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Tissue Fractionation Studies

12. INTRACELLULAR DISTRIBUTION OF SOME DEHYDROGENASES, ALKALINE DEOXYRIBONUCLEASE AND IRON IN RAT-LIVER TISSUE

By H. BEAUFAY,* D. S. BENDALL,† P. BAUDHUIN AND C. DE DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

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In a previous publication from this Laboratory, a new scheme of fractionation of rat-liver homogenates permitting a more detailed analysis of the large granules has been described (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). In the present investigations, this scheme has been applied to a study of the intracellular distribution of a few enzymes which were either known or suspected to be associated with the large granules: the diphosphopyridine nucleotide-linked dehydrogenases of glutamic acid, malic acid and β -hydroxybutyric acid and alkaline deoxyribonuclease (deoxyribonuclease I). In addition, we have studied the distribution of iron to verify the possibility, suggested by electron-microscope observations, that particles rich in ferritin might be concentrated in the same rat-liver fraction as the lysosomes (Novikoff, Beaufay & de Duve, 1956). In all these experiments, cytochrome oxidase, acid phosphatase and glucose 6-phosphatase were measured simultaneously to serve as reference enzymes. Measurements were also made of acid deoxyribonuclease (deoxyribonuclease II), which has been studied before (de Duve *et al.* 1955) but was assayed here by an improved technique. Some of the results obtained have been reported previously in preliminary communications (de Duve & Beaufay, 1957; Baudhuin, 1959).

* Chargé de recherches du FNRS.

† Present address: Department of Biochemistry, Cambridge.

METHODS

The tissue fractionations and determinations of reference enzymes were performed according to the methods described by Appelmans, Wattiaux & de Duve (1955), de Duve *et al.* (1955) and Wattiaux & de Duve (1956), except that cytochrome oxidase was assayed at 25° instead of at room temperature.

Glutamic dehydrogenase was measured by the following modifications of the methods described by Hogeboom & Schneider (1953). For the forward reaction [diphosphopyridine nucleotide (DPN) reduction] the final reaction mixture contained 0.02 M-potassium phosphate buffer, pH 7.7, 0.03 M-nicotinamide, 0.4 mM-sodium cyanide, 0.1% of Triton X-100, mM-cysteine, 13 mM-potassium glutamate, 1.4 mM-DPN and enzyme in a total volume of 3 ml. The reaction was started by the addition of enzyme and the increase in extinction at 340 m μ and 25° was followed in a Beckman model DU spectrophotometer with a reference cell containing all components of the system except glutamate. Triton X-100 was used to release the enzyme; it also causes a reversible inhibition (about 28%) which was ignored in these experiments since the conditions were the same for all fractions. Cysteine could be replaced by mM-ethylenediaminetetra-acetic acid (EDTA).

For the back reaction the reaction mixture contained 0.02 M-potassium phosphate buffer, pH 7.7, 0.03 M-nicotinamide, 0.4 mM-sodium cyanide, 0.1% of Triton X-100, mM-EDTA, 0.01 M-sodium α -oxoglutarate, 0.05 M-ammonium chloride, 0.28 mM-reduced DPN (DPNH) and enzyme in a total volume of 1.8 ml. In order to avoid an initial lag-phase in the reaction, it was found necessary to pre-incubate the enzyme with all components of the reaction mixture except α -oxoglutarate at 25° for 15 min. The reaction was then started by the addition of 0.18 ml. of

0.1M-sodium α -oxoglutarate and the decrease in extinction at 340 m μ and 25° was followed in the spectrophotometer against a blank containing 0.22 mM-DPNH and all other components of the system except α -oxoglutarate. Under these conditions, the activity was proportional to enzyme concentration.

Malic dehydrogenase was assayed at 25° by following the increase in extinction at 340 m μ in 1.8 ml. of a reaction mixture containing 0.02M-2-amino-2-hydroxymethylpropane-1,3-diol (tris)-HCl buffer, pH 8.5, mM-EDTA, 0.01M-sodium cyanide, pH 8.5, 0.03M-nicotinamide, 0.1% of Triton X-100, 0.7 mM-DPN and 0.05M-sodium L-malate. This mixture, in which the high concentration of cyanide was necessary to trap the oxalacetate, was allowed to stand at 25° for at least 1 hr. to allow the DPN and cyanide to equilibrate. The reaction was started by the addition of 0.05–0.1 ml. of the enzyme preparation. The reference cell contained all the components of the system except L-malate. The rates measured in this manner were constant with time up to a change in extinction of 0.1, and proportional to the concentration of enzyme.

The assay of β -hydroxybutyric dehydrogenase was carried out in a similar manner with 0.05M-sodium DL- β -hydroxybutyrate as substrate. Only the D-isomer will have been oxidized since the L-isomer is only oxidized after conversion into the coenzyme A derivative (Lehninger & Greville, 1953), which could not have been formed under the experimental conditions used. The reaction mixture was made up as for malic dehydrogenase, with the exception that the cyanide concentration was 0.4 mM and that Triton X-100 was omitted. This, as well as a number of other detergents, was found to inhibit the enzyme strongly. Activation of the enzyme was obtained by leaving the preparations in the deep freeze overnight and then, after thawing, treating them for 10 min. in a cooled Blender (Measuring and Scientific Equipment Ltd., London) turning at full speed. It is not certain that this treatment gave 100% activation, and this may help to explain the low recovery obtained. The progress curve was linear up to a difference in extinction of about 0.25, and the measured rates were proportional to the enzyme concentration.

Alkaline deoxyribonuclease was measured in a manner similar to that adopted for the acid enzyme (de Duve *et al.*

1955; Wattiaux & de Duve, 1956) by measuring spectrophotometrically the amount of acid-soluble oligonucleotides formed at 37° in 2 ml. of a reaction mixture containing 0.05M-tris-HCl buffer, pH 7.6, mM-magnesium chloride, 0.1% of Triton X-100 and 1.5 mg. of potassium deoxyribonucleate (DNA). When followed in this manner, the reaction progresses along a typical sigmoid curve, shown in Fig. 1. In practice, the assays were performed with three different amounts of enzyme and the value or values falling on the linear part of the reference curve (approximately between 0.5 and 1.5 units) were used to estimate the activity graphically.

Acid deoxyribonuclease was measured as before (Wattiaux & de Duve, 1956), except that the medium contained 0.2M-KCl. This ensures optimum activity (Rotherham, Schottelius, Irvin & Irvin, 1956; Shack, 1957) and has the additional advantage of inhibiting the alkaline enzyme completely.

Easily detachable iron, representing essentially ferritin and haemosiderin, was determined according to a technique adapted from those of Kitzes, Elvehjem & Schnette (1944) and of Watson (1952). The tissue samples were extracted for 10 min. in a boiling-water bath with 0.5M-sodium acetate-acetic acid buffer, pH 4.7, 0.06M-L-ascorbic acid and 0.05% of 2:2'-dipyridyl. The extracts were filtered and their extinction was measured colorimetrically with a light-filter of maximal transmittance at 500 m μ . Total iron was determined similarly after digestion in conc. H₂SO₄.

In conformity with previous publications (de Duve *et al.* 1955) units of enzymic activity are defined as the amount of enzyme causing the decomposition of 1 μ mole of substrate/min. under the conditions of the assays (except for cytochrome oxidase, see Appelmans *et al.* 1955). In these calculations, a molar-extinction coefficient at 340 m μ of 6.22×10^4 cm.² mole⁻¹ was used for DPNH (Horecker & Kornberg, 1948) and the breakdown of DNA was evaluated in mononucleotides on the basis of an average molar-extinction coefficient at 260 m μ of 8.5×10^4 cm.² mole⁻¹ (Stimson & Reuter, 1945).

The materials used in these experiments were the following: Triton X-100 (Rohm and Haas, Co., Philadelphia, Pa., U.S.A.); DL-sodium β -hydroxybutyrate (British Drug Houses Ltd.), DPN (Sigma Chemical Co., St Louis, Mo., U.S.A.); tris (Sigma Chemical Co.); L-glutamic acid (Pfaffstiehl Chemical Co., Waukegan, Ill., U.S.A.); L-cysteine hydrochloride (Hofmann-La Roche and Co., Basle, Switzerland); α -oxoglutaric acid (Sigma Chemical Co.). Other substrates were those described by de Duve *et al.* (1955).

RESULTS

Distribution of enzymes

The enzyme distributions found are listed in Table 1 and shown graphically in Fig. 2. The results obtained on the reference enzymes and on acid deoxyribonuclease were essentially similar to those recorded previously (de Duve *et al.* 1955), except that the nuclear fraction was more heavily contaminated with cytoplasmic components. There is also a somewhat greater variability in the values obtained for the heavy mitochondrial (M) and light

Fig. 1. Kinetics of alkaline deoxyribonuclease. Results of different experiments in which enzyme concentration (●) or incubation time (○) were varied.

Table 1. Intracellular distribution of enzymes

Absolute values are given in mg./g. for nitrogen; in units/g. for enzymes. Statistics refer to the means $\pm$ s.d. These are given rather than the s.e.m. since they are a measure of individual variability, the most interesting information in the present case. E, Cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant.

	No. of experiments	Absolute values	Percentage values						
			E+N	N	M	L	P	S	Recovery
Nitrogen	4	35.4 $\pm$ 5.2	100	22.3 $\pm$ 4.3	16.3 $\pm$ 2.3	5.4 $\pm$ 1.8	22.3 $\pm$ 3.8	34.1 $\pm$ 3.8	100.4 $\pm$ 4.0
Cytochrome oxidase	3	21.2 $\pm$ 7.4	100	14.7 $\pm$ 4.5	50.2 $\pm$ 4.8	18.4 $\pm$ 8.1	6.1 $\pm$ 3.1	—	89.4 $\pm$ 9.2
Glutamic dehydrogenase	2	13.4 (59.4)*	100	17.9 $\pm$ 8.1	56.3 $\pm$ 8.6	14.4 $\pm$ 10.1	4.9 $\pm$ 0.3	2.2 $\pm$ 1.4	95.7 $\pm$ 5
Malic dehydrogenase	1	53.4	100	16.4	60.0	11.4	1.5	11.2	100.5
β -Hydroxybutyric dehydrogenase	1	11.4	100	14.4	54.7	6.2	4.4	0.3	80.0
Alkaline deoxyribonuclease	3	0.927 $\pm$ 0.26	100	20.6 $\pm$ 3.6	52.8 $\pm$ 8.0	12.7 $\pm$ 7.3	15.7 $\pm$ 5.8	3.6 $\pm$ 6.2	105.4 $\pm$ 9.1
Acid phosphatase	4	6.40 $\pm$ 0.98	100	11.9 $\pm$ 3.1	17.2 $\pm$ 6.9	33.2 $\pm$ 5.5	23.2 $\pm$ 4.6	9.7 $\pm$ 3.4	95.2 $\pm$ 4.2
Acid deoxyribonuclease	3	0.716 $\pm$ 0.20	100	17.9 $\pm$ 7.5	25.4 $\pm$ 3.8	32.0 $\pm$ 15.4	15.1 $\pm$ 1.6	6.9 $\pm$ 6.9	97.3 $\pm$ 13.4
Glucose 6-phosphatase	4	22.2 $\pm$ 4.7	100	17.1 $\pm$ 6.5	3.2 $\pm$ 0.6	3.7 $\pm$ 0.9	80.6 $\pm$ 17.6	2.0 $\pm$ 0.8	106.6 $\pm$ 17.6

* Value in parentheses is for DPNH oxidation (back reaction); the other value is for DPN reduction (forward reaction).

mitochondrial (L) fractions in individual experiments. This is due to slight differences in the manner in which the supernatant was decanted from the M sediment.

As could already be suspected from the data of Hogeboom & Schneider (1953) and of Allard, de Lamirande & Cantero (1957), the distribution of glutamic dehydrogenase paralleled that of cytochrome oxidase. So did those of malic dehydrogenase, β -hydroxybutyric dehydrogenase and alkaline deoxyribonuclease, except for a 10% excess of alkaline deoxyribonuclease in the microsomes and of malic dehydrogenase in the soluble fraction.

Distribution of iron

In Table 2 and Fig. 3 are shown the results of five experiments in which the distribution of easily detachable iron was measured. In conformity with the data of Thiers & Vallee (1957) and of Kuff & Dalton (1957), the largest proportion of the hepatic iron was recovered in the final supernatant, from which fraction Kuff & Dalton (1957) have been able to separate ferritin particles by prolonged ultracentrifuging. The remainder of the iron was distributed diffusely amongst the particulate fractions, with, however, a small but significant peak in the light mitochondrial fraction (L), which is richest in lysosomes (Fig. 3). As shown by Fig. 4, there is a distinct correlation between the iron concentration and the specific activity of acid phosphatase in this fraction, an indication that the two components are purified simultaneously. In

Fig. 2. Distribution patterns of enzymes. Ordinate: mean relative specific activity of fractions. Abscissa: fractions are represented by their relative nitrogen content, in the order in which they are isolated. Numbers in parentheses refer to numbers of experiments. Abbreviations are defined in Table 1.

Table 2. *Intracellular distribution of iron*

Results of five experiments: absolute values are given in mg./g. for nitrogen and iron; in units/g. for enzymes. Statistics refer to the means $\pm$ s.d. Abbreviations are defined in Table 1.

Component	Absolute values E+N	Percentage values						Recovery
		E+N	N	M	L	P	S	
Nitrogen	34.9 $\pm$ 2.7	100	15.8 $\pm$ 3.6	17.8 $\pm$ 2.1	3.7 $\pm$ 0.5	20.8 $\pm$ 2.6	35.7 $\pm$ 2.1	93.8 $\pm$ 2.9
Cytochrome oxidase	26.1 $\pm$ 6.0	100	13.7 $\pm$ 7.3	57.4 $\pm$ 5.6	13.3 $\pm$ 9.0	7.2 $\pm$ 1.9	—	91.6 $\pm$ 11.8
Acid phosphatase	6.54 $\pm$ 0.66	100	6.5 $\pm$ 1.3	22.5 $\pm$ 4.2	37.8 $\pm$ 2.6	18.1 $\pm$ 3.2	14.5 $\pm$ 2.0	99.4 $\pm$ 2.5
Glucose 6-phosphatase	22.0 $\pm$ 2.1	100	7.6 $\pm$ 1.7	2.5 $\pm$ 0.6	2.0 $\pm$ 0.4	85.2 $\pm$ 12.7	2.9 $\pm$ 1.4	100.2 $\pm$ 12.7
Easily detachable iron	0.281 $\pm$ 0.088	100	5.3 $\pm$ 0.9	6.6 $\pm$ 1.8	4.4 $\pm$ 0.8	10.9 $\pm$ 2.5	68.2 $\pm$ 4.3	95.4 $\pm$ 3.5
Total iron*	0.244 (0.211)	100	9.4 (5.1)	5.6 (6.3)	5.2 (4.9)	12.3 (9.0)	65.4 (75.1)	97.9 (100.4)

* Result of a single experiment. The values for easily detachable iron in the same experiment are given in parentheses.

one of the above experiments, the distribution of total iron was also measured. As indicated in Table 2, excess of iron, originating mostly from haemoglobin, was found essentially in the nuclear and microsomal fractions.

In another experiment, the animal was given 20 mg. of iron in the form of saccharated iron

Fig. 3. Distribution patterns of easily detachable iron and of acid phosphatase. Ordinate: mean relative specific content or activity of fractions. Abscissa: fractions are represented by their relative nitrogen content, in the order in which they are isolated. Results of five experiments. Abbreviations are defined in Table 1.

Fig. 4. Correlation between relative specific iron content and relative specific acid phosphatase activity of light mitochondrial (L) fractions. Results from five fractionations and three other purification experiments.

Table 3. Intracellular distribution of easily detachable iron after administration of iron

Fractionation of liver from rat injected with 20 mg. of iron in the form of saccharated iron oxide 3 days before being killed. Absolute values are given in mg./g. for nitrogen and iron, in units/g. for enzymes. Abbreviations are defined in Table 1.

Component	Absolute values E + N	Percentage values						
		E + N	N	M	L	P	S	Recovery
Nitrogen	27.7	100	15.2	11.8	3.8	18.3	42.2	91.3
Cytochrome oxidase	17.8	100	16.6	43.6	11.7	10.6	—	82.5
Acid phosphatase	3.49	100	7.6	19.7	28.2	20.9	13.7	90.1
Glucose 6-phosphatase	8.05	100	5.6	1.8	1.3	66.3	0.5	75.5
Iron	1.74	100	31.7	19.8	3.3	6.4	32.9	94.1

Fig. 5. Distribution patterns of easily detachable iron and of acid phosphatase in liver of rat injected with 20 mg. of iron in the form of saccharated iron oxide 3 days before being killed. For explanation see Fig. 3.

oxide (Hausmann Laboratories, Saint-Gall, Switzerland) administered intravenously 3 days before it was killed. The results obtained on the liver of this animal are given in Table 3 and Fig. 5. They show that the extra iron tends to accumulate in the form of gross haemosiderin particles, which, from the data of Gabrio, Shoden & Finch (1953), may be expected to sediment largely with the nuclei.

DISCUSSION

The method of fractionation applied in this work was primarily devised to distinguish between truly mitochondrial enzymes and others, such as the lysosomal hydrolases and uricase, which are also concentrated in the mitochondrial fraction when the classical method is followed (de Duve *et al.* 1955). The truly mitochondrial enzymes, like

cytochrome oxidase, occur in larger amounts and with a higher specific activity in the heavy (M) than in the light (L) mitochondrial fraction, whereas the reverse is true for the others, which follow the distribution of acid phosphatase.

The results obtained thus indicate that the three dehydrogenases studied, as well as alkaline deoxyribonuclease, are essentially located in the mitochondria, whereas the acid deoxyribonuclease is associated with the lysosomes. As far as the dehydrogenases are concerned the observed association is in conformity with the well-known metabolic properties of mitochondria. More surprising are the results obtained on alkaline deoxyribonuclease. It now appears that of the two intracellular enzymes known to attack DNA, one is present in the lysosomes and the other in the mitochondria.

The presence of excess of iron in fraction L and the correlation observed between the iron content of this fraction and its acid phosphatase activity must be considered in conjunction with the observation that lysosome-rich fractions from rat liver contain a large number of particles (peribiliary dense bodies) loaded with electron-dense granules (Novikoff *et al.* 1956). The present findings confirm the interpretation based on morphological criteria that these electron-dense granules are made of ferritin. They afford additional support to the possibility that lysosomes and peribiliary dense bodies may be identical. Until purer fractions can be isolated this identity must, however, remain hypothetical.

SUMMARY

1. The intracellular distribution of a number of components has been investigated in rat liver, according to a fractionation scheme in which the mitochondrial fraction is divided into two sub-fractions. In most experiments, cytochrome oxidase, acid phosphatase and glucose 6-phosphatase were measured as reference enzymes for mitochondria, lysosomes and microsomes respectively.

2. Glutamic, malic and β -hydroxybutyric dehydrogenases, together with alkaline deoxyribonuclease (DNase I), were found to be associated essentially with the true mitochondria. In addition, the microsomal fraction contained about 10% excess of alkaline deoxyribonuclease over what could be expected from mitochondrial contamination, and a similar excess of malic dehydrogenase was found in the final supernatant. As observed before, acid deoxyribonuclease (DNase II), which was measured here by an improved technique, showed a lysosomal distribution.

3. About two-thirds of the easily detachable iron of liver tissue was recovered in the final supernatant. The remainder was distributed diffusely amongst all particulate fractions, with a significant peak in the light mitochondrial fraction, which is richest in lysosomes. The iron content of this fraction was correlated with its acid phosphatase activity. These results support the contention, based on electron-microscope observations, that particles loaded with ferritin are concentrated together with lysosomes.

4. After administration of iron, the iron content of all fractions was augmented; the greatest increase occurred in the nuclear and heavy mitochondrial fractions, in which the iron was probably present in the form of gross haemosiderin granules.

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Tissue Fractionation Studies

13. ANALYSIS OF MITOCHONDRIAL FRACTIONS FROM RAT LIVER BY DENSITY-GRADIENT CENTRIFUGING

By H. BEAUFAY,* D. S. BENDALL,† P. BAUDHUIN, R. WATTIAUX AND C. DE DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

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In the investigations described in this paper an attempt has been made to analyse in a more detailed manner the distribution of various enzymes in mitochondrial fractions from rat liver, with the help of density-gradient centrifuging. The results obtained, which have been briefly reported before (Beaufay, Baudhuin & Bendall, 1958; Baudhuin, 1959), fully confirm the distinction between mitochondria and lysosomes and

provide additional information about both groups of particles.

Density-gradient centrifuging, especially when applied to cell particles, requires specialized equipment and fairly elaborate mathematical treatments. These aspects, as well as the theoretical bases of the method, have recently been examined in detail by de Duve, Berthet & Beaufay (1959). To avoid lengthy duplication of material, we must refer to the above mentioned paper for all details which are not strictly essential to the understanding of the present account.

* Chargé de recherches du FNRS.

† Present address: Department of Biochemistry, Cambridge.