybrid. tRNA was eluted from three 50 μ g mitochondrial DNA filters to which was bound a total of 396 cts/min of [3 H] fMet tRNA as described in Methods. A blank (no DNA) filter bound 23 cts/min. Approximately 280 cts/min were recovered from the Sephadex column and 225 cts/min were applied to the paper. The peaks obtained by paper electrophoresis were identified as in Figure 1. Counting efficiency of the paper strips was 40% of that of a liquid sample. Approximately 75% of the radioactivity, originally on the filters, could be accounted for in this experiment when values are corrected for reduced counting efficiency. The following samples were examined: (a) [3 H] fMet tRNA eluted from the hybrid with mitochondrial DNA; (b) mitochondrial [3 H] fMet tRNA used for hybridization; (c) [3 H] methionine; (d) a mixture of *E. coli* fMet and Met tRNA and free [3 H] methionine as marker.

DNA. Competition of the hybridization by unlabeled mitochondrial tRNA but not by unlabeled extramitochondrial tRNA is further evidence for the specificity of the results. The extramitochondrial tRNA^{Met} that is formylated by *E. coli* transformylase, and initiates cytoplasmic protein synthesis, does not hybridize to mitochondrial DNA. *E. coli* tRNA^{fMet} also does not hybridize to mitochondrial DNA.

It should be theoretically possible to estimate the number of cistrons for fMet tRNA present in the mitochondrial genome from the saturation-hybridization data, assuming the specific activity of the tRNA to be that of the [3 H] methionine used. Such calculations, however, are rendered invalid by uncertainty regarding degree of acylation of the tRNA, and by the short periods of incubation used for hybridization, which may prevent the reaction from going to completion.

Weiss et al¹⁸ had previously noted that there was a considerably lower saturation level when the tRNA was labeled in the amino-acid, than when the nucleotides were labeled with [³⁵S].

Nass and Buck have shown²⁰ the hybridization of several mitochondrial tRNA species including tyrosyl, seryl, phenylalanyl and leucyl tRNA in rat liver. Leucyl tRNA was observed to hybridize only with the heavy strand of the mitochondrial DNA. Our laboratory now has shown that yeast mitochondrial DNA specifies the synthesis of several tRNA molecules including tRNA^{fMet} (this communication), tRNA^{Met} (Halbreich and Rabinowitz, unpublished), tRNA^{Leu5} and tRNA^{Val}.⁶ The two mitochondrial ribosomal RNA species also show sequence homology with mitochondrial DNA.^{7,21,22} Other mitochondrial tRNA's are being tested to determine whether mitochondrial DNA codes for all, or for only a few, of the mitochondrial tRNA's. It is of interest that several cytoplasmic petite yeast strains retain cistrons for valyl and leucyl tRNA.^{5,6} It is hoped that the hybridization of several mitochondrial tRNA species with mitochondrial DNA of a series of yeast petite strains, combined with information obtained from the genetic analysis of cytoplasmically controlled antibiotic resistance markers,²³ will offer an opportunity for preliminary mapping of the mitochondrial genome.

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A PROTEIN COMPONENT OF SERUM HIGH DENSITY LIPOPROTEIN WITH CO-FACTOR ACTIVITY AGAINST PURIFIED LIPOPROTEIN LIPASE*

By

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Lipoprotein lipase (LPL) (EC 3.1.1.3), either extracted from tissues or highly purified from post-heparin plasma, has been shown to hydrolyze artificial triglyceride emulsions only in the presence of a plasma lipoprotein co-factor.¹⁻³ The high density lipoprotein class contains co-factor activity and this appears related to its protein moiety.⁴ Since this can now be fractionated into several proteins^{5,6} it was of interest to determine whether all or only some of the fractions are involved in lipoprotein lipase activity. This problem was examined with lipid-free and lipid-rich products, the latter obtained by a sonication procedure recently developed in this laboratory.⁷

MATERIALS AND METHODS

Preparation and fractionation of delipidated HDL₂.[§] HDL₂ was separated from whole human serum by ultracentrifugal flotation and was delipidated with 3:2 ethanol:ether at -10°C, as described previously.⁸ The dried product was re-dissolved in 0.05 M NH₄Cl-NH₄OH buffer, pH 8.3, for immediate use. When sub-fractions of apo HDL₂ were required, the powder was dissolved in 0.05 M Tris-HCl buffer, pH 8.3, containing 8 M urea, and fractionated through Sephadex G200 columns pre-equilibrated with the same buffer.⁶ The fractions obtained, previously defined as I, III, IV and V (Fraction II was inconstantly seen) were freed from urea by extensive dialysis into the ammonia buffer mentioned above, and their purity was checked by acrylamide gel electrophoresis in 8 M urea.

Relipidation of apo HDL₂ and sub-fractions by sonic irradiation. The method of re-lipidation has been previously described.⁷ After sonic irradiation, the mixtures of apo HDL₂ (and sub-fractions) and HDL₂ lipids were separated by preparative ultracentrifugation into three products of $d < 1.063$, $d 1.063-1.21$ and $d > 1.21$, and then dialyzed against the ammonia buffer before use. A similar fractionation procedure was carried out on HDL₂ preparations that had been sonicated under conditions identical to those followed in the re-lipidation experiments.

Preparation and assay of lipoprotein lipase. LPL was purified from human post-heparin plasma by centrifugation of the enzyme-substrate complex followed by detergent treatment and calcium phosphate gel fractionation to remove residual lipoproteins.^{2,3} The enzyme product was without significant activity against a triolein emulsion in the absence of added lipoproteins. LPL

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[§] Abbreviations: HDL - high density lipoprotein $d 1.063-1.21$; HDL₂ - subclass of HDL of $d 1.063-1.125$; apo HDL and apo HDL₂ - delipidated protein moieties of HDL and HDL₂; VLDL - very low density lipoprotein of $d < 1.006$; LPL - lipoprotein lipase.

was assayed using the medium previously described⁹ except that a radioactive triglyceride emulsion was used as substrate. Glycerol tri-1(¹⁴C)-oleate, 33.5 mC/mmol (Radiochemical Centre, Amersham) was diluted with unlabeled triolein (Applied Science Laboratories) in ether to a specific activity of 0.01 mC/mmol, and the triolein was mixed with lecithin (Nutritional Biochemicals, Cleveland) in the proportion 100/6, w/w. After removal of the organic solvent under nitrogen, the lipid was dispersed in 0.145 M NaCl then sonicated in a Branson sonifier (Heat Systems Company, Melville, N. Y.) for three one-minute periods at 75 watts, using the standard microtip. Further periods of sonication up to 10 minutes, or at increased power up to 100 watts, did not increase the rate of enzymatic hydrolysis in the subsequent assay. To this substrate, co-factors in the form of lipoproteins or derivatives were added as described under Results. The unesterified radioactive fatty acid released into the assay medium was measured according to Schotz et al.¹⁰ Sufficient enzyme was added to each ml of assay medium to produce release of 1 μ mol fatty acid/hour at 37°C when whole HDL₂ (0.05 mg protein/ml) was the only lipoprotein added.

RESULTS

As shown in Figure 1, apo HDL₂ permitted a significantly lower maximal rate of enzymatic hydrolysis as compared with the starting product HDL₂. In addition, the activity of apo HDL₂, upon reaching a maximum, decreased as its concentration was raised above 0.01 mg/ml.

To determine whether each of the protein sub-fractions of apo HDL₂ as separated by gel

Figure 1. Effects of HDL₂ and apo HDL₂ upon co-factor activity of LPL. Each assay contained the following: 0.2 ml of sonicated ¹⁴C-triglyceride emulsion (10 mg/ml); 0.4 ml of 15% bovine serum albumin in Krebs-Ringer solution, pH 8.3; 0.2 ml of lipoprotein solution (HDL₂ or apo HDL₂) in ammonia buffer; 0.025 ml of enzyme solution in ammonia buffer containing 0.5 mM potassium linolenate (see ref. 2); 0.175 ml of 0.145 M sodium chloride solution. Incubation was for 2 hours at 37°C.

chromatography had co-factor activity, each was added to the assay system at final concentrations of from 0.001 to 0.20 mg/ml (Figure 2). A strong activity was found only with Fraction V. Such activity was essentially absent from Fractions III and IV. Fraction I, which consists of a mixture of all the apo HDL₂ fractions,⁶ had an activity of 5-10 per cent of that of Fraction V at the same concentration.

Figure 2. Cofactor activity of fractions of apo HDL₂. Sephadex fractions in ammonia buffer replaced apo HDL₂ in the assay medium as in Figure 1.

In view of these results, co-factor activity of sonicated HDL₂ and relipidated apo HDL₂ was investigated. It has been previously shown that after such procedures a moiety rich in Fraction V can be separated by ultracentrifugation.⁷ Apo-HDL₂ was sonicated with an aqueous suspension of the lipid which had been previously removed from it by extraction with organic solvents. The product was separated by ultracentrifugation into fractions of $d < 1.063$, $d 1.063-1.21$ and $d > 1.21$ (Figure 3A). The principal activity was obtained in the $d < 1.063$ cut which contained mainly Sephadex peak V.⁶ Some activity was found between 1.063-1.21, whereas the material sedimenting at $d > 1.21$ and containing principally Fraction III was totally inactive. Experiments were also carried out with HDL₂ that had been sonicated and fractionated by ultracentrifugation as in Figure 3A. Very similar results were obtained (Figure 3B).

DISCUSSION

The present results using a purified preparation of LPL from post-heparin plasma confirm previous observations^{3,4} that serum HDL* has co-factor activity for this enzyme. They also show that in the system employed, this property is greatly reduced if HDL₂ is replaced by its lipid-free derivative, apo HDL₂ (Figure 1). The most important aspect of our findings was,

* Such a generalization is justified by the fact that we have found that both sub-classes of HDL, HDL₂ and HDL₃ ($d 1.125-1.21$) exhibit co-factor activity.

Figure 3. Co-factor activity of (A) sonically irradiated HDL₂ and (B) sonicated mixtures of apo HDL₂ and extracted HDL₂ lipid. Experimental details are described in the text. Assay conditions as in Figure 1.

however, the observation that essentially all co-factor activity of apo HDL₂ was contained in its Sephadex Fraction V, representing only 5 per cent of this protein (Figure 2) and containing at least three polypeptide chains of molecular weight about 10,000.⁶ The co-factor activity of this fraction was also exhibited by its lipid-enriched forms (Figures 3A and 3B). We should like to emphasize that the observed similarity in the activity of Fraction V under these different conditions does not necessarily imply that lipid is not required for LPL activation, since phospholipid was a component of our substrate emulsion. Further information on this point may assist in interpreting the differences in the activity curves of HDL₂ and apo HDL₂ observed but not explained by this study. There is evidence that at least some of the polypeptide chains found in apo HDL₂ may be present also in the protein moiety of VLDL.¹¹ It may be relevant that the concentration curve for activation of LPL by VLDL is of the same form as that observed for apo HDL.¹²

The elucidation of the importance of such polypeptides in the LPL-mediated degradation of lipoprotein triglyceride will require a better understanding of the structure-function relationships of all protein components of apo-VLDL and apo HDL. Studies in this direction are currently being pursued in this laboratory.

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ISOELECTRIC FRACTIONATION AND CHARACTERIZATION OF POLYPEPTIDES FROM HUMAN SERUM VERY LOW DENSITY LIPOPROTEINS*

By

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A newly developed method of isoelectric focusing in a narrow pH gradient has been applied to the separation of Tris-soluble very low density lipoprotein (VLDL) apoproteins. The technique offers the advantages of high resolving power, excellent reproducibility and direct determination of isoelectric points. While this work was in progress, two papers appeared in which the VLD apolipoproteins were isolated by Sephadex and DEAE chromatography. Three major VLD apoproteins were described in these investigations.^{1,2} The present investigation describes two polypeptides obtained from Tris-soluble VLD apoprotein. One of these, apo LP-ala, designated from the COOH-terminal residue, was found to exist in three different forms. These forms contained 2 moles, 1 mole, and no moles of sialic acid per mole of protein respectively. The second polypeptide appears identical to a polypeptide recently described.² The amino acid composition of this polypeptide is presented for the first time. The relative concentrations of these polypeptides obtained from the healthy fasting individual are also presented.

MATERIALS AND METHODS

Preparation of VLD apolipoprotein. VLDL was isolated from the sera of healthy male Caucasian donors who had fasted overnight. In general, VLDL was isolated from a pool of two donors, although two experiments were performed with VLDL isolated from single donors. The sera were centrifuged at 10,000 rev/min for 30 min to remove the chylomicrons. Subsequently, the chylomicron-free sera containing 0.05 per cent EDTA were centrifuged in the L2-65B ultracentrifuge at 30,000 rev/min for 24 hours. The top VLDL fraction was removed and resuspended in 0.85 per cent NaCl, 0.05 per cent EDTA solution (pH 7.4). The purified VLDL was delipidated by extraction with ethanol-diethyl ether, 3:1, for 16 hours at -10°. The solvent was decanted and the protein was re-extracted with ethanol-diethyl ether, 3:2, for 8 hrs. The insoluble protein residue was washed three times with ether and dried under nitrogen. The VLD-apoprotein was partially solubilized in 0.1 M Tris-HCl (pH 8.8). The insoluble residue was removed by centrifugation at 4,000 rev/min for 15 min. The soluble protein remaining (approximately 45 per cent of the total protein) will be referred to as Tris-soluble VLD apoprotein. The soluble apoprotein was dialyzed against 6 M urea (Mann) overnight with three changes of dialysate using cellulose casing (Union Carbide). No losses of apoprotein were obtained by this procedure.

Isoelectric focusing. The proteins were electrofocused by the method of Vesterberg and Svensson,³ using carrier ampholytes (Ampholine, LKB-Produkter AB, Stockholm). Solutions

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and density gradient were prepared as described by LKB Instruments.⁴ A 110 ml capacity electrofocusing column (No. 8101) and gradient mixer (No. 8121) were used in these experiments (LKB Instruments). The column was coated with silicone prior to use. Initially, an electrofocusing run was performed without a sample but containing 6 per cent ampholine (pH range 4-6) in the presence of 6 M urea (Mann). The ampholine was electrofocused for 60 hr at 15° with a potential of 900-1,000 volts. After the run, approximately 55 two ml portions were collected and fractions that contained ampholine in the pH range 4.35-4.85 were pooled. (This pH range, 4.35-4.85, was chosen because preliminary experiments with electrofocusing of tris-soluble VLD-apoprotein indicated that approximately 90 per cent of the protein was found within this pH range.) This prefocused 6 per cent ampholine solution (pH range 4.35-4.85) was subsequently used for the electrofocusing run with the sample. All solutions again contained 6 M urea. From 3-10 mg of Tris-soluble VLD apoprotein was electrofocused for 64-66 hrs at 15°. The potential was raised gradually over a 16 hr period to a final potential of 900 V. After each run, approximately ninety 1.2 ml portions were collected and the pH and optical density (280 m μ) were determined. All pH measurements were made at 25° with a Radiometer pH meter (Copenhagen, PHM 26) and Radiometer electrode (GK 2322C). Prior to measuring the pH, each tube was diluted 1:1 with distilled water to give a final concentration of urea of 3 M. The desired portions were pooled and passed through a G-50 column equilibrated with 4M urea, 0.5M NaCl, 0.1M Tris-HCl (pH 8.6). The protein that appeared in the void volume was subsequently used for chemical characterization. All solutions were concentrated using the Diaflo ultrafiltration cell model 52 and UM-2 membrane (Amico Corp.).

Amino acid analysis. Protein samples (0.3-1.5 mg) were hydrolyzed in constant boiling 6N HCl for 24 hours at 110°. Seventy-two hr hydrolysis was performed whenever sufficient material was available. Quantitative amino acid analysis was determined with a Beckman 120C automatic analyzer equipped with a scale expander and digital integrator according to the procedure of Spackman, Stein and Moore.⁵ Cystine was independently determined as cysteic acid in performic acid oxidized samples.⁶ Tryptophan was determined according to Goodwin and Morton.⁷ Corrections were made for the loss of serine and threonine during hydrolysis by extrapolating data to zero hydrolysis time and assuming first order kinetics for decomposition.

Terminal amino acid analysis. NH₂-terminal residues were identified by the dansyl chloride procedure.⁸ The dansyl derivatives were separated on polyamide sheets (Cheng Chin Trading Co., Ltd.) according to Woods and Wang.⁹

COOH-terminal residues were determined using carboxy peptidase A (COA DFP, Worthington).¹⁰

Sialic acid determination. Sialic acid was determined by the thiobarbituric acid assay of Warren¹¹ after its release from the protein by hydrolysis in 0.1N sulfuric acid at 80° or after release by neuraminadase. Sialic acid was removed enzymatically by incubation of the apoprotein in 0.1 M sodium acetate buffer, pH 5.5, containing 0.005M CaCl₂ at 37° with neuraminadase (25 units/mg protein) from *Vibrio Cholera* (Calbiochem) for 24 hrs.

Other methods. Molecular weight determinations were made by polyacrylamide gel electrophoresis in the presence of SDS as described by Shapiro, Vinuela and Maizell.¹²

Standard polyacrylamide gel electrophoresis was performed essentially according to Davies¹³ with some modification to allow for gel polymerization in the presence of 8M urea.

Protein was measured by the method of Lowry et al¹⁴ or by summing the quantities of the amino acids obtained from the amino acid analyzer and obtained spectroscopically as in the case of tryptophan.

RESULTS

Isoelectric focusing. A typical isoelectric focusing profile from one of five experiments of Tris-soluble LD apoprotein is shown in Figure 1. Four peaks (A, B, C, and D) were consistently obtained. These four fractions represented at least 90 per cent of the protein applied to the column. The remaining protein (less than 10 per cent) was present as a broad minor peak between fraction D and the basic lock (tubes 66-75). This fraction was not investigated in this study.

Figure 1. Isoelectric focusing profile of Tris-soluble VLD apoprotein. Solid line, optical density at 280 mμ; dotted line, pH gradient.

The isoelectric points and protein distribution of the isoelectric focusing fractions are shown in Table 1. The four protein peaks with pI's of 4.53, 4.70, 4.79 and 4.93 respectively have a protein distribution of 26, 50, 17 and 7 per cent respectively.

Polyacrylamide gel electrophoresis. Each fraction obtained from isoelectric focusing (A, B, C, and D) gave a single band on polyacrylamide electrophoresis (Figure 2). Fractions C and D had nearly identical electrophoretic mobilities. An equal mixture of Fractions A, B, and D which had previously been treated with neuraminadase for 24 hours at 37° gave a single band on polyacrylamide gel electrophoresis (not shown).

Amino acid analysis. The amino acid composition of fractions A, B, and D were indistinguishable within the experimental error (Table 2). A, B, and D contained no isoleucine, cysteine, or cystine. The amino acid composition of C differed from A, B, and D, and from all previously

Table 1

ISOELECTRIC POINTS AND PROTEIN DISTRIBUTION OF
VLD-APOLIPOPROTEIN FRACTIONS

Values represent the average of five determinations obtained from different preparations. The first column shows the standard error of the mean and the last two columns the ranges obtained from the five determinations.

Fraction	pI	% protein (A,B,C,D)*	% protein (A,B,D)†
A	4.53 ± .02	26 (19-32)	31 (23-38)
B	4.70 ± .02	50 (42-56)	61 (51-68)
C	4.79 ± .01	17 (12-22)	
D	4.93 ± .03	7 (4-10)	8 (5-11)

* Values represent % protein assuming that fractions A, B, C, and D equal 100%.

† Values represent % protein assuming that fractions A, B, and D equal 100%.

Figure 2. Polyacrylamide gel electrophoresis of isoelectric focusing fractions (A,B,C,D) performed in 8M urea (pH 8.9) and 7.5% acrylamide. Tris-soluble VLD apoprotein is shown for comparison (V).

reported fractions obtained from low density lipoprotein and high density lipoprotein. Fraction C contained no histidine, cysteine, or cystine.

Terminal amino acid analysis. A and B contained NH_2 -terminal serine. NH_2 -terminal threonine was identified in C. No NH_2 -terminal analysis was performed on D. Carboxypeptidase released 2 moles of alanine per mole of valine from fractions A, B, and D. No amino acid was released from fraction C by 24 hrs digestion with carboxypeptidase A at pH 8.5.

Table 2

AMINO ACID COMPOSITION OF THE ISOELECTRIC FOCUSING FRACTIONS

Values shown in the columns are the means and ranges of four analyses each of A and B, three analyses each of C and D. Values are expressed as moles of amino acid/100,000 g of protein.

Amino acid	A	B	C	D
Lysine	71 (69-74)	70 (68-72)	70 (67-73)	71 (68-75)
Histidine	11 (10-12)	11 (9-12)	0	10 (9-11)
Arginine	23 (19-25)	24 (21-27)	13 (12-13)	24 (20-26)
Aspartic acid	85 (82-88)	84 (81-89)	64 (62-67)	86 (80-90)
Threonine	58 (53-62)	58 (53-61)	101 (95-105)	58 (54-62)
Serine	117 (111-122)	119 (114-123)	99 (93-104)	119 (113-123)
Glutamic acid	120 (119-122)	119 (117-122)	164 (160-168)	118 (116-121)
Proline	26 (24-27)	26 (24-28)	42 (41-43)	26 (24-27)
Glycine	46 (43-49)	44 (42-48)	28 (26-29)	45 (42-47)
Alanine	118 (115-121)	117 (114-122)	75 (71-80)	117 (113-121)
Cysteine & cystine*	0	0	0	0
Valine	69 (63-72)	72 (65-74)	45 (43-46)	70 (68-72)
Methionine	19 (18-19)	19 (18-20)	21 (21-22)	19 (18-19)
Isoleucine	0	0	10 (10-11)	0
Leucine	58 (57-60)	60 (58-61)	92 (88-98)	59 (56-61)
Tyrosine	21 (19-22)	20 (17-22)	53 (50-57)	21 (19-21)
Phenylalanine	44 (42-46)	46 (43-48)	24 (22-26)	45 (42-47)
Tryptophan†	27	27	‡	28

* No cysteic acid was present following performic acid oxidation.

† Determined spectroscopically on a single preparation.

‡ Not determined.

Sialic acid determination. Fraction A yielded 2 moles of sialic acid per mole of protein (assuming a molecular weight of 10,000). Fraction B yielded 1 mole of sialic acid per mole of protein. Fractions C and D contained no sialic acid.

Molecular weight determination. The approximate molecular weight of the fractions, A, B, C, and D as determined by polyacrylamide gel electrophoresis in SDS was 10,000 for A, B, and D, and 9,000 for C.

DISCUSSION

Recent investigations of VLD apolipoprotein have shown that it contains three major proteins, apo LP-val, apo LP-glu, and apo LP-ala,^{1,2} designated by their respective COOH-terminal amino acids. These apoproteins were isolated by a combination of Sephadex and DEAE chromatography. Apo LP-ala was shown to exist in two forms, designated D₃ and D₄.^{1,2} Apo LP-glu (purified D₂) had NH₂-terminal threonine, 18 mole per cent glutamic acid, but no histidine, cysteine or cystine. The COOH-terminal of apo LP-glu was resistant to carboxypeptidase A. The amino acid analysis of apo LP-glu was not published in these studies.

In the present study the VLD-apolipoproteins have been separated by a newly developed technique of preparative isoelectric focusing in a narrow pH gradient. Fraction A appears to be essentially identical to fraction D₄ and fraction B essentially identical to fraction D₃ on the basis of amino acid composition, NH₂- and COOH-terminal analysis, and position on polyacrylamide gel electrophoresis. Apo LP-glu from purified D₂ appears to be identical to fraction C since it contains 18 mole per cent glutamic acid, no histidine, cysteine or cystine; it has NH₂-terminal threonine, COOH-terminal resistant to carboxypeptidase; and it has the same relative mobility on disc electrophoresis as fraction C.

Fraction D, which was identical in amino acid composition to fractions A and B and has COOH-terminal alanine but no sialic acid, was not obtained in the previous investigations. Three forms of apo LP-ala were present in all the preparations studied in the approximate ratio of 31:61:8 (Table 1). We shall designate these forms as apo LP-ala₂, apo LP-ala₁ and apo LP-ala₀, using the subscript to denote the moles of sialic acid per mole of protein. The possibility that apo LP-ala₀ was formed during the isoelectric focusing procedure by acid hydrolysis is unlikely since less than 0.5 per cent of the total protein would be expected to be in contact with the acid lock solution. Furthermore, the polyacrylamide electrophoretic pattern of Tris-soluble VLD apoprotein that had been previously incubated with an equal volume of the acid lock solution at 15° for 60 hrs appeared identical to untreated VLD apoprotein. The fact that neuraminidase treated apo LP-ala₂ and apo LP-ala₁ gave a single band in the position of apo LP-ala₀ supports the concept that the charge differences of the polymorphic forms of apo LP-ala are due to differences in the sialic acid residues. The possibility that other differences exist between the three forms of apo LP-ala, e.g., differences in the carbohydrate prosthetic group, must be considered.

Thus far, the protein distribution of the four polypeptides have been obtained from only five preparations of VLDL from healthy fasting donors; two preparations were obtained from individual donors. It may be noted, however, that for the Tris-soluble VLD-apolipoproteins analyzed, the relative concentrations of the four fractions were in the order B > A > C > D (Table 1). The similarity in distribution of these polypeptides among the five VLDL preparations indicates that further studies are needed to evaluate the factors that maintain or alter this distribution, e.g., the metabolic state of the donor, or the lipolytic activity of the donor's serum. Second, a comparison of the distribution of these polypeptides in the normal individual with the distribution that may exist in disease states, e.g., hyperlipoproteinemias, abetalipoproteinemia, and Tangier's disease, may give us an insight into the function and genetic control of these polypeptides. When the Tris-insoluble VLD apoprotein residue was dissolved in 8M urea only a small fraction (less than 10 per cent) of these four polypeptides was present. Thus, the percentage of these four polypeptides found in the Tris-soluble VLD-apolipoprotein preparation should reflect the distribution of these peptides in whole VLDL. The distribution of these polypeptides in individual VLDL molecules, however, remains open to investigation.

The small polypeptides of HDL have recently been shown to activate post-heparin lipoprotein lipase.¹⁵ Some of these polypeptides have similar if not identical electrophoretic mobilities to apo LP-ala₂ (A), apo LP-ala₁ (B) and apo LP-glu (C). Apo LP-ala has already been shown to be present in HDL.¹⁶ One or more of the polypeptides isolated in this study would therefore be expected to play a role in the activation of lipoprotein lipase. Indeed, preliminary evidence in this

laboratory indicates that fraction C, apo LP-glu, may be the principal polypeptide responsible for the activation of post-heparin lipoprotein lipase.

The method of isoelectric focusing in a narrow pH gradient as developed for Tris-soluble VLD-apolipoprotein offers some definite advantages over other fractionation procedures. The separation of fraction B from fraction C with a pI difference of only 0.09 pH units indicates a high resolving power. Again, excellent reproducibility and direct determination of isoelectric points are two additional features of the method. Since, however, the pH was taken in the presence of 3M urea, the isoelectric point obtained does not necessarily refer to the pI at zero ionic strength.

The isoelectric focusing method in narrow pH gradients should be useful for the separation of other apolipoproteins that have proved difficult to separate by the more conventional chromatographic methods.

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ISOLATION AND CHARACTERIZATION OF SUBUNIT POLYPEPTIDES FROM APOPROTEINS OF RAT SERUM LIPOPROTEIN*

By

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Recent reports on the isolation and partial characterization of the protein moieties of human serum HDL^{¶1-3} and VLDL^{4,5} indicated the probable existence of several distinct polypeptides in each apoprotein class, based on the heterogeneity of the band pattern by disc gel electrophoresis in 8 M urea. In the present study the various subunit polypeptides were separated on a preparative scale and characterized by physical, chemical, and immuno-chemical methods.

MATERIALS AND METHODS

Preparation of apoproteins. Lipoproteins were separated from the sera of Sprague-Dawley male rats (300-350 g of body weight) as previously reported.⁶ The animals were fasted for 16 hours before bleeding. Density criteria for each lipoprotein class were as follows: $d > 1.006$ for VLDL, $d 1.006-1.040$ for LDL and $d 1.063-1.21$ for HDL, unless otherwise specified. HDL was delipidated with ethanol-ether, 3:2, at -10°C ⁷ and dissolved in 0.01 M Tris-HCl buffer (pH 8.6) containing 8 M urea, 0.001 M EDTA, and 200 mg/l of sodium azide for further fractionation by Sephadex G-200. VLDL and LDL were incubated with 200 mM of SDS for 60 minutes at 40°C and then delipidated with ethanol-ether, 3:1. Those delipidated products were soluble in the buffer mentioned above. Removal of SDS during delipidation and following Sephadex gel filtration was measured using SDS-³⁵S (New England Nuclear Corp., Boston, Mass.).

Gel filtration studies. For most of the experiment Sephadex G-200 was packed in a 2.5 x 92 cm column that was equilibrated and eluted by ascending flow with 0.01 M Tris-HCl buffer (pH 8.6) containing 8 M urea, 0.001 M EDTA and 200 mg/l of sodium azide.¹ For apo-HDL studies, two columns of 2.5 x 92 cm were connected sequentially to extend their effective length to 184 cm. Molecular weight was estimated by measuring the elution volume of each component.⁸ Human- γ -globulin (MW, 160,000), bovine serum albumin (crystal, 67,000), ovalbumin (2X cryst., 45,000), chymotrypsinogen A (from beef pancreas, 6X cryst., 25,000), myoglobin (from sperm whale, cryst., 17,800) and cytochrome C (from horse heart, 12,400) were used as molecular weight standards, and were purchased as a kit from Mann Research Laboratories, Inc., New York, N. Y.

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[¶] Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo VLDL, apo LDL, apo HDL, apoprotein of VLDL, LDL and HDL, respectively; SDS, sodium dodecyl sulfate.

DEAE-cellulose chromatography. Microgranular type DEAE-cellulose (Whatman DE52, W and R Balston, Ltd., London, England) was degassed in 0.5 N HCl, washed twice with 0.5 N NaOH and then equilibrated in 0.01 M Tris-HCl buffer at pH 8.0. After packing, the column (0.9 x 30 cm) was equilibrated again with the starting buffer which was 0.015 M Tris-HCl (pH 8.0) containing 8 M urea and 0.001 M EDTA. Protein samples were extensively dialyzed against the starting buffer. Two sets of linear gradient of ionic strength were utilized for elution in most of the experiments. The first gradient was formed by 165 ml of 0.015 M Tris-HCl and 165 ml of 0.05 M Tris-HCl, the second gradient by 100 ml of 0.05 M Tris-HCl and 100 ml of 0.25 M Tris-HCl. All eluting buffer contained 8 M urea and 0.001 M EDTA, at pH 8.0. All chromatographic operations were performed in a controlled temperature room at 15°C.

Disc electrophoresis. Polyacrylamide gel of 7.5 per cent in 8 M urea was prepared either at pH 8.9⁹ or pH 4.5.¹⁰ The electrophoresis was conducted in a Canalco Model 1200 bath (Canalco, Rockville, Md.) using 0.5 x 9.0 cm tube with a constant current of 3 ma per tube at 4°C. The gels were stained with 1 per cent Amido Schwarz in 7 per cent acetic acid with continuous stirring and frequent changes of the solution.

Immunological studies. Antisera were prepared in rabbits as previously reported.⁶ Immunological analysis was carried out by the technique of double diffusion¹¹ and immunoelectrophoresis¹² using 1 per cent (w/v) agarose in 0.05 M veronal buffer at pH 8.6.

Other analyses. Amino acid composition was analyzed as previously reported.⁶ Lipids were extracted from a subfraction of HDL with chloroform-methanol, 2:1.¹³ Extracts were analyzed by thin-layer chromatography also as previously described.⁶ Total protein was determined by the method of Lowry, Rosebrough, Farr and Randall,¹⁴ with bovine serum albumin as a standard. No corrections were attempted.

Chemicals. Ultra pure urea (Mann Research Laboratories, Orangeburg, N. Y.) was used for DEAE-cellulose chromatography. For Sephadex gel filtration studies, reagent grade urea (J. T. Baker Chemical Co., Phillipsburg, N. J.) was filtered through a column of mixed bed resin (AG 50 1-X8[D], 20-50 mesh, Bio-Rad Laboratories, Richmond, Calif.) at the concentration of 8.9 M and then the buffer made up by adding the other components. SDS was crystallized from ethanol before use. All other chemicals were reagent grade.

RESULTS

Solubilization of apo VLDL and apo LDL. Apoproteins of VLDL and LDL obtained by the delipidation with SDS were completely and easily soluble in 0.01 M Tris-HCl buffer, pH 8.6, containing 8 M urea. Usually no vigorous shaking or agitation was required. Only one preparation of apo LDL obtained from lipoprotein density between 1.019 and 1.040 required additional small amount of SDS. Removal of SDS during the delipidation procedure was calculated as 97.7 per cent using SDS-S³⁵. Estimated final concentration of SDS in the apoprotein solution was in the range of 10-20 mM. This quantity was almost completely removed by gel filtration with Sephadex G-200. Almost all radioactivity was recovered in the fraction collected after elution of the protein peaks (after peak III in Figure 1). The SDS fraction showed also some UV absorption at 280 mμ and its elution volume was 430-440 ml from 2.5 x 92 cm column.

Fractionation of apoproteins by Sephadex G-200. Apo VLDL and apo LDL were fractionated into three components (Figures 1 and 2). Apo HDL yielded five fractions (Figure 3). Molecular weights of each component, estimated by the elution volume are shown in Table 1.

1

2

3

Figure 1. Gel filtration (Sephadex G-200 in 8 M urea) of apo VLDL. Column: 2.5 x 92 cm. For this and other figure legends, see text for more details.

Figure 2. Gel filtration (Sephadex G-200 in 8 M urea) of apo LDL. Column: 2.5 x 92 cm.

Figure 3. Gel filtration (Sephadex G-200 in 8 M urea) of apo HDL. Column: 2.5 x 18.4 cm.

Table 1

APPARENT MOLECULAR WEIGHTS OF APO HDL AND APO
VLDL SUBFRACTIONS AS ESTIMATED BY GEL
FILTRATION (SEPHADEX G-200 COLUMNS,
8 M UREA)

	Apo HDL (4)*		Apo VLDL (5)
PII	76,300 ± 3,700 [†]		
PIII	44,300 ± 2,200	PII	44,500 ± 900
PIV	36,600 ± 1,400		
PV	18,600 ± 600	PIII	18,300 ± 400

* Values in parentheses indicate the number of observations.

[†] Molecular weights are averages ± standard error of the mean.

The relative amounts of each component were estimated on the basis of UV absorption at 280 m μ . One apo VLDL sample was fractionated into 39 per cent of PI, 32 per cent of PII and 29 per cent of PIII. Apoprotein obtained from lipoprotein d > 1.019 yielded 24 per cent, 55 per cent and 21 per cent of PI, PII and PIII, respectively. From apo LDL, 70 per cent of PI, 14 per cent of PII and 16 per cent of PIII were obtained. Approximate amounts of each fraction from apo HDL were 23 per cent, 6 per cent, 24 per cent, 38 per cent and 9 per cent for PI, PII, PIII, PIV and PV, respectively.

Disc electrophoretic studies of the components isolated by Sephadex G-200. Disc electrophoretic patterns of the apoproteins and their fractionated components are shown in Figures 4, 5 and 6. PI-(VLDL) and PI-(LDL) stayed at the origin of the gel. PII-(VLDL) and PII-(LDL) migrated as a single band into the gel. PIII-(VLDL) and PIII-(LDL) were separated into several bands. Their interrelationship will be discussed later.

Apo HDL was fractionated into five components (Figures 6 and 7). The largest portion of PI-(HDL) stayed at the origin and was associated with several faster moving faint bands (Figure 7). Some of those bands in the gel showed identical mobility to PII, PIII and PIV of apo HDL. PII-(HDL) and PIII-(HDL) showed more than one component, indicating some cross contamination. PIV-(HDL), the dominant component of apo HDL, migrated as a single band. Disc electrophoretic differences between PII, PIII and PIV peptide were more clearly identified using pH 4.3 gel instead of pH 8.9 gel (compare Figure 6 and Figure 7). PV-(HDL) was separated into several bands, which were clearer in pH 8.9 gel.

Immunological studies of the isolated subfraction peptides. Each isolated subfraction was tested against several kinds of antisera. The results are summarized in Table 2. Immunological reactions of PI, PII and PIII of apo LDL against all kinds of antisera tested, were identical to those of PI, PII and PIII from apo VLDL, respectively. Totally identical reactions were observed between PII-(VLDL) and PIV-(HDL), and also between PII-(VLDL) and PIII and PIV-(HDL) against anti-apo HDL antiserum (Figure 8A). On the other hand, subfractions of these PIV-(HDL) reacted neither against anti-VLDL (Figure 8B) nor anti-apo VLDL serum suggesting that PIV-(HDL) peptide may not exist in VLDL.

PIII and PIV from apo HDL showed only one precipitin line against anti-apo HDL. They exhibited a reaction of total identity with PII-(VLDL) against the same antiserum (Figure 8A). PIII and PIV-(HDL) were also indistinguishable by immunoelectrophoresis using anti-apo HDL serum (Figure 9).

PV-(HDL), PIII-(LDL) and PIII-(VLDL) showed a reaction of total identity against anti-PV-(HDL) serum by the double diffusion technique.

Amino acid analysis. Amino acid composition of fractionated peptide by Sephadex G-200 from apo VLDL, apo LDL and apo HDL are listed in Tables 3 and 4. Some distinguishable features observed were: (a) a high glutamic acid and relatively high arginine content in PII-(VLDL) and PII-(LDL) with alanine being relatively higher in those fractions than any other components of apo VLDL or apo LDL; (b) low histidine content in PII-(VLDL) and PII-(LDL); (c) amino acid composition of PIII-(HDL) close to that of PII-(VLDL) or PII-(LDL); (d) high content of aspartic acid and relatively high amount of lysine in PIV-(HDL); (e) relatively high serine in PI-(VLDL), PI-(LDL), PIII-(VLDL) and PV-(HDL); (f) valine and histidine relatively low in PIII-(VLDL) and PV-(HDL); (g) relatively high amount of isoleucine in PI-(VLDL) and PI-(LDL).

Fractionation by DEAE-cellulose chromatography. Since PIII-(HDL) was not obtained free

Figure 4. Disc electrophoresis of apo VLDL and its subcomponents fractionated by Sephadex G-200 chromatography. The left panel is a photograph of the original gel patterns.

Figure 5. Disc electrophoresis of apo LDL and its subcomponents fractionated by Sephadex G-200 chromatography.

Figure 6. Disc electrophoresis of apo HDL and its subcomponents fractionated by Sephadex G-200 chromatography.

Figure 7. Disc electrophoresis of apo HDL and its subcomponents fractionated by Sephadex G-200 chromatography.

Table 2

IMMUNOLOGICAL PROPERTIES OF PEPTIDE FRACTIONS FROM RAT SERUM APO HDL AND APO VLDL

Antisera	Anti-apo HDL	Anti-apo LDL	Anti-apo VLDL	Anti-PIII & PIV (HDL)	Anti-PV (HDL)	Anti-albumin
Apo-HDL subfractions						
PI	+++	-	+	+++	-	-
PII	+++	-	+	+++	-	-
PIII	+++	-	+	+++	-	-
PIV	+++	-	-	+++	-	-
PV	++	-	+	-	+++	-
Apo-VLDL subfractions						
PI	-	+	+	-	-	-
PII	+	-	+	+	-	-
PIII	+	-	++	-	++	-

Anti-serum

Antibody

A: Anti-apo HDL
B: Anti-apo VLDL

1 and 4: PIV-(HDL)
2: PIII and PIV-(HDL)
3: PII-(VLDL)

8

Figure 8. Immunological characteristics of PIII-(HDL), PIV-(HDL) and PII-(VLDL). Antiserum: A, anti-apo HDL; B, anti-apo VLDL. Antigen: 1 and 4, PIV-(HDL); 2, PIII and PIV-(HDL); 3, PII-(VLDL).

Upper well contains PIV-(HDL) and lower well PIII and PIV-(HDL). Middle trough contains anti-apo HDL sera.

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Figure 9. Immunoelectrophoresis of two major components from apo HDL. Upper well contains PIV-(HDL) and lower well PIII and PIV-(HDL). Middle trough contains anti-apo HDL sera.

from PIV-(HDL) component, a further purification was attempted by DEAE-cellulose chromatography. The overlapping part of PIII and PIV-(HDL) from Sephadex G-200 was applied to a DEAE-cellulose column. Six fractions were obtained, two major (Nos. 4 and 5), one small (No. 6) and three minor ones (Nos. 1, 2 and 3) (Figure 10). Disc electrophoretic patterns of these isolated peaks are shown in Figure 11. The amounts of peaks 1, 2 and 3 were so small that they could not be identified by electrophoresis. Peaks 4A and 4B were pure PIV-(HDL) peptide. Peak 5 was predominantly PIV peptide with a little contamination by PIII. Peak 6 was also a mixture of PIII and PIV. Consequently, pure PIII-(HDL) polypeptide was not obtained by the conditions utilized.

Fractionation of PII-(VLDL) yielded four fractions (Figure 12). Peaks 1 and 4 were too small to analyze. The disc electrophoretic pattern of peak 3 was the same as the original PII-(VLDL). Peak 2 failed to be fixed on the gel by either 7 per cent acetic acid or 12.5 per cent TCA.

PV-(HDL) fractionated on DEAE-cellulose column yielded several peaks (Figure 13). Disc electrophoretic patterns of isolated fractions are shown in Figure 14. Peak 2 was too small to identify. Peak 5, as peak 2 from PII-(VLDL), was not stained for protein with either Amido Schwarz or Coomassie Blue after fixation with 7 per cent acetic acid or 12.5 per cent TCA. Peak 1 consisted of two strong and one faint band which corresponded to bands 1 and 2 in Figure 15. Peak 3 was band 5 and peak 4 was band 6. Peak 6 was one of the major components of

Table 3

AMINO ACID COMPOSITION OF FRACTIONS FROM APO VLDL AND APO LDL

	(apo VLDL)			(apo LDL)	
	PI	PII	PIII	PI	PII
Aspartic	86.8*	63.7	75.4	87.7	72.6
Threonine	48.7	44.0	53.5	51.3	41.8
Serine	76.3	35.7	73.0	70.1	38.8
Glutamic	104.4	179.3	133.1	93.3	162.1
Proline	28.8	19.4	25.8	35.5	13.6
Glycine	56.0	45.9	59.1	39.9	40.6
Alanine	48.7	70.2	54.7	47.1	66.8
1/2-Cystine	†	†	†	†	†
Valine	40.4	46.1	22.3	47.8	44.2
Methionine	7.0	20.7	28.6	11.0	6.8
Isoleucine	39.9	19.5	25.9	44.2	23.4
Leucine	83.4	85.3	70.0	94.7	88.2
Tyrosine	19.7	10.5	14.2	26.0	14.4
Phenylalanine	25.4	9.7	32.4	38.4	16.2
Lysine	46.3	24.9	40.3	56.7	37.3
Histidine	9.8	2.8	5.7	16.0	6.4
Arginine	26.1	74.5	36.3	29.1	66.9
Tryptophane	†	†	†	†	†
Number of preparations analyzed	2	2	1	1	1

* Figures are moles of amino acid/100,000 g of protein.

† Not determined.

PV-(HDL), corresponding to band 8 shown in Figure 15. Peak 7 was a mixture of bands 8 and 9, and peak 8 was pure band 9. Peaks 9 and 10 were a mixture of bands 9 and 10, and peak 11 was band 10.

Peaks 6 and 8 from PV-(HDL) were the main components of this group of peptides and showed a reaction of total identity against anti-PV(HDL) and anti-apo VLDL sera. Other components (peaks 1-5) failed to react against those antisera or against anti-apo HDL and anti-PIII and PIV (HDL), suggesting they were not contaminants of PIII or PIV-(HDL).

DISCUSSION

The present studies indicate that several peptides can be separated by combining gel filtration and ion exchange chromatography, starting from delipidated preparations of VLDL, LDL and HDL. A list of these peptides is presented in Table 5. They will be discussed separately in order of observed molecular weight.

PI-(LDL). This is the predominant and distinctive component of apo LDL and also exists in VLDL, representing 25-40 per cent of its protein moiety. It does not migrate into 7.5 per

Table 4

AMINO ACID COMPOSITION OF SUBUNIT PEPTIDE FROM APO HDL

	PI	PII	PIII	PIV	PV
Aspartic	86.2*	91.8	84.7	103.3	71.2
Threonine	42.9	39.9	40.6	38.6	56.7
Serine	45.9	39.0	37.5	32.1	75.6
Glutamic	115.8	133.8	159.4	144.1	127.4
Proline	32.3	34.8	24.1	26.8	33.6
Glycine	42.8	44.6	42.8	36.3	51.2
Alanine	59.0	52.6	69.1	57.9	64.6
1/2-Cystine	2.5	†	†	†	†
Valine	45.1	47.0	49.0	44.2	23.4
Methionine	18.6	20.5	21.3	21.6	23.3
Isoleucine	27.0	21.3	18.0	13.4	14.6
Leucine	87.2	92.6	90.7	98.4	81.6
Tyrosine	19.1	14.6	16.0	17.2	27.1
Phenylalanine	26.4	20.7	18.0	21.5	32.6
Lysine	45.1	50.1	41.2	66.1	43.0
Histidine	14.8	13.3	10.5	17.2	5.2
Arginine	38.0	33.9	59.4	40.6	26.9
Tryptophane	†	†	†	†	†
Number of preparations analyzed	1	1	2	2	3

* Figures are moles of amino acid/100,000 g of protein.

† Not determined.

cent polyacrylamide gel (Figures 4 and 5) and has an amino acid composition characterized by a relatively high content of isoleucine and serine and relatively low amounts of methionine and arginine. This protein reacts against anti-LDL or anti-VLDL sera.

PII-(VLDL). Although a major component of apo VLDL, this fraction is also found as a minor component of apo-LDL. All the available data (molecular weight, disc electrophoresis, immunology and amino acid analysis) also indicate that it is identical with PIII-(HDL), the second dominant fraction of apo HDL. Its amino acid composition is characterized by a low content of histidine and high amounts of glutamic acid and arginine.

While this manuscript was in preparation, a communication in abstract form concerning rat serum apoproteins was published by Burst et al.¹⁵ On the basis of the limited information provided, it is not possible to compare their findings with ours, although it is probable that their VS-2 fraction corresponds to our PII (VLDL).

In human VLDL this type of protein has not been clearly identified, and this may indicate that it is a unique component characteristic of rat serum VLDL.

PIV-(HDL). This dominant fraction of HDL can be isolated from apo HDL either by Sephadex G-200 or DEAE cellulose chromatography. This fraction migrates as a single sharp band

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12

11

13

Figure 10. DEAE-Cellulose chromatography of Sephadex fractions PIII and PIV from HDL. For details, see text.

Figure 11. Disc electrophoresis of DEAE fraction of PIII and PIV-(HDL). From left to right, peaks 4, 5 and 6. Gel pH 4.3.

Figure 12. DEAE-Cellulose chromatography of Sephadex fraction PII from VLDL.

Figure 13. DEAE-Cellulose chromatography of Sephadex fraction PV from HDL.

14

15

Figure 14. Disc electrophoresis of isolated subcomponents from PV-(HDL) by DEAE-cellulose column chromatography. Left to right: peaks 1, 3, 4, 6 and 8. Gel pH 8.9.

Figure 15. Disc electrophoresis of smaller peptide components from rat serum lipoproteins.

Table 5

POLYPEPTIDES ISOLATED AND IDENTIFIED IN RAT SERUM LIPOPROTEINS

Isolated fraction	Distribution*			Possible human counterpart
	VLDL	LDL	HDL	
PI-(LDL)	++	+++	-	B-protein, R-Ser (16) [†]
PII-(VLDL)	+++	+	++	SF 2 (5)
				? Fraction IV (1), or R-Glu (2)
PIV-(HDL)	±~-	±~-	+++	Fraction III (1), or R-Thr (2)
Peptides M.W. less than 20,000				
Peak 1 (from DEAE)	+++	+	+	R-Val (4)
Peak 3	+	+	++	
Peak 4	+	+	+	
Peak 6	+++	+++	+++	R-Ala (4)
Peak 8	+++	+++	+++	R-Ala (4)

* Assumed by disc electrophoresis and immunology.

[†] Figures in parenthesis indicate the reference number.

in a polyacrylamide gel at pH 4.3, containing 8 M urea or as a single broader band in pH 8.9 gels (Figures 6 and 7) and has an apparent molecular weight of 36,600. Bands with identical disc electrophoretic mobility to PIV-(HDL) were seen in apo VLDL and in its Sephadex fraction PII-(VLDL) (Figure 16), and had an amino acid composition featuring relatively high amounts of aspartic acid and lysine.

Figure 16. Comparison of PII-(VLDL), PIII-(HDL), and PIV-(HDL). Left to right: PII-(VLDL), PII and PIV-(HDL) and PIV-(HDL). Gel pH 4.3.

Smaller polypeptides showing apparent molecular weights less than 20,000. This group of smaller peptides is obtained from all three classes of lipoprotein by gel filtration with Sephadex G-200 and exhibits fast electrophoretic mobility (Figure 15). Bands 8, 9 and 10 are the prominent components of this fraction whereas bands 1 and 2 (Figure 15) were minor in PIII (VLDL), PIII-(LDL) or in PV-(HDL). At pH 8.9 there were several other relatively weak bands (bands 3, 4, 5, 6, and 7 in Figure 15). Among them, No. 3 was characteristic of apo VLDL and No. 5 was marked in apo HDL and could be separated by DEAE-cellulose chromatography.

Other components of apo HDL. PII-(HDL) is a minor but distinct component having apparent molecular weight about 76,000. It is obtained by Sephadex G-200 column chromatography with a little contamination by PIII- and PIV-(HDL), with characteristic immunological reactivity (Table 3) and amino acid composition (Table 5).

The other fraction, PI-(HDL), contains about 2 per cent of phospholipid by weight, compared with an average phospholipid content in apo HDL of less than 1 per cent,⁶ and upon rechromatography partially dissociates into PIII-, PIV- and PV-(HDL) types of fractions. The dissociation of PI-(HDL) into several bands (PII-, PIII and PIV-(HDL)) was also seen by Page though a large portion of the protein remained near the origin (Figure 7).

It is thus apparent that, as in the case of human products,¹⁻³ rat serum apoproteins con-

tain several components differing in physical, chemical and immunological properties. The structural and functional significance of these fractions remains yet unknown and waits investigation.

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