

## INTRACELLULAR LOCALIZATION OF ENZYMES IN SPLEEN\*

### I. REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE CYTOCHROME *c* REDUCTASE, CYTOCHROME *c* OXIDASE, AND SUCCINIC DEHYDROGENASE IN THE RAT AND GUINEA PIG

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In 1949, Hogeboom (1) reported that in rat liver homogenates which were prepared in 0.88 M sucrose and separated into four fractions by differential centrifugation, 32 and 58 per cent of the total DPNH<sup>1</sup> cytochrome *c* reductase activity of the original homogenates were recovered in the mitochondria and microsomes, respectively. He also found that, based on activity per mg. of nitrogen, the enzyme was concentrated 3.3 times in the microsome fraction and only 1.3 times in the mitochondria with respect to the homogenate. In 1951, as part of a study of the DPNH cytochrome *c* reductase of rabbit liver and heart mitochondria, Eichel (2) observed a 1.6- and 2.9-fold concentration of the enzyme in mitochondrial fractions prepared from isotonic sucrose homogenates of liver and heart, respectively. Cytochrome *c* oxidase assays on these (and other) fractions and the original homogenates confirmed the mitochondrial nature of the isolated fractions. Subsequently, Brody *et al.* (3) studied the distribution of DPNH cytochrome *c* reductase in various fractions obtained from homogenates of rat brain cerebral cortex. In contrast to the results with rat liver, they found a 2.8-fold concentration of the enzyme in the mitochondria with respect to the original homogenate, while the specific activity of the microsomal fraction was slightly less than that of the original homogenate. No data were given for the recovery of total enzyme activity in each of the particulate fractions. Strittmatter and Ball (4) and de Duve *et al.* (5) have also studied the distribution of the reductase in rat liver fractions. Their results are in essential agreement with those reported previously.

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<sup>1</sup>The following abbreviations will be used: DPNH = reduced diphosphopyridine nucleotide, DPN = oxidized diphosphopyridine nucleotide, DNA = deoxyribose nucleic acid.

Preparatory to initiating a study of the effect of whole-body x-irradiation on the DPNH cytochrome *c* reductase activity of the particulate components of rat spleen, an investigation of the enzyme distribution in fractions of normal spleens was undertaken. The present paper describes the results of this study. For comparative purposes, the distribution of DPNH cytochrome *c* reductase has also been studied in guinea pig spleen homogenates. In addition, data are presented for the distribution of succinic dehydrogenase and cytochrome *c* oxidase in spleen fractions. A preliminary report of this work has appeared (6).

### Methods

*Preparation and Fractionation of Spleen Homogenates.*—Male rats (100 to 175 gm.) of the Wistar strain, obtained directly from the Wistar Institute, were used. They were maintained on a stock commercial diet (Purina dog chow checkers). The guinea pigs (American breed) were all males weighing from 200 to 300 gm. and were fed Purina rabbit pellets. In each fractionation experiment, two spleens (wet weight per organ = 0.4 to 0.7 gm.) from either rats or guinea pigs were excised under ether anesthesia, washed free of adhering blood, and homogenized in cold 0.25 M sucrose with a Ten Broeck glass homogenizer. Exsanguination of the rats via heart puncture did not affect the results and was discontinued in the early phase of the investigation. The homogenate was diluted to 10 ml. with sucrose, 2 ml. were removed for nitrogen analysis, and the remainder was fractionated, with some changes, according to the method of Hogeboom *et al.* (7) as modified by Schneider and Hogeboom (8). A description of the operations, which were carried out at 0–4°, follows.

Eight ml. of homogenate were added to a 15 ml. conical tube and centrifuged at 600  $g^2$  for 10 minutes (International refrigerated centrifuge, Model PR-2, horizontal yoke No. 269). The supernatant was withdrawn carefully with the aid of a pipette with an attached rubber bulb. The bright red sediment was resuspended with the aid of a homogenizer in about 6 to 8 ml. of sucrose and recentrifuged at 600  $g$  for 10 minutes. The supernatant was withdrawn and added to the first supernatant. The pellet was again resuspended in sucrose, centrifuged, and the supernatant removed. The sediment was dispersed by homogenization in sucrose, made up to 15 ml. with sucrose, and termed the nuclear fraction. The combined supernatants were centrifuged at 5,000  $g$  for 15 minutes (Spinco model L ultracentrifuge, rotor No. 40). The supernatant, and as much as possible of the loosely packed, pink "fluffy layer" (see p. 402) were removed, and the mitochondrial pellet was resuspended by homogenization in 6 to 8 ml. of sucrose and centrifuged at 5,000  $g$  for 15 minutes. The supernatant above the original mitochondrial pellet was also recentrifuged at 5,000  $g$  for 15 minutes. The supernatants were withdrawn and combined for another centrifugation at 5,000  $g$  for 15 minutes. All sediments were combined, resuspended by homogenization in sucrose, and centrifuged at 6,600  $g$  for 10 minutes. The pellet was resuspended by homogenization in sucrose and made up to 10 ml. to give the tan mitochondrial fraction. The combined supernatants were centrifuged at 110,800  $g$  for 60 minutes. The red gelatinous microsomal pellet, identical in appearance with the liver fraction sedimenting at the same speed, was resuspended by homogenization in sucrose and made up to 10 ml. to give the microsomal fraction. The final supernatant, usually about 25 to 30 ml., represents the soluble or so called supernatant fraction. Four fractions were prepared in the same manner from homogenates of rat liver. Each fractionation procedure required about 3½ hours to complete.

<sup>2</sup> All values for centrifugal force were calculated for the bottom of the tubes.

Isolation of nuclei from homogenates of rat spleen was performed according to the method of Hogeboom *et al.* (9) which makes use of a sucrose-CaCl<sub>2</sub> layering technique. Two spleens (0.65 to 1.08 gm.) were homogenized with about 8 ml. of cold 0.25 M sucrose containing 0.0018 M CaCl<sub>2</sub>, and the homogenate was made up to 10 ml. with the same mixture. An 8 ml. aliquot of the homogenate was layered over 20 ml. of 0.34 M sucrose-0.00018 M CaCl<sub>2</sub> and centrifuged at 2,000 R.P.M. for 10 minutes (International refrigerated centrifuge, horizontal yoke No. 269). The centrifuge was accelerated and decelerated as slowly as possible in order to avoid mixing of the two layers. The entire supernatant was withdrawn, and the pellet (containing the nuclei) was resuspended in about 3 ml. of 0.25 M sucrose-0.00018 M CaCl<sub>2</sub> with the aid of a glass rod. The resuspended pellet was transferred quantitatively to a 7 ml. Ten Broeck homogenizer and homogenized for about 30 seconds to disperse clumps of nuclei. The homogenate was then taken up in a 5 ml. pipette and layered very slowly above 10 ml. of 0.34 M sucrose-0.00018 M CaCl<sub>2</sub>. A small volume of sucrose mixture was used to wash the walls of the homogenizer; this was also introduced above the sucrose mixture. In all, about 5 ml. of solution were layered. The mixture was centrifuged at 2,000 R.P.M. for 10 minutes. The procedure of homogenization of the pellet, layering, and centrifugation was repeated twice more. The final sediment of nuclei was dispersed by homogenization in 5 ml. of 0.25 M sucrose-0.00018 M CaCl<sub>2</sub>. The combined supernatants (70 to 78 ml.), containing mitochondria, microsomes, and the soluble material of the homogenate, were saved for analysis. The whole procedure was completed within a little less than 2 hours.

*Determination of Enzyme Activities.*—DPNH cytochrome *c* reductase activity was determined spectrophotometrically (Beckman DU spectrophotometer) at 25° essentially according to the method of Potter and Albaum (10). The following components were added to each cuvette in the order given: 0.025 to 0.2 ml. of a suitable dilution of homogenate or fraction, 0.2 ml. of 0.03 M KCN, 0.3 to 0.5 ml. of 0.1 M K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, water to make a volume of 3 ml., 1 mg. of cytochrome *c* in 0.25 to 0.75 ml. of water, and 0.3 to 0.4 micromole of DPNH in 0.5 to 0.7 ml. of 0.1 M K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. Throughout a large part of this work, each cuvette also contained 10 mg. of nicotinamide. This component was omitted later when it was observed that its absence did not affect reductase activity. Succinic dehydrogenase activity (literally succinic-cytochrome *c* reductase) was measured spectrophotometrically at 25° by a slightly modified method of Potter and Albaum (10). Each cuvette contained the following components: a suitable volume of homogenate or fraction which was incubated for 2 minutes with 8.1 mg. of sodium succinate in 0.1 ml. of water, 0.2 ml. of 0.03 M KCN, 1 ml. of 0.1 M K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, water to make a final volume of 3 ml., and 1 mg. of cytochrome *c* in 0.25 ml. of water. Cytochrome *c* oxidase was also assayed spectrophotometrically at 25° (10); each cuvette contained 1 mg. of cytochrome *c* previously reduced with sodium hydrosulfite (and aerated to destroy any excess of the latter) in 2 ml. of 0.1 M K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, water to make a final volume of 3 ml., and 0.025 to 0.05 ml. of a suitable dilution of either homogenate or isolated fraction. Rat spleen homogenate was further diluted 1:5 in 0.25 M sucrose for all enzyme assays. Guinea pig spleen homogenate was further diluted 1:40 in sucrose for assays of DPNH cytochrome *c* reductase and 1:5 for cytochrome *c* oxidase assays. Rat liver homogenate, originally diluted 1:10 prior to fractionation, was further diluted 1:25 for DPNH cytochrome *c* reductase and cytochrome *c* oxidase assays.

All enzyme activities were reasonably proportional to the amount of tissue present. Specific activities (*Q*) were calculated with the value  $1.96 \times 10^7$  sq. cm. per mole as the difference between the molecular extinction coefficients of oxidized and reduced cytochrome *c*. For DPNH cytochrome *c* reductase and succinic dehydrogenase, the reaction rates were zero order with respect to cytochrome *c* concentration, and the specific activities were expressed as *micromoles of cytochrome c reduced per minute per mg. of N.* For cytochrome *c*

oxidase, the reaction was first order with respect to cytochrome *c* concentration, and the specific activity was expressed as *per minute per mg. of N.*

*Determination of DNA and Total Nitrogen.*—DNA was extracted from spleen homogenates and fractions by the method of Schneider (11) and measured spectrophotometrically by the diphenylamine reaction (12) with the aid of the recent suggestions of Dische (13). Nitrogen was determined by a micro-Kjeldahl procedure.

*Materials.*—The DPNH used in these studies was prepared chemically with sodium hydrosulfite (14) from DPN of about 80 per cent purity obtained from the Sigma Chemical

TABLE I  
*Comparison between Data of Hogeboom (1) and This Paper of the Distribution of DPNH Cytochrome c Reductase in Rat Liver*

Fraction	DPNH cytochrome <i>c</i> reductase					Cytochrome <i>c</i> oxidase		
	Hogeboom*		This paper†			This paper‡		
	Q§	Recovery <i>per cent</i>	Total	Q§	Recovery <i>per cent</i>	Total¶	Q**	Recovery <i>per cent</i>
Homogenate	2.9	(100)	54.7	2.9	(100)	2789	147	(100)
Nuclei			6.8	1.9	12.5	690	188	24.7
Mitochondria	3.5	32	20.5	4.6	37.5	1843	417	66.1
Microsomes	9.2	58	26.4	7.5	48.4	44	11	1.6
Supernatant	0.4	5	1.4	0.2	2.5	0	0	0
Total recovery		95	55.1		100.9	2577		92.4

\* Fractionation carried out in 0.88 M sucrose.

† Each value for this report represents the amount found in, or derived from, 750 mg. of liver.

§ Micromoles of cytochrome *c* reduced per minute per mg. of N.

|| Micromoles of cytochrome *c* reduced per minute.

¶ Per minute.

\*\* Per minute per mg. of N.

Company. However, the DPNH was made up in 0.1 M  $K_2HPO_4$ - $KH_2PO_4$ , pH 7.4, instead of  $NaHCO_3$ - $Na_2CO_3$ . Cytochrome *c* of about 70 per cent purity was obtained from the Sigma Chemical Company.

## RESULTS

*Microscopic Examination.*—Under the phase microscope, preparations of rat and guinea pig spleen mitochondria appeared to consist predominantly of short rods and spheres. There was very little contamination of this fraction with either nuclei or blood cells. The microsome preparations contained many particles which were much smaller than those of the mitochondrial fractions. Tissue fragments, blood cells, nuclei, and some mitochondria were visible in the nuclear preparations. The supernatant fluids contained only a few small particles.

*DPNH Cytochrome c Reductase Activity and Nitrogen in Fractions of Rat Spleen.*—Data from a typical experiment showing the distribution of DPNH cytochrome *c* reductase in fractions of rat liver are presented in Table I. For

comparative purposes, values reported by Hogeboom (1) are also given. The results indicate that in our hands the technique of differential centrifugation yielded essentially the same data as are found in the literature except for small differences in the  $Q$  values of the mitochondria and microsomes. Simultaneous assays for cytochrome  $c$  oxidase were carried out, and these data are also listed in Table I. In agreement with numerous reports in the literature, the bulk of the activity was recovered in the mitochondria, and on the basis

TABLE II

*Distribution of Nitrogen and DPNH Cytochrome  $c$  Reductase, Cytochrome  $c$  Oxidase, and Succinic Dehydrogenase Activities in Subcellular Fractions Isolated from Rat Spleen*

The numbers represent the average values and average deviations. The numbers in parentheses next to each of the four components studied represent the number of preparations assayed.

Fraction	Nitrogen (10)		DPNH cytochrome $c$ reductase (10)			Cytochrome $c$ oxidase (5)			Succinic dehydrogenase (3)		
	Total	Recovery	Total*	$Q$ †	Recovery	Total‡	$Q$	Recovery	Total*	$Q$ †**	Recovery
	mg.	per cent¶			per cent¶			per cent¶			per cent¶
Homogenate	19.7	(100)	4.72	0.240±.018	(100)	768	35.9±2.5	(100)	2.41	0.105±.001	(100)
Nuclei	9.5	48.2±3.0	1.21	0.128±.014	25.6±2.3	141	13.9±3.1	18.3±5.5	0.50	0.044±.006	20.7±3.6
Mitochondria	1.6	8.4±1.1	1.41	0.910±.097	30.2±3.0	319	206.0±41.0	41.6±7.4	1.57	1.089±.125	65.1±3.9
Microsomes	2.6	12.5±2.0	1.89	0.791±.081	39.8±4.0	52	19.5±4.1	6.8±1.9	0.19	0.063±.002	7.9±0.5
Supernatant	5.6	28.6±3.4	0.39	0.068±.013	7.2±2.2	0	0	0	0	0	0
Reconstructed homogenate				0.243±.024			25.0±4.4			0.100±.002	
Total recovery	19.3	97.7±3.6	4.90		102.8±4.9	512		66.7±3.9	2.26		93.7±3.1

\* Micromoles of cytochrome  $c$  reduced per minute.

† Micromoles of cytochrome  $c$  reduced per minute per mg. of N.

‡ Per minute.

|| Per minute per mg. of N.

¶ Homogenate taken as 100 per cent.

\*\* All values in this column corrected for endogenous activity.

of nitrogen, there was a 3-fold concentration of the enzyme in the mitochondria with respect to the original homogenate.

Table II summarizes the results of experiments on the distribution of nitrogen and DPNH cytochrome  $c$  reductase activity in fractions of rat spleen. It can be seen that about half of the nitrogen was recovered in the nuclear fraction; in rat liver, about 22 per cent of the total nitrogen can be accounted for in this fraction. It will be shown later that isolation of nuclei by the sucrose- $\text{CaCl}_2$  layering technique reduced the nitrogen recovered in this fraction considerably without removing DNA; hence, it is likely that nuclei separated in the usual manner were contaminated with mitochondria and possibly adsorbed microsomes. Enzymatic data on isolated nuclei, to be presented later, would appear to strengthen this view. Only 8.4 and 12.5 per cent of the total nitrogen were recovered in the mitochondria and microsomes, respectively, while in rat liver, the mitochondria and microsomes contain about 25 and 20

per cent respectively of the total cellular nitrogen. The results of Table II are in good agreement with the data of Maver and Greco (15) for rat spleen and those of Maxwell and Ashwell (16) for mouse spleen.

The  $Q$  values for DPNH cytochrome  $c$  reductase indicate that the enzyme was concentrated to the greatest degree in the mitochondria and to a slightly lesser extent in the microsomes. About 40 per cent of the total activity was recovered in the microsomes and 30 per cent in the mitochondria. The  $Q$  value of the nuclear fraction was only half that of the original homogenate from which it was derived, suggesting that the activity was due to the presence of mitochondria and possibly adsorbed microsomes. Because of the large percentage of nitrogen represented by the nuclear fraction, the per cent recovery of enzyme in this fraction was rather high. The  $Q$  value of the supernatant fraction was very low and was due primarily to the small but significant rate of reduction of cytochrome  $c$  by various soluble endogenous substrates and probably also the presence of some microsomes. Centrifugation of the supernatant fraction for an additional 30 minutes at 110,800  $g$  did not alter this activity. The  $Q$  values for the reductase were uncorrected for the ability of each fraction to reduce cytochrome  $c$  in the absence of added DPNH (endogenous activity). However, these endogenous activities were studied during subsequent assays for succinic dehydrogenase, and in general, it can be said that for the supernatant fractions they were of about the same order as reported in Table II with DPNH as substrate. It should be pointed out that the endogenous rates of the supernatant fractions were not always directly proportional to the volume of fraction tested, and this made an accurate calculation of the absolute activities difficult. The reason for this phenomenon is not known. The total recovery of DPNH cytochrome  $c$  reductase was generally excellent. Aliquots of the nuclei, mitochondria, microsomes, and supernatant were also combined in the proportions in which they were present in the original homogenate to give a reconstructed homogenate. The latter, after mixing, was allowed to stand for 5 minutes at  $0^\circ$  and then assayed for DPNH cytochrome  $c$  reductase activity. The data of Table II show that the activities of the reconstructed homogenate and the original whole homogenate were the same.

It should be noted here that the so called "fluffy layer," which is found on top of the well packed mitochondrial pellet obtained from rat liver homogenate (17-21), was also observed as a pinkish layer on top of the more densely packed "tan" mitochondria separated from rat and guinea pig spleen homogenates. De Duve and Berthet (22) and Hogeboom and Schneider (23) have recently considered the evidence in support of either the mitochondrial or microsomal nature of the "fluffy layer." In the present report, the enzymatic properties of the "fluffy layer" were not investigated. However, when

it was retained with the mitochondria, rather than added to the microsomes which was the usual procedure, no striking shift in DPNH cytochrome *c* reductase activity was observed.

*Cytochrome c Oxidase Activity in Fractions of Rat Spleen.*—Various lines of evidence in the literature indicate that cytochrome *c* oxidase is localized exclusively in the mitochondria of the rat liver cell and other tissues. As a means of verifying the mitochondrial nature of the fraction from spleen homogenate sedimenting at 5,000 *g*, the distribution of cytochrome *c* oxidase activity was also studied in the homogenate and all fractions. The data in Table II show that, on the basis of nitrogen, cytochrome *c* oxidase was concentrated 6-fold in the mitochondria with respect to the whole homogenate. On the other hand, the low *Q* values of the microsomes and nuclei suggested that these activities were due to mitochondrial contamination. With respect to the nuclear fraction, the low specific and total activities found in isolated nuclei (as presented later) supported this view.

It should be noted that an average of only 67 per cent of the total cytochrome *c* oxidase activity was recovered among the four fractions. Furthermore, the *Q* values of reconstructed homogenates were only 55 to 75 per cent of those of the original homogenates. The fact that incomplete recoveries of the oxidase were accompanied consistently by complete recoveries of DPNH cytochrome *c* reductase suggested that the mitochondrial oxidase was unstable. Four separate fractionation experiments were carried out to determine if the recovery could be increased by assaying all fractions, and particularly the mitochondria, immediately after their preparation. With this precaution, recoveries of cytochrome *c* oxidase activity were 72, 88, 88, and 66 per cent, suggesting that some loss of activity might have occurred in the earlier work during the time that elapsed between the completion of the mitochondrial preparations and their assay. Nevertheless, the data presented in connection with Table III show clearly that 90 to 100 per cent of the oxidase was recovered in preparations of spleen homogenate from which only nuclei were isolated; in these cases, the mitochondria were not separated from the remainder of the splenic tissue. In addition, reconstitution of the homogenate gave a *Q* value which was 95 per cent of that of the original homogenate. Thus, it would appear that the low oxidase recoveries resulted from the separation of the mitochondria from the microsomes and supernatant.

The total recovery of cytochrome *c* oxidase activity among the four fractions obtained from rat liver was good (Table I). Schneider and Hogeboom (8) have reported an 80 per cent recovery of the succinoxidase activity of normal mouse liver and hepatoma and de Duve *et al.* (5) a 73 per cent recovery of the succinic dehydrogenase activity of rat liver. On the other hand, both groups recovered close to 100 per cent of the liver cytochrome *c* oxidase

activity. Brody *et al.* (3) found consistent recoveries of 108 to 120 per cent of the original cytochrome *c* oxidase activity among the fractions isolated from homogenates of rat liver and brain.

TABLE III

*Distribution of DNA, Nitrogen, and DPNH Cytochrome c Reductase and Cytochrome c Oxidase Activities between Isolated Nuclei and Remainder of Rat Spleen Homogenate*

The numbers for DNA, nitrogen, and the reductase represent the average values and ranges; for the oxidase, the numbers represent the average and actual values. The numbers in parentheses next to each of the four components studied represent the number of preparations assayed.

Fraction	DNA (5)			Nitrogen (5)		DPNH cytochrome <i>c</i> reductase (3)			Cytochrome <i>c</i> oxidase (2)		
	Total*	Recovery	Per mg. N	Total*	Recovery	Total†	Q‡	Recovery	Total	Q¶	Recovery
	µg.	per cent	µg.	mg.	per cent			per cent			per cent
Homogenate	7,693 (5,203-9,520)	(100)	393 (327-450)	20.1 (11.6-27.9)	(100)	5.12 (3.86-6.56)	0.254 (0.232-0.266)	(100)	587 (388, 786)	24.3 (20.7, 27.8)	(100)
Isolated nuclei	6,948 (4,573-8,184)	90.9 (79.3-99.7)	937 (701-1,041)	7.8 (3.8-10.2)	38.8 (31.9-52.6)	0.25 (0.20-0.31)	0.050 (0.042-0.059)	4.9 (4.7-5.2)	22 (6, 38)	3.3 (1.5, 5.1)	3.2 (1.6, 4.8)
Combined supernatants	603 (161-1,671)	7.5 (2.2-17.6)	33 (17-53)	12.3 (7.4-15.7)	61.2 (50.0-68.6)	4.70 (3.55-6.37)	0.367 (0.353-0.388)	91.8 (84.4-97.1)	550 (339, 761)	35.2 (28.1, 42.2)	92.0 (87.2, 96.8)
Isolated nuclei + Combined supernatants							0.250 (0.231-0.260)			22.9 (19.4, 26.3)	
Total recovery	7,551 (4,965-9,216)	98.4 (95.4-101.8)		20.1 (11.2-27.3)	100.0 (95.7-108.0)	4.95 (3.75-6.68)		96.7 (89.5-101.8)	572 (345, 798)		95.2 (88.8, 101.6)

\* The values represent the amounts found in, or derived from, five pairs of spleens whose wet weights were 674, 811, 984, 425, and 860 mg.

† Micromoles of cytochrome *c* reduced per minute. The values represent the amounts found in, or derived from, three pairs of spleens whose wet weights were 640, 688, and 1,044 mg.

‡ Micromoles of cytochrome *c* reduced per minute per mg. of N.

§ Per minute. The values represent the amounts found in, or derived from, two pairs of spleens whose wet weights were 688 and 1,044 mg.

¶ Per minute per mg. of N.

*Succinic Dehydrogenase Activity in Fractions of Rat Spleen.*—In view of the low cytochrome *c* oxidase recoveries that were observed in rat spleen, it was of interest that Maxwell and Ashwell (16) reported an 89 per cent recovery of succinoxidase activity in fractionation experiments with mouse spleen homogenates. At least two enzymes are involved in the succinoxidase system, succinic dehydrogenase and cytochrome *c* oxidase, and since the latter is generally present in excess over the dehydrogenase, of the two, the dehydrogenase is probably the rate-limiting component. Hence, complete recovery



of the dehydrogenase could be expected to result in complete recovery of over-all succinoxidase activity even though the recovery of cytochrome *c* oxidase were incomplete. Another explanation for the difference in the recoveries of spleen succinoxidase and cytochrome *c* oxidase might lie simply in a difference between preparations from rat and mouse spleen. These possibilities were tested by studying the distribution of succinic dehydrogenase in fractions of rat spleen homogenates. Ninety-four to 100 per cent of the total succinic dehydrogenase activity was recovered in the four fractions (Table II), while the activity of the reconstructed homogenate equalled that of the original homogenate. In simultaneous assays for cytochrome *c* oxidase, the total recovery of this enzyme was again only 65 to 70 per cent. Since it can be calculated for rat spleen homogenate that the rate of oxidation of ferrocyanochrome *c* by cytochrome *c* oxidase is about four times the rate of reduction of ferricytochrome *c* by succinic dehydrogenase, the latter is rate-limiting with respect to cytochrome *c* oxidase in the succinoxidase system. Therefore, the complete recovery of succinic dehydrogenase is consistent with the succinoxidase recovery reported by Maxwell and Ashwell.

On a nitrogen basis, succinic dehydrogenase activity was concentrated about 10 times in the mitochondria (Table II), and 65 per cent of the total activity was recovered in this fraction. The presence of 8 per cent of the activity in the microsomes was undoubtedly due to the incomplete removal of mitochondria. This was probably also true of the nuclear activity, where the 21 per cent recovery paralleled the results with DPNH cytochrome *c* reductase and cytochrome *c* oxidase.

*Experiments with Nuclei Isolated From Rat Spleen Homogenates.*—In view of the relatively large recovery of enzyme activities in the nuclear fraction, rat spleen nuclei were isolated by the sucrose-CaCl<sub>2</sub> layering technique in an effort to remove mitochondria. With preparations of rat liver nuclei, this procedure has been shown to reduce contamination with free mitochondria to less than 0.5 per cent (9). A marked lowering of enzyme activity in the nuclear fraction would suggest that the nuclei themselves contain little, if any, of the three enzymes studied. As indicated in Table III, an average of 91 per cent of the original spleen DNA was found in the nuclear fraction, and except for one case, the over-all recovery was close to 100 per cent. The isolated nuclei accounted for about 32 per cent of the total nitrogen in three of the five preparations summarized; in three other preparations whose results were not tabulated, only 25 per cent of the nitrogen was recovered in the nuclear fraction. These data are to be compared with the value of 48 per cent total nitrogen recovered in "unpurified" nuclear fractions (Table II). Similarly, in studies with nuclei isolated from liver (9), the nitrogen content was shown to be markedly reduced as compared with values found when no special attempt (7) was made to obtain a complete separation of nuclei and mitochondria (22 per cent reduced to 12 per cent). For the homogenate, the

average ratio for micrograms of DNA per mg. of nitrogen was 393, and the DNA per mg. wet wt. ratio was 10.2. These figures fall within the range of values reported in the literature. DNA per mg. wet wt. ratios of 16.5 (24), 15.9 (25), 8.6 (26), and 7.7 (27) for rat spleen homogenates have been found, and a ratio of 12.8 has been described in mouse spleen homogenates (16). The explanation for this wide variation in DNA content undoubtedly lies in the use of different methods of extraction and determination.

The data of Table III also indicate that the DPNH cytochrome *c* reductase specific activity of the nuclear fraction was reduced to  $\frac{1}{2}$  that of the homogenate, and the total activity was lowered to about 5 per cent. Since 84 to 97 per cent of the total reductase activity was recovered in the combined supernatants (mitochondria, microsomes, and the soluble portion), and since little nuclear material was lost from the nuclear fraction as shown by the DNA analysis, it is very likely that little, if any, of the enzyme is present in spleen cell nuclei. The results listed in Table III demonstrate that this holds for cytochrome *c* oxidase, as well.

*Distribution of Nitrogen and Enzyme Activities in Fractions of Guinea Pig Spleen.*—The distribution of nitrogen, DPNH cytochrome *c* reductase, and cytochrome *c* oxidase in guinea pig spleen homogenates and fractions derived from them is given in Table IV. The nitrogen content of each fraction was very similar to the nitrogen content of the respective rat fractions. Per mg. of nitrogen, the reductase was concentrated 4-fold in the microsomes, but only 1.7 times in the mitochondria, compared to the whole homogenate. Fifty to 60 per cent of the activity was recovered in the microsomes, and most of the remainder was split between the mitochondria and nuclei. The latter fraction, oddly, consistently had somewhat more total enzyme than the mitochondria, and as in the case of rat spleen, it is likely that the nuclear activity represented mitochondrial contamination. The total recovery of reductase activity among the four guinea pig fractions was excellent, as was the specific activity of the reconstructed homogenate. The cytochrome *c* oxidase data resembled those of rat spleen in all respects. It can also be calculated that the specific activity of DPNH cytochrome *c* reductase of guinea pig spleen homogenate is equal to, or slightly greater than, that of its cytochrome *c* oxidase.

*Comparison of the Concentration of DPNH Cytochrome *c* Reductase Activity in Mitochondria and Microsomes from Various Sources.*—It may be of interest to compare the DPNH cytochrome *c* reductase concentration (ratio of specific activity of fraction to specific activity of homogenate) of rat and guinea pig spleen mitochondria and microsomes with each other as well as with similar fractions isolated from other organs of other animals. These data are compiled in Table V. First, it will be noted that the reductase concentration of rat spleen mitochondria is considerably different from that of rat liver mitochondria (compare 3.8 with 1.25), while the enzyme concentrations of guinea pig spleen

mitochondria and microsomes resemble those of both rat liver particulates (compare 1.68 with 1.25 and 3.95 with 3.29). In addition, 50 to 58 per cent of the total activity of guinea pig spleen reductase was recovered in the microsomes, while 48 to 58 per cent of the total activity of rat liver reductase was recovered in this fraction. Thus, we find that the intracellular distribution

TABLE IV

*Distribution of Nitrogen and DPNH Cytochrome c Reductase and Cytochrome c Oxidase Activities in Subcellular Fractions Isolated from Guinea Pig Spleen*

The numbers for nitrogen and the reductase represent the average values and ranges; for the oxidase, the numbers represent the average and actual values. The numbers in parentheses next to each of the three components studied represent the number of preparations assayed.

Fraction	Nitrogen (3)		DPNH cytochrome c reductase (3)			Cytochrome c oxidase (2)		
	Total	Recovery	Total*	Q†	Recovery	Total‡	Q	Recovery
	mg.	per cent			per cent			per cent
Homogenate	16.6 (15.0-19.0)	(100)	22.4 (19.6-26.5)	1.38 (1.24-1.42)	(100)	493 (473,512)	32.0 (29.8,34.2)	(100)
Nuclei	6.7 (5.8-7.9)	40.2 (36.4-42.8)	4.8 (3.6-7.1)	0.71 (0.59-0.91)	21.0 (17.8-26.9)	31 (24,38)	5.0 (4.1,5.9)	
Mitochondria	1.5 (1.2-1.8)	8.8 (8.4-9.2)	3.2 (2.7-3.8)	2.16 (1.91-2.41)	14.1 (13.9-14.3)	186 (185,187)	141.0 (129.9,152.1)	
Microsomes	2.3 (2.0-2.7)	13.8 (13.5-14.4)	12.4 (10.6-15.1)	5.36 (5.24-5.53)	55.2 (50.3-58.1)	37 (39,35)	17.8 (18.3,17.2)	
Supernatant	5.0 (4.7-5.7)	30.9 (30.1-31.6)	1.0 (0.8-1.5)	0.20 (0.17-0.31)	4.8 (2.8-7.8)	0	0	
Reconstructed homogenate				1.40 (1.27-1.47)			18.0 (16.5,19.5)	
Total recovery	15.5 (14.3-18.1)	93.7 (89.8-96.4)	21.4 (18.2-26.8)		95.1	254 (248,260)		51.6 (50.8,52.4)

\* Micromoles of cytochrome c reduced per minute.

† Micromoles of cytochrome c reduced per minute per mg. of N.

‡ Per minute.

|| Per minute per mg. of N.

pattern of an enzyme in an organ of one species (guinea pig spleen) may resemble that pattern more closely in a different organ of a different species (rat liver) than it does the pattern in the same organ of a different species (rat spleen). These comparisons do not, of course, consider that the cytochrome reductases cited in Table V may not be identical.

Other workers have reported on differences in the distribution patterns, either with respect to activity per mg. of nitrogen or total activity, of what appears to be the same enzyme in the same organ of different animals. For example, Nyberg *et al.* (28) showed that, on a nitrogen basis, guinea pig liver catalase was concentrated to an extremely high degree in a combined micro-

somes-supernatant fluid fraction which also contained 70 per cent of the total activity. In rat liver, however, 60 per cent of the catalase was recovered in the mitochondria, and the activity per mg. of nitrogen was only four times greater than that of the homogenate. Fumarase activity was found largely in

TABLE V  
*Ratios of Specific Activity of DPNH Cytochrome c Reductase in Mitochondria/Homogenate and Microsomes/Homogenate from Different Animals and Organs*

Animal	Organ	Mitochondria* Homogenate	Reference in Literature	Microsomes* Homogenate	Reference in Literature
Rat	Liver	1.25	(1)	3.29	(1)
Mouse	Liver	1.20	(36)	2.57	(36)
Rabbit	Liver	1.6	(2)		
Rat	Spleen	3.80	This paper	3.30	This paper
Guinea pig	Spleen	1.68	This paper	3.95	This paper
Rat	Brain	2.82	(3)	1.24	(3)
Rabbit	Heart	2.9	(2)		

\* All ratios are based on specific activity calculated as micromoles of cytochrome *c* reduced per minute per mg. of N at 25°.

TABLE VI  
*DPNH Cytochrome c Reductase Activity in Spleen, Liver, and Heart Homogenates from Different Sources*

Animal	DPNH cytochrome <i>c</i> reductase activity*		
	Liver	Heart	Spleen
Rat	2.30 (2) ‡	1.15 (3)	0.24 (11)
Guinea pig	0.77 (4)	0.63 (4)	1.49 (7)
Rabbit	1.19 (5)	0.36 (5)	

\* Micromoles of cytochrome *c* reduced per minute per mg. of N.

‡ Numbers in parentheses represent the number of separate determinations.

the supernatant fraction of human liver (29) and the mitochondria of rabbit brain (30) and mouse liver (31).

*Comparison of DPNH Cytochrome c Reductase Activity in Homogenates from Various Sources.*—Another point of interest is the comparison between the specific activities of DPNH cytochrome *c* reductase of the two types of spleen homogenate studied here and the reductase activities of other tissues from the same and other species. Some time ago, we reported (2) that the DPNH cytochrome *c* reductase activity of rabbit liver homogenate was three times greater than that of rabbit heart homogenate. This was of particular interest because other components of the electron transport chain are usually far more active, or present in greater concentration, in heart than in liver, *i.e.*, cyto-

chrome *c* oxidase activity of rat heart is five times that of liver (32), while the cytochrome *c* concentration of rat heart is from 3 to 8 times that of liver (32, 33). A summary of the DPNH cytochrome *c* reductase activity in spleen, liver, and heart from the rat, guinea pig, and rabbit is presented in Table VI. Since the rabbit data were obtained five years earlier, they are strictly comparable with the figures for the rat and guinea pig only insofar as liver is compared with heart. The absolute values for the two rabbit tissues may actually be somewhat higher. First, it can be seen that guinea pig spleen is considerably more active than rat spleen and twice as active as guinea pig liver, whereas in the rat, liver is far more active than spleen. And finally, in the three animals, heart enzyme activity is less than, or about equal to, that of liver. The significance, if any, of these somewhat unexpected cytochrome reductase activities cannot be explained within the limits of our present knowledge.

#### DISCUSSION

Because of the marked heterogeneity of the spleen cell population, it is not possible to state at this time which cell type (or types) gives rise to the particles of the isolated fractions studied here. However, a recent study<sup>3</sup> has revealed that no destruction of DPNH cytochrome *c* reductase activity occurs in homogenates and their derived fractions obtained from the spleens of rats exposed to 700 r of whole-body x-irradiation. Assays were carried out on preparations from animals sacrificed 13 to 85 hours after irradiation. A detailed report of the histopathology (34) of spleens from rats given 600 r of x-rays to the whole body has indicated drastic reduction in the absolute as well as the relative amount of the white pulp, obliteration of the germinal centers of the white pulp after 3 hours, almost complete destruction of medium sized lymphocytes and somewhat lesser disappearance of small lymphocytes, extensive degeneration of the erythroblasts of the red pulp between 8 hours and 2 days after exposure, and reduction of the number of megakaryocytes. Moderate reconstitution of the white pulp was observed only after 3 weeks. It is possible that the reticular cells, which become very prominent in appearance and show clumped nuclear chromatin and occasional giant nuclei but are never seen to die (34), are a major source of the particles, and hence the DPNH cytochrome *c* reductase and other enzymes, studied here. The importance of electron microscope studies for the cytologic characterization of cell fractions is well known. In the final analysis, a correlated electron microscope study of whole spleen sections as well as isolated spleen fractions will be required for this characterization. Nevertheless, it would seem that the high level of reproducibility of the data obtained and the close conformity of the enzyme distribution patterns with those reported for fractions which sediment from liver at similar centrifugal forces, reflect the state of

<sup>3</sup> Eichel, H. J., in press.

intracellular enzyme distribution in the spleen under normal physiological conditions. While the chemical and enzymatic data are quite uniform from preparation to preparation, this is not taken to imply either morphological or biochemical homogeneity of the isolated granules. Even in the case of liver, a far more homogeneous organ than spleen with regard to cell type, experiments from a number of laboratories have suggested that isolated mitochondria and microsomes are biochemically heterogeneous. This evidence has been reviewed recently (23). Indeed, by ultracentrifugation Petermann and Hamilton (35) have isolated several different groups of macromolecular particles from preparations of submicroscopic particles obtained from hypertonic sucrose homogenates of normal and leukemic mouse spleen.

#### SUMMARY

1. The intracellular distribution of nitrogen, DPNH cytochrome *c* reductase, succinic dehydrogenase, and cytochrome *c* oxidase has been studied in fractions derived by differential centrifugation from rat and guinea pig spleen homogenates.

2. In the spleens of each species, the nuclear fraction accounted for 40 to 50 per cent of the total nitrogen content of the homogenate, and the mitochondrial, microsome, and supernatant fractions contained about 8, 12, and 30 per cent of the total nitrogen, respectively.

3. Per mg. of nitrogen, DPNH cytochrome *c* reductase was concentrated in the mitochondria and microsomes of both rat and guinea pig spleens. Seventy per cent of the total DPNH cytochrome *c* reductase activity was recovered in these two fractions. The reductase activity associated with the nuclear fraction was lowered markedly by isolating nuclei from rat spleens with the sucrose-CaCl<sub>2</sub> layering technique. The lowered activity was accompanied by the recovery of about 90 per cent of the homogenate DNA in the isolated nuclei, indicating that little, if any, of the reductase is present in spleen cell nuclei.

4. Per mg. of nitrogen, succinic dehydrogenase was concentrated about 10-fold in the mitochondria of rat spleen, and 65 per cent of the total activity was recovered in this fraction.

5. Cytochrome *c* oxidase was concentrated, per mg. of nitrogen, in the mitochondria of both rat and guinea pig spleens. The activity associated with the nuclear fraction was greatly diminished when this fraction was isolated from rat spleens by the sucrose-CaCl<sub>2</sub> layering technique. Only 50 to 70 per cent of the total cytochrome *c* oxidase activity of the original homogenates was recovered among the four fractions from both rat and guinea pig spleens, while the specific activities of reconstructed homogenates were only 55 to 75 per cent of those of the original whole homogenates. This was in contrast to the results with DPNH cytochrome *c* reductase and succinic dehydrogenase where the recovery of total enzyme activity approached 100 per cent, and the

specific activities of reconstructed homogenates equalled those of the original homogenates. The recovery of cytochrome *c* oxidase was greatly improved when only the nuclei were separated from rat spleen homogenates.

6. Data were presented comparing the concentrations (ratio of activity per mg. of nitrogen of the fraction to activity per mg. of nitrogen of the homogenate) of DPNH cytochrome *c* reductase in mitochondria and microsomes derived from different organs of different animals.

7. Data were presented comparing the activities per mg. of nitrogen of DPNH cytochrome *c* reductase in homogenates from several organs of various animals.

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