Calf Thymus Composition. A Comparison of Differential Centrifugation and Chemical Fractionation Procedures*

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ABSTRACT

The extraction behavior of thymus and the composition of fractions prepared from this organ has been studied. Sequential extraction methods using 0.15 M NaCl followed by water gave information with respect to the weight fraction of cytoplasmic and nuclear constituents. Lipide, nucleic acid, and electrophoretic analysis of the extracts provided additional information. A less complex electrophoretic pattern was obtained from subsequent extracts in the sequence.

Sucrose and saline dispersates obtained from tissue fragmented with either the Potter-Elvehjem homogenizer or in a Waring blendor were fractionated, using standard differential sedimentation methods. The fractions obtained by means of four different dispersion procedures were compared in terms of yield, chemical analysis, and electrophoretic composition.

The quantity of material in thymus having the sedimentation characteristics of liver mitochondrial and microsomal fractions was remarkably small. Both the suspension medium employed and the method used to bring about a disruption of the cells in the tissue affected the yield of "particulate" material. The components present in the later extracts in the sequence, E_4 to E_7, in the case of sequential extraction study resembled with respect to chemical composition and electrophoretic characteristics, the microsome fraction prepared by differential sedimenta tion methods.

About 76 per cent of the PNA in the tissue appeared to be in the cytoplasm. The remaining 24 per cent PNA was found in the nucleus and accounted for 1.7 per cent of nucleus on a dry weight basis.

From 75 to 88 per cent of cytoplasmic PNA was extracted from the tissue and 76 to 94 per cent of the PNA in the extract was found in the final supernatant solutions, depending upon the dispersion methods and suspension medium used in the extraction procedure.

The composition of the final supernatant fractions using differential sedimentation methods were comparable in terms of electrophoretic properties, protein concentration, nucleic acid content, and fractionation behavior to saline extracts E_1 to E_3, of thymus used in earlier studies.

INTRODUCTION

The composition of the nuclei of cells, chiefly lymphocytes, found in mammalian thymus, has been the subject of investigation in many laboratories (1–5). The composition of the cytoplasm from these cells has had much less attention.

Electrophoretic patterns obtained from extracts of calf thymus and human tonsils have been published by Abrams and Cohen (6). Petermann and coworkers have investigated the composition of mouse spleen dispersates (7, 8). Laird has demonstrated that a preponderance of the pentosenucleic acid (PNA) in rat thymus brei remained in the supernate after removal of the mitochondrial and microsome fractions by centrifugation (9).

Previous studies from this laboratory have been confined to the macromolecular composition of

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saline extracts of lymphatic organs (10–14). Approximately 45 per cent of the dry weight of the tissue was found in the saline supernates, depending upon the number of extractions (11). For lack of explicit evidence to the contrary we have assumed that this material is chiefly cytoplasmic in origin.[1]

Since the lymphocyte is known to have but a thin layer of cytoplasm, 0.45 would appear to represent a remarkably large weight fraction comprising the cytoplasmic portion of the cell. It is not, however, unreasonable when one considers the geometry of a sphere. If it is assumed that lymphocytes, which comprise more than 95 per cent of the cells of normal thymus (15), are spherical in shape, and have a diameter of 6 μ (16) occupied by a nucleus of 4.8 μ diameter (17), it can be readily calculated that the volume fraction of the cell occupied by the cytoplasm amounts to 0.49.

Since experience indicated that the composition of a particular extract depended upon its position in the extraction sequence, we have analyzed a sequence of extracts prepared from calf thymus. In addition we have compared fractions isolated by chemical methods with fractions prepared by current cell fractionation procedures based on differential centrifugation (18–22). This report summarizes the results of these studies.

Experimental

The details of the extraction procedure employed in the sequential extraction method have been discussed (11, 14). In the case of sequential extracts, the tissue was dispersed in 0.15 M NaCl, 2 ml. per gram of tissue, using the Waring blendor; stirred, and centrifuged at 20,000 *g* for 60 minutes. The sediment was resuspended in the same volume of 0.15 M saline, dispersed, stirred, and centrifuged as before. The operation was repeated seven times to yield seven separate extracts labeled E_1 to E_7, and a final sediment.

In the studies employing differential sedimentation methods, with 0.15 M saline as a solvent, the procedures outlined by Barnum and Huseby (19) were followed. When isotonic sucrose was used the procedure suggested by Schneider (20) and outlined in "Manometric Techniques" (23) was followed. The tissue was dispersed either with a Waring blendor or by means of a Potter-Elvehjem tissue homogenizer equipped with a teflon pestle. The so called "particulate" fractions were washed with the appropriate solvents and the supernates from the washings included with subsequent fractions. All operations were carried out in a cold laboratory held at approximately 2°C., using cold equipment and solvents.

The four methods for the preparation of dispersates used in this study are listed below.

Method A: dispersion with a Potter-Elvehjem homogenizer in 0.25 M sucrose.

Method B: dispersion in a Potter-Elvehjem homogenizer in 0.15 M NaCl.

Method C: dispersion by means of a Waring blendor in 0.15 M NaCl.

Method D: dispersion in a Potter-Elvehjem homogenizer in 0.25 M sucrose containing 0.0033 M $CaCl_2$.

Dispersion of the thymus in 0.25 M sucrose with the Waring blendor was unsatisfactory. The swollen nuclei were so completely ruptured by the blendor that the nucleohistones dissolved in the extract.

The various analytical procedures used in this study have been previously described (11–14). In the case of lipide analyses, lyophilized dry solids were extracted with a 3:1 alcohol-ether mixture using a Soxhlet extractor. The extracted material remaining after evaporation of the alcohol-ether was taken up in chloroform. Material which dissolved in chloroform was considered lipide.

Three procedures were used to determine nucleic acid. Total nucleic acid was determined with the Schneider method (24). The nucleic acid content of the trichloracetic acid extract was determined spectrophotometrically using an extinction coefficient of 248 at the wave length 260 mμ. PNA was measured using the Schmidt-Tannhauser method (25), and DNA was determined quantitatively using the diphenylamine reagent of Dische (26). In the case of the diphenylamine test a commercial sample of purified DNA obtained from Nutritional Biochemicals Corporation, Cleveland, was used as a standard. The phosphorus and total nucleic acid content indicated this sample contained 78 per cent DNA. The optical densities were read at 600 mμ using a model 6A Coleman spectrophotometer. In samples containing both forms of nucleic acid the total nucleic acid content determined with the Schneider procedure agreed well with the sum of PNA and DNA employing the Dische and the Schmidt-Tannhauser procedures.

RESULTS

Sequential Saline Extraction.—The results of the sequential extraction studies have been tabulated in Tables I and II. In this study a substantially larger quantity of thymus protein dissolved in the first two extracts (E_1, E_2) than reported previously (11). As a consequence, the total quantity of

[1] This weight fraction is too large by the amount of extracellular material present in the tissue and presumed to be the normal proteins found in lymph. The quantity of such material can be estimated from the amount of serum albumin present, assuming that the other serum proteins are present in the same relative amounts as in blood serum.

TABLE I

Optical Concentrations (O.C.) and Dry Weight Values of Saline Extracts for 100 gm. Wet Tissue

Extract	Concentration of protein	Total non-dialyzable solids	O.C.* 260 mμ	O.C. 280 mμ	280/260 Ratio
	gm./100 ml.	*dry wt. gm.*			
E_1	1.5	3.44	68.4	44.0	0.642
E_2	0.67	1.45	29.9	19.7	0.643
E_3	0.24	0.49	8.2	5.5	0.672
E_4	0.15	0.31	5.7	3.8	0.67
E_5	0.11	0.18	3.1	2.1	0.69
E_6	0.06	0.12	2.2	1.5	0.72
E_7	0.05	0.10	1.8	1.3	0.74
Total..		6.1			

* The term Optical Concentration has been defined (11).

TABLE II

Chemical Analyses of Sequential Extracts and Fractions

Fraction	Lipide	Nucleic acid (PNA)	
		Original	Recovered*
	per cent dry wt.	*per cent dry wt.*‡	*per cent dry wt.*‡
E_1	7.4	6.7	6.1
E_2	9.2	6.5	5.2
E_3	11.2	5.8	4.7
E_4	13.5	5.9	3.4
E_5	14.0	5.8	2.7
E_6	—	5.6	—
E_7	—	5.3	—
5.1 P	14.1	9.9	8.7
5.1 S	3.6	0.6	0.6

* Column designated "recovered" refers to material dissolved in μ 0.10 pH 8.6 veronal buffer for electrophoretic analysis. After the electrophoretic analysis the material was dialyzed against water in order to remove the buffer salts and lyophilized. The lyophilized sample was analyzed for nucleic acid. We interpret the decrease in PNA as evidence for the presence of ribonuclease in the fraction.

‡ Including lipide removed before nucleic acid analysis.

soluble material in the combined extracts (E_1 to E_3) in this study resembled earlier results obtained with palatine tonsils (11). We attribute the difference to the fact that thymus from younger animals was used in the present work.[2]

[2] In the case of thymus the relative number of lymphocytes and reticular cells varies with the age and condition of the animal (5, 15). In the case of calf thymus procured from commercial slaughter houses such variations affect the quantity of material extracted. In general, a larger quantity of material can be extracted from the thymus of young weanling calves than can be obtained from the thymus of older animals.

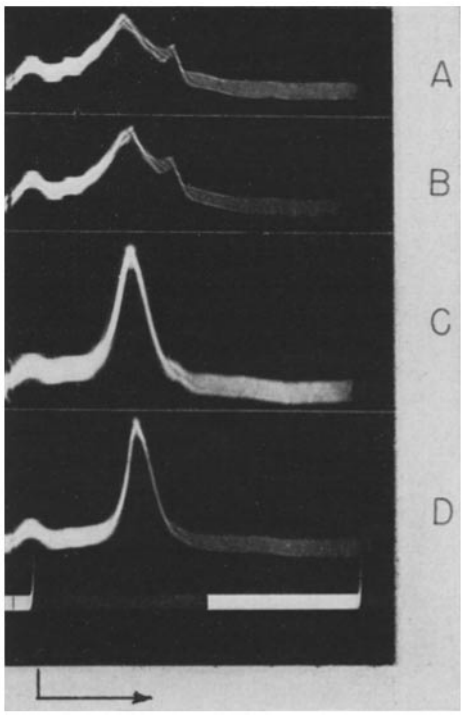

FIG. 1. Electrophoretic patterns obtained from successive extracts of bovine thymus. Photographs made from descending limb of the cell after 120 minutes under a potential gradient of 6.4 volts $cm.^{-1}$ in veronal buffer pH 8.6 ionic strength 0.10.

A. First extract, E_1, protein concentration 1.4 per cent, diagonal slit angle 35°, mobility of main peak -4.3×10^{-5} $cm.^2$ $volt^{-1}$ $sec.^{-1}$.

B. Second extract, E_2, protein concentration 1.4 per cent, diagonal slit angle 35°, mobility of main peak -4.4×10^{-5} $cm.^2$ $volt^{-1}$ $sec.^{-1}$.

C. Third extract, E_3, protein concentration 1.1 per cent, diagonal slit angle 50°, mobility of main peak -4.6×10^{-5} $cm.^2$ $volt^{-1}$ $sec.^{-1}$.

D. Fourth extract, E_4, protein concentration 1.25 per cent, diagonal slit angle 40°, mobility of main peak -4.9×10^{-5} $cm.^2$ $volt^{-1}$ $sec.^{-1}$.

Fifth extract identical to E_4.

The electrophoretic patterns obtained from the extracts are shown in Fig. 1. It was apparent that subsequent extracts represented less complex mixtures than the preceding. Although less obvious, but nonetheless significant, the mobility of the main peak was greatest in E_4, and the least in E_1.

The electrophoretic patterns obtained from E_4 and E_5 were indistinguishable.

All seven extracts were tested for the presence of deoxypentose by means of the diphenylamine reagent of Dische (26). A negative test was obtained in each instance, indicating that less than 20γ per ml. of DNA was present in the extract.

Extracts E_1, E_2, E_3, were combined and fractionated according to Step 1 of the procedure previously published (11). The material which precipitated at an ionic strength 0.025 and pH 5.1, the ribonucleoprotein fraction, amounted to 3.7 gm. The electrophoretic pattern obtained from this fraction called 5.1 P, was essentially the same as previously published (11). Fraction 5.1 P and extracts four and five, therefore, had very similar electrophoretic properties. As can be seen in Table II, these fractions are also quite similar with respect to lipide content. The nucleic acid content of fraction 5.1 P has been found to be consistently somewhat higher than that found in extracts E_4 and E_5.

Fraction 5.1 P, and E_4 and E_5 were also similar with respect to fractionation behavior. Fraction 5.1 P has been separated into two fractions called 3.0 P and 3.0 S (11). Fraction 3.0 P[3] was a PNA type nucleoprotein, whereas in fraction 3.0 S, which does not contain nucleic acid, was component L3, and the materials found in fraction 6.2 S (12). Extracts four and five have been separated in the same manner into fractions 3.0 P and 3.0 S. The subfractions prepared from E_4 and E_5 have electrophoretic and chemical properties similar to the fractions prepared from fraction 5.1 P. The conclusion based upon fractionation behavior, that E_4 and E_5 contained the same components as fraction 5.1 P appeared significant from the viewpoint of cell structure and organization and will be discussed below.

Extraction with Water.—If it is assumed that the materials found in extracts E_1 to E_7 represent the cytoplasmic constituents of the cells, the sediment from extract E_7 contained the nuclei. The Dische test, as mentioned above, confirmed the absence of detectable amounts of DNA in extracts E_1 to E_7.

[3] Fraction 5.1 P and Fraction 5.1 S represent the precipitate and supernate respectively when the extracts (20,000 *g* supernates) are brought to pH 5.1 at an ionic strength of 0.025. Fractions 3.0 P and 3.0 S represent the precipitate and supernate respectively when a solution of Fraction 5.1 P is dialyzed against 0.10 μ sodium monochloracetate at pH 3.0.

TABLE III

Weight Relationships and Nucleic Acid Content of Thymus Fractions/100 gm. Wet Tissue

Fraction	Dry weight	Weight fraction	DNA	PNA
	gm.		*per cent dry wt.*	*per cent dry wt.*
E_1 to E_7 (non-dialyzable)	6.1	0.35	none	6.5
E_1 to E_7 (dialyzable)	1.8	0.10	none	0.8
Water extract (insoluble 0.15 M NaCl)	6.6	0.38	42	0.9
Water extract (soluble 0.15 M NaCl)	0.6	0.03	21	4.0
Final sediment	2.4	0.14	4.0	1.7
Total	17.5	1.00		

The possibility remained that nuclear constituents lacking DNA were present in the extracts. This possibility will be discussed subsequently. In order to determine the amount of nuclear material in the tissue, the sediment from the seventh extract was suspended in water, stirred several hours, then centrifuged 60 minutes at 20,000 *g*. The sediment from this step was also suspended in water, stirred, and centrifuged as before. The final sediment was suspended in water and lyophilized. On the basis of 100 gm. wet weight of tissue, this final sediment amounted to 2.4 gm. dry weight or about 14 per cent of the total dry weight of the tissue. This sediment presumably contained connective tissue, cell debris, and as shown by chemical analysis, unextracted nucleoprotein.

The two supernatant solutions obtained from the water extraction steps were brought to 0.15 molarity with solid NaCl. The precipitate was in each instance removed by centrifugation, suspended in water, dialyzed against water, and lyophilized. A total of 5.5 gm. of material was obtained from the first water extract and 1.1 gm. from the second. The supernatant solutions from the above precipitation steps were tested with the Dische reagent, dialyzed against water, and lyophilized. A positive reaction was obtained in both instances with the Dische test. On the basis of 100 gm. wet tissue, 0.6 gm. of dry material was found in the supernatant solutions. The weight relationships of the various fractions have been tabulated in Table III.

Assuming the materials in extracts E_1 to E_7 represent the cytoplasm, the weight fraction of cytoplasm in the tissue on a dry weight basis, is

0.45.[1] The two water extracts represented nuclear elements of the tissue and on a dry weight basis comprised a weight fraction of 0.41.

The 100 gm. wet thymus contained 0.53 gm. of PNA of which 0.40 gm. was found in E_1 to E_7. The same tissue contained 3.0 gm. of DNA. From the above data it was calculated that 4 per cent of the nucleic acid found in the nucleus was the PNA type, and that PNA amounted to 1.7 per cent of the nucleus. The PNA content of the nucleus, as indicated above, agrees well with the values reported by Allfrey, Mirsky, and Osawa (5).

Differential Sedimentation Studies.—The purpose of the differential sedimentation studies was to determine the quantity of material in extracts of thymus possessing the sedimentation characteristics of the mitochondrial and microsomal fractions found in liver. Inasmuch as the method employed to disrupt cells, as well as the medium in which the cells have been disrupted, affects the yield and properties of the fraction (27), several commonly used procedures were employed. Information was desired also, about the nature of the materials which remained in the supernatant fraction after the so called "particulate" elements of the cell were removed.

Results from the above studies have been tabulated in Table IV. The supernatant materials, designated S3 in Table IV, were fractionated chemically according to the earlier procedure (11), in order to make comparisons with the results of the sequential extraction method. With the exception of method A, the quantity of fraction 5.1 S was nearly identical to that obtained using the sequential extraction procedures described above and to earlier published results (11). In the case of method A, a somewhat smaller yield of 5.1 S was obtained, which amounted to about 70 per cent of the yield obtained with the other methods.

The yield of fraction 5.1 P on the other hand, was least in the case of method C and greatest in the case of methods A and B. From this observation we concluded that the material in the microsome fraction, obtained in the case of methods C and D, represented material present in the 5.1 P fraction in the case of methods A and B. The higher PNA content of the microsome fraction using methods C and D likewise supported this conclusion. The lipide and nucleic acid contents of the various fractions have been listed in Table V.

As can be seen in Table IV, the total yield of mitochondrial, microsome, and supernatant materials was less when 0.25 M sucrose was used as a dispersion medium. This can be attributed to swelling of the nuclei which occurs when the cells are dispersed in 0.25 M sucrose. The larger volume occupied, as well as the poorer sedimentation characteristics of the swollen nuclei, renders difficult a quantitative removal of supernatant fluid. In this connection it is of interest to note that the swollen nuclei retain sucrose tenaciously. The sucrose was

TABLE IV

Weight Relationships Amongst Various Fractions Prepared by Differential Sedimentation Procedures

Fraction	Dry weight of fraction in gm./100 gm. wet thymus			
	A*	B	C	D
Mitochondrial‡	0.17	0.41	0.61	0.67
Microsomal§	0.42	0.64	1.34	0.95
Supernate (S3)	4.23	4.56	3.70	4.10
Total extract	4.82	5.71	5.65	5.72
Residue	11.72‖	10.00	10.10	10.00
Total dry weight¶	16.55	15.71	15.75	15.76

A, dispersed in 0.25 M sucrose with a Potter-Elvehjem homogenizer.

B, dispersed in 0.15 M NaCl with a Potter-Elvehjem homogenizer.

C, dispersed in 0.15 M NaCl with a Waring blendor.

D, dispersed in 0.25 M sucrose 0.0033 N $CaCl_2$ with a Potter-Elvehjem homogenizer.

* When thymus was dispersed in 0.25 M sucrose, the nuclei became swollen and occupied a larger volume fraction of the suspension than was the case when the tissue was dispersed in saline. As a consequence a smaller volume of S3 supernate was obtained in the case of sucrose extraction. The concentration of dissolved material in the supernatant solution was greater, however, in the case of sucrose extraction, as shown by optical density measurements. In order to avoid the swelling effect, and as suggested by Allfrey, Mirsky and Osawa (5), the thymus was also dispersed in 0.25 M sucrose containing 0.0033 M $CaCl_2$.

‡ Obtained as sediment after 10 min. at 8500 *g* in case of sucrose or after 30 min. at 2000 *g* in case of saline.

§ Obtained as sediment after 90 min. at 23,000 *g*.

‖ This total includes sucrose which had not been removed after 96 hours dialysis against eight changes of distilled water.

¶ These totals do not include the dialyzable materials present in the extracts.

TABLE V

Chemical Analysis of Various Fractions

	Lipide in per cent dry weight of fraction				Nucleic acid (PNA)* in per cent dry weight of fraction			
	A	B	C	D	A	B	C	D
Mitochondrial fraction	57	48	39	45	3.8	3.5	5.1	4.7
Microsome fraction	53	49	43	43	2.2	2.0	5.1	5.3
Supernate fraction	14	10	11.1	11.5	6.9	7.2	6.6	6.2
Fraction 5.1 P	17	13	16	18	9.0	10.5	10.6	8.7
Fraction 5.1 S	5	3	3	4	0.7	0.9	0.6	0.7

* The figures represent the per cent nucleic acid in the fraction including the lipides.

not readily removed from the residue by dialysis against water. This matter will be discussed below.

In the case of methods B, C, and D the total amount of material in the extracts was remarkably uniform. It is of interest that this total is comparable to the combined total of materials in extracts E_1 to E_4 in the case of sequential extracts, as shown in Table I. Also of interest, and as shown in Table IV, the dry weight of residue in case of methods B, C, and D, agreed almost exactly with the quantity of sediment remaining after the fourth extract in the sequential extraction study as can be seen from Tables I and III. Since the quantity of material found in E_5 to E_7 was small, it seems reasonable to conclude that the residues from B, C, and D resemble, with respect to chemical composition, the sediment from E_7 as seen in Table III.

There was a remarkable uniformity in the quantity of PNA in extracts B, C, and D, as can easily be calculated from the data in Tables IV and V. The amount of PNA in the extracts varied from 335 to 357 mg. and represented 82 to 88 per cent of the total PNA present in E_1 to E_7. Of this PNA, from 71 to 95 per cent was present in the supernate S3; the largest percentage was found with method A, and the lowest with method C.

As shown in Table IV, the amount of material having the sedimentation characteristics of mitochondria and microsomes was strikingly dependent upon both the dispersion medium and dispersion technique used. Where an increased quantity of mitochondrial and microsome material was obtained, it occurred at the expense of the supernate (S3).

TABLE VI

The Effect of Redispersion Methods on the Yield of Microsomal Fraction

	Weight of fraction in gm./100 gm. wet thymus			
	C	C1	C2	C3
Microsome fraction*	1.34	0.96	0.92	0.58
Supernate S3	3.70	3.90	4.06	4.34
Total	5.04	4.86	4.98	4.92

* The microsome fraction prepared according to Method C was redispersed:

C1, with a Waring blendor in 0.15 M NaCl.

C2, with a Potter-Elvehjem homogenizer in 0.15 M NaCl.

C3, with a Potter-Elvehjem homogenizer in 0.25 M sucrose.

This matter was studied further in the following manner. Microsome fractions prepared according to method C, were dispersed in: (1) 0.15 M NaCl with the Waring blendor (method C1); (2) 0.15 M NaCl with the Potter-Elvehjem homogenizer (method C2); and (3) in 0.25 M sucrose with a Potter-Elvehjem homogenizer (method C3). The material which sedimented after 90 minutes at 20,000 *g* was dispersed in the same manner a second time and again centrifuged. The materials remaining in the supernates were added to the supernate S3. The results of this study have been tabulated in Table VI. The results suggested that the materials found in the microsome fraction in the case of method C were present in the supernatant solutions when methods A, B were employed to disperse the tissue. The results shown in Table VI suggest, also, that for this fraction, sucrose was a more effective dispersing medium than was saline.

It was observed that the microsome pellet prepared by each method could be dispersed in water by means of the Waring blendor or a Potter-Elvehjem homogenizer followed by dialysis against water. The fraction remained in "solution" when dialyzed against pH 8.6 μ 0.10 veronal buffer. Similar electrophoretic patterns were obtained irrespective of method of preparation of the microsome fraction. The patterns furthermore, closely resembled with respect to composition and mobility, the pattern shown in Fig. 1 *D*, obtained from extract E_4 in the sequential extraction study.

The electrophoretic patterns obtained from the final supernate fractions (S3) in the case of the

differential sedimentation studies were comparable to the patterns obtained from extracts E_1 to E_3 previously published (11).

DISCUSSION

In the case of the differential sedimentation studies, standard procedures (19–22) for the isolation of fractions, commonly called mitochondria and microsomes and referred to as "particulate" elements, have been applied to the thymus. In so doing, we have assumed that the sedimentation behavior of these cellular elements was similar, irrespective of cell types. Admittedly this assumption is tenuous. It must be recognized, however, that the assumption is implied in all tissue fractionation studies employing the differential sedimentation technique, inasmuch as no tissue represents an homogeneous cell population. For comparative purposes, an operational definition of the "particulate elements," in terms of sedimentation properties, seemed both appropriate and expeditious.

It was clear from the results that the quantity of material in thymus having the sedimentation characteristics of liver mitochondrial and microsome fractions was remarkably small. It is also apparent that both the suspension medium employed and the method used to bring about a disruption of the cells affected the yield of "particulate" material. It was found that the amount of microsome fraction obtained using one method could be altered by subjecting the fraction to further dispersion. These results, shown in Table VI, as well as the electrophoretic behavior, support the conclusion that extracts E_4 to E_7 in the successive extraction study contained essentially the same materials found in the microsome fraction when method C or D was employed. The electrophoretic mobility of the microsome fraction and extracts E_4 and E_5, -4.9×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, was identical with that previously reported (11) for fraction 5.1 P. Petermann and coworkers, reported this same mobility for the microsome fraction obtained from mouse spleen (8).

Not only was the weight fraction of particulate matter in the thymus dispersate low, which is in accord with results of Petermann and coworkers (28) using spleen, but the lipide and nucleic acid content of this material differed considerably from the values reported for lipides and nucleic acid in mitochondria and microsome fractions separated from liver extracts (19, 29).

It seems unlikely that microsomal particles of such size as to have sedimented with the nuclei were present to any great extent in the dispersed tissue. Only a small amount of PNA remained in the sediment after removal of extracts E_1 to E_7, in the sequential extraction studies. If we consider the total PNA present in E_1 to E_7 as representing the cytoplasmic PNA, then 80 per cent of the cytoplasmic PNA was found in the final supernate in the case of method B. The quantity of residue obtained with methods B, C, and D, furthermore, was gravimetrically comparable to that which remained from E_7 in the sequential extraction procedure, as mentioned above. It should be pointed out, also, that we have not detected any materials of high PNA content referred to as "ultramicrosomes" (19), or "macromolecules of ribonucleoprotein" (30), in any of the fractions we have obtained using either differential centrifugation or chemical fractionation methods.

The fact that the microsome fraction, irrespective of the method of preparation, exhibited essentially the same electrophoretic behavior and that this behavior closely resembled that of extract E_4 and fraction 5.1 P seemed significant. Inasmuch as the nucleic acid content of the microsome fraction, E_4, and fraction 5.1 P, differed somewhat, the conclusion seems justified that the electrophoretic characteristics of the particle could be attributed to the protein moiety. This conclusion carries with it ramifications with respect to structure which await further investigation.

The appearance of the microsome fraction in tissue suspensions has been attributed to fragmentation of endoplasmic reticulum (31, 32) by the device used to disperse the tissue. The variation in the quantity of the microsome fraction obtained from thymus using various dispersing methods allows several interpretations. Either the endoplasmic reticulum is split into smaller fragments when inorganic ions are absent from solution or the fragmented reticulum aggregates in the presence of ions. Comparison of yields of the fraction in the case of methods B and C suggested that the Potter-Elvehjem homogenizer was a more efficient device for fragmenting endoplasmic reticulum than was the Waring blendor. The very large fraction of PNA remaining in the final supernate suggests that the endoplasmic reticulum in the lymphocyte differs from that of the liver parenchymal cell in being more extensively frag-

mented during dispersion. An alternative possibility is that the lymphocyte possesses a poorly developed endoplasmic reticulum and a large amount of "free ribonucleoprotein particles." If the former interpretation is correct, the lymphocyte resembles the exocrine cell of the pancreas. Palade and Siekevitz (33) noted the extensive fragmentation of the endoplasmic reticulum in the case of pancreas.

It is of interest that Kuff and Hogeboom (34) found that over 50 per cent of the cytoplasmic PNA of the strain L fibroblast was associated with a fraction which had not cleared the meniscus after 1 hour at 40,000 R.P.M. The observation of Kuff and Hogeboom is of interest because of the viewpoint of some histologists (16) that lymphocytes under certain conditions transform into fibroblasts.

A question can be raised concerning how much material would have sedimented if the supernatant solutions had been subjected to greater centrifugal fields (35). It is of course a truism that when higher centrifugal forces and longer times of sedimentation are employed a greater quantity of material will be removed from solution. This becomes a complex matter involving partial specific volumes, solution densities, diffusion properties, as well as other hydrodynamic factors. The individual worker is limited to centrifugal forces of available equipment, which makes any procedure somewhat arbitrary. Even sucrose and potassium chloride have sedimentation coefficients measurable with existing equipment under suitable conditions (36).

If an optical criterion, the ability to refract light, is invoked, these supernatant solutions fulfilled the requirement of being true solutions. In the electrophoretic patterns the area under the curve was a linear function of the quantity of solids in the system and consistent with the refractive index increments commonly ascribed to proteins. Since this area is also linearly dependent upon the refractive index of the solution, the solids in the system contributed to the refractive properties and were, therefore, according to the optical criterion, dissolved. The light-scattering properties of these systems, furthermore, were consistent with solution behavior rather than suspension behavior, as can be seen in Fig. 1.

The question can be raised, as mentioned above, as to whether extracts E_1 to E_7 do not contain, in addition to cytoplasmic materials, proteins which originate in the nucleus, do not contain DNA, and are extractable with 0.15 M NaCl. Allfrey, Mirsky, and Osawa (5) have emphasized this viewpoint as a complicating factor in cell fractionation procedures. Our studies do not exclude the possibility.

It seems reasonable to assume that protein originating in the nucleus, and extractable with 0.15 M NaCl, would be present in relatively larger proportion with respect to cytoplasmic constituents, in the later extracts in a sequence. Extracts E_4 to E_7, therefore, could be expected to reflect the nature of these proteins. The electrophoretic behavior of the materials in extracts E_4 and E_5 (Fig. 1 *D*) was indistinguishable from the patterns obtained from the dispersed microsomal fractions and resembled very closely, as mentioned earlier, the electrophoretic behavior of Fraction 5.1 P obtained from both extracts E_1 to E_3, and supernates (S3). The materials in E_4 and E_5 also resembled Fraction 5.1 P with respect to subsequent chemical fractionation behavior, as stated before. The nucleic acid analysis, shown in Table II, indicated that these materials are PNA type nucleoproteins. For these reasons we have considered that the materials in extracts E_4 to E_7 originate in the cytoplasm and result from fragmentation of the endoplasmic reticulum due to repeated dispersion with the Waring blendor.

Osawa, Takata, and Hotta (39, 40) have isolated nuclear PNA proteins and microsomal PNA proteins from thymus indistinguishable from each other in terms of electrophoretic mobility and PNA content. Our findings are in accord, therefore, with the observations of Osawa, Takata, and Hotta (39). It seems to us, however, equally plausible that what Osawa, Takata, and Hotta consider "nuclear PNA protein indistinguishable from microsomal PNA protein" could represent fragments of endoplasmic reticulum of such size as to have sedimented with nuclei in their isolation procedure. According to our interpretation, the more vigorous dispersion techniques we employed in the sequential extraction procedure resulted in such fragmentation of endoplasmic reticulum that the PNA protein appeared in extracts E_4 to E_7.

The striking retardation in the rate of dialysis of the sucrose in the presence of the DNA-type nucleoprotein can be attributed to at least three possible causes operating separately or in combination: (*a*) There was an interaction between sucrose and the nucleoprotein to give a "binding" effect. (*b*) The nucleoprotein molecule served as an obstruction around which the diffusing sucrose

molecule had to flow. (*c*) The sucrose molecules diffused more slowly as a consequence of the high solution viscosity contributed by the nucleoprotein. The obstruction theory has been developed by Wang (37). The work of Biancheria and Kegeles (38) suggests that viscosity may be less important than the other factors, or even negligible.

A point of some importance should be emphasized with respect to the results of the Dische test. When the DNA-type nucleoproteins were dissolved in water and precipitated from 0.15 M NaCl, sufficient DNA remained in the supernatant solution to give a visual color with the diphenylamine reagent. In the seven successive extracts of the tissue using 0.15 M NaCl no visual color was observed in the case of the diphenylamine test. The absence of color when these extracts were tested cannot therefore be attributed to the lack of intrinsic solubility of the nucleohistones. The above considerations support the viewpoint that even under the exhaustive extraction procedures employed, the nucleohistone structures were not disrupted sufficiently to permit solution. The fact that the sediment from E_7 contained 1.7 per cent PNA suggested that the nuclear PNA had not been removed from the nucleus during the sequential extractions.

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