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REFERENCES

- Anderson, N. G. (1955). *Science*, **121**, 775.
 Davidson, J. N., Frazer, S. C. & Hutchison, W. C. (1951). *Biochem. J.* **49**, 311.
 Dickens, F. (1951). *Biochem. J.* **48**, 385.
 Dounce, A. L. (1948). *Ann. N.Y. Acad. Sci.* **50**, 982.
 Dounce, A. L. & Beyer, G. T. (1948). *J. biol. Chem.* **173**, 159.
 Fiske, C. T. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Harrison, M. F. (1953). *Nature, Lond.*, **171**, 611.
 Hogeboom, G. H., Schneider, W. C. & Striebich, M. J. (1952). *J. biol. Chem.* **196**, 111.
 Holter, H., Ottesen, M. & Weber, R. (1953). *Experientia*, **9**, 346.
 Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
 Kennedy, E. P. & Lehninger, A. L. (1949). *J. biol. Chem.* **179**, 957.
 Kensler, C. J. & Langermann, H. (1951). *J. biol. Chem.* **192**, 551.
 Kuff, E. L. & Schneider, W. C. (1954). *J. biol. Chem.* **206**, 677.
 Lang, K. & Siebert, G. (1950). *Biochem. Z.* **320**, 402.
 Margoliash, E. (1954). *Biochem. J.* **56**, 529.
 Meyerhof, O. & Lohmann, K. (1934a). *Biochem. Z.* **271**, 89.
 Meyerhof, O. & Lohmann, K. (1934b). *Biochem. Z.* **273**, 413.
 Novikoff, A. B., Podber, E., Ryan, J. & Noe, E. (1953). *J. Histochem. Cytochem.* **1**, 27.
 Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
 Roodyn, D. B. (1956). *Biochem. J.* **64**, 368.
 Schein, A. H., Podber, E. & Novikoff, A. B. (1951). *J. biol. Chem.* **190**, 331.
 Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
 Schneider, W. C. (1946). *J. biol. Chem.* **165**, 585.
 Schneider, W. C. & Hogeboom, G. H. (1950). *J. nat. Cancer Inst.* **10**, 969.
 Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.
 Shepherd, J. A. & Kalnitsky, G. (1951). *J. biol. Chem.* **192**, 1.
 Sibley, J. A. & Lehninger, A. L. (1949). *J. biol. Chem.* **177**, 859.

The Enzymic Properties of Rat-liver Nuclei

2. FACTORS AFFECTING THE ALDOLASE ACTIVITY OF RAT-LIVER NUCLEI

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It was established in the previous paper (Roodyn, 1956) that rat-liver nuclei prepared in sucrose-calcium chloride media possess aldolase activity, but that the amount recovered in the nuclear fraction in different experiments was very variable, although apparently the same method of isolation of the nuclei had been used. During the course of an investigation into the origins of this variability certain effects were found to produce changes in the aldolase activity of the nuclei.

METHODS

Maleate buffers. These were prepared by the neutralization with N-NaOH of boiled solutions of maleic anhydride. The buffers were brought to the required ionic strength by adding NaCl.

Preparation of nuclei. Unless indicated otherwise, nuclear fractions were prepared by the method of Hogeboom, Schneider & Striebich (1952) carried out as described

previously (Method 1 of Roodyn, 1956). All other methods, materials and terms used were as described previously (Roodyn, 1956).

RESULTS

Effect of method of cell breakage

Pretreatment of the liver by forcing it through a Latapie mincer caused some fall in activity. (Unminced liver gave a nuclear fraction with aldolase activity of 52.1 units/mg. of N while minced liver from the same rat gave a nuclear fraction with an activity of 46.2 units/mg. of N.) A similar effect was observed by Hogeboom & Schneider (1952) with the diphosphopyridine nucleotide-synthesizing enzyme.

The time of homogenization in the Potter-Elvehjem apparatus was not very critical. Treatment for 6 sec. gave a nuclear fraction with a relative concentration [see Roodyn (1956) for a definition of this term] of 0.75; a further 24 sec. caused this to fall to 0.63 and the same activity was obtained with up to 4 min. homogenization of the liver.

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Table 1. *Effect of treatment of homogenate with Waring Blendor on distribution of aldolase in cell fractions*

Both homogenates were fractionated by Method 1 (Roodyn, 1956) and are from the same liver. Values in cols. 2 and 3 are given for the amount of homogenate that contained 1 mg. of N or for the amount of fractions derived from this. Figures in parentheses are percentages of value for appropriate homogenate. HDP, Hexose diphosphate.

Treatment	Fraction	Nitrogen (mg.)	Aldolase activity (μ moles of HDP/hr.)	Aldolase activity/mg. of N	Relative concn.
Potter-Elvehjem homogenizer	Homogenate	1.00	31.5	—	—
	Supernatants	0.901	19.8 (62.9)	—	—
	Nuclear fraction	0.078	7.22 (23.0)	92.9	2.95
	Recovery	0.979	(85.9)	—	—
Potter-Elvehjem homogenizer followed by Waring Blendor	Homogenate	1.00	29.0	—	—
	Supernatants	0.895	26.6 (91.9)	—	—
	Nuclear fraction	0.064	2.46 (8.5)	38.5	1.33
	Recovery	0.959	(100.4)	—	—

Fig. 1. Effect of concentration of liver suspension during cell breakage. ○, Expt. 1: the three homogenates used were from the same liver and had aldolase activities of 27.6, 23.3, and 27.6 μ moles of hexose diphosphate/hr./mg. of N. After homogenization all homogenates were diluted to 10% (w/v) for fractionation by Method 1 (Roodyn, 1956). ●, Expt. 2: relative concentrations calculated from aldolase activity of the 20% (w/v) homogenate, which was 24.9 μ moles of hexose diphosphate/hr./mg. of N. Suspensions were prepared from liver mince.

traction of liver suspension (about 25%, w/v) during cell breakage. However, there is a wide difference in the range of aldolase activity observed in the two experiments, so that it is clear that some other effects must operate.

The use of a Waring Blendor produced a large difference in the distribution of enzyme (Table 1). Treatment for 30 sec. was given in an instrument fitted with a plastic head cooled by an ice bath. The machine was run for three intervals of 10 sec. with a pause for 2 min. between each blending. The temperature of the homogenate did not rise significantly. Deoxyribonucleic acid analyses on the two fractions showed that the yield of nuclei was almost the same in both. It can be seen that relatively short treatment of the homogenate with a Waring Blendor caused the total enzyme recovered in the nuclear fraction to fall from 7.22 to 2.46 units, which is equivalent to a loss of 66%.

Effect of delaying the fractionation procedure

Preliminary experiments indicated that delay in starting the fractionation after preparation of the homogenate had an effect on the aldolase recovered in the nuclear fraction. However, the effect was not reproducible in its extent nor in its direction, since sometimes a 30% increase in relative concentration was observed if the first centrifuging was delayed for 15 min., and sometimes a 15% fall was seen. It was possible that this effect was associated with alterations in the pH of the homogenate at 0° since it was found (Fig. 2a) that the pH first fell, then remained stable for a variable period, and then rose.

This view was confirmed by simultaneous determinations of the pH of the homogenate and of the aldolase activity of nuclei derived from the homogenate at different times (Table 2 and Fig. 2b). There is a reasonable correlation between the fall in activity in the nuclear fraction and the rise in pH of the homogenate, although it must be stressed that the time scale of the experiment is based on the

The concentration of the liver suspension at the moment of homogenization was more important (Fig. 1), and it appeared from two experiments that in order to obtain maximum aldolase activity in the nuclear fraction one has to use an optimum concn-

arbitrary assumption that the bulk of the supernatant has been separated by the end of the second sedimentation.

Effect of pH of the isolation medium

Nuclei were isolated from homogenates that had been buffered with dilute maleate buffers. The pH range chosen was similar to that observed in unbuffered homogenates (Fig. 2*a*). (Serious clumping of particulate material into the nuclear fraction occurred at pH 5.75.) It can be seen from Table 3 that raising the pH of the homogenate from 5.75 to 6.75 caused the enzyme recovered in the nuclear fraction to fall from 31.1 to 5.2% of the total. It may be noted that this range of activities is very similar to the previously observed variation in nuclear aldolase activity (Roodyn, 1956).

The reversibility of the pH effect was studied by isolating nuclei from homogenates at pH 6.0 and 6.85 and then resuspending them in, and reisolating them from, each other's supernatants. In each case (Table 4) the nuclei, on reisolation, gained or lost about 33% of the initial difference between them. This experiment once more confirmed the direction of the pH effect since (a) the initial fractionation at pH 6.0 gave a nuclear fraction with about twice the

Fig. 2. Variation in pH of homogenates in 0.25 M sucrose-0.0018 M CaCl_2 at 0° , and in aldolase of nuclei. (a) Δ , $\blacktriangle$, Rats fed for 15 hr. before death; $\times$, rat starved for 24 hr. before death. (b) $\bullet$, pH of liver homogenate; $\circ$, relative concentration of nuclear aldolase. (See Table 2 for details of the fractionation.)

Table 2. *Effect of leaving nuclei in presence of homogenate before isolation*

Samples of homogenate were taken for fractionation after increasing times at 0° . It was assumed that the nuclei were freed from the bulk of the supernatant at the end of the second centrifuging. Results in cols. 4 and 5 are given for that amount of homogenate that contains 1 mg. of total nitrogen or for the amount of fraction derived from this. Figures in parentheses give values as percentage of that for homogenate which had an aldolase activity of $27.2 \mu\text{moles}$ of hexose diphosphate (HDP)/hr./mg. of N.

Fractionation	Time between homogenization and removal of second supernatant (min.)	Fraction	Nitrogen (mg.)	Aldolase activity (μmoles of HDP/hr.)	Aldolase activity/mg. of N	Relative concn.
1	20	Supernatants	0.910	21.7 (80.0)	—	—
		Nuclei	0.098	5.30 (19.6)	54.0	1.98
		Recovery	1.008	(99.6)	—	—
2	32	Supernatants	0.915	24.4 (90.0)	—	—
		Nuclei	0.0885	2.79 (10.3)	31.5	1.16
		Recovery	1.003	(100.3)	—	—
3	55	Supernatants	0.895	23.1 (85.0)	—	—
		Nuclei	0.087	2.41 (8.9)	27.8	1.02
		Recovery	0.982	(93.9)	—	—
4	75	Supernatants	0.935	24.7 (90.5)	—	—
		Nuclei	0.0795	1.59 (5.9)	20.0	0.73
		Recovery	1.015	(96.4)	—	—
5	90	Supernatants	0.918	24.0 (88.0)	—	—
		Nuclei	0.0795	1.78 (6.55)	22.4	0.82
		Recovery	0.998	(94.5)	—	—
6	102	Supernatants	0.918	24.6 (90.5)	—	—
		Nuclei	0.0815	1.78 (6.55)	21.9	0.81
		Recovery	0.999	(97.0)	—	—

Table 3. *Effect of pH of isolation medium on aldolase activity of nuclear fraction*

Values in cols. 5 and 6 are given for that amount of homogenate that contains 1 mg. of total nitrogen or for the amount of fraction derived from this. Figures in parentheses are percentage of value for the appropriate homogenate. Fractionation as in Method 1 (Roodyn, 1956) except that all media were 0.005M with respect to maleate buffer and were adjusted to an ionic strength of 0.017. All homogenates were prepared from the same liver.

Fractionation	pH of medium	pH of homogenate	Fraction	Nitrogen (mg.)	Aldolase activity (μ moles of HDP/hr.)	Aldolase activity/mg. of N	Relative concn.
1	5.70	5.75	Homogenate	1.00	23.1	—	—
			Supernatants	0.575	11.9 (51.4)	—	—
			Nuclear fraction	0.398	7.19 (31.1)	18.0	0.78
			Recovery	0.973	(82.5)	—	—
2	5.95	6.00	Homogenate	1.00	24.5	—	—
			Supernatants	0.888	19.4 (79.1)	—	—
			Nuclear fraction	0.109	4.82 (19.7)	44.2	1.81
			Recovery	0.997	(98.8)	—	—
3	6.40	6.42	Homogenate	1.00	24.0	—	—
			Supernatants	0.88	21.5 (89.5)	—	—
			Nuclear fraction	0.082	2.4 (10.0)	29.3	1.22
			Recovery	0.962	(99.5)	—	—
4	6.72	6.75	Homogenate	1.00	28.0	—	—
			Supernatants	0.90	26.0 (92.8)	—	—
			Nuclear fraction	0.07	1.45 (5.2)	20.7	0.74
			Recovery	0.97	27.4 (98.0)	—	—

Table 4. *Partial reversibility of effect of pH of homogenate on nuclear aldolase activity*

Homogenates (25%, w/v) were prepared from the same liver, with the addition of 0.005M maleate buffer at the appropriate pH to all media. In other respects, fractionation was by Method 1 (Roodyn, 1956). Nuclei were isolated and re-suspended in supernatants from opposite fractionation. They were reisolated by Method 1. Results in col. 2 are for the amount of nuclei derived from 1 ml. of the appropriate homogenate.

	Aldolase activity (μ moles of HDP/hr.)	Aldolase as percentage of total in homogenate	Percentage reversal of pH effect*
Nuclei prepared at pH 6.0	2.80	10.0	—
Nuclei prepared at pH 6.85	1.25	4.7	—
Difference	1.55	—	—
Nuclei prepared at pH 6.0 and reisolated at pH 6.85	2.29	—	—
Activity lost from these nuclei on reisolation	0.51	—	32.6
Nuclei prepared at pH 6.85 and reisolated at pH 6.0	1.79	—	—
Activity gained by these nuclei on reisolation	0.54	—	34.9

* Reversal is 100% if nuclei gain or lose 1.55 units.

activity of that obtained at pH 6.85, (b) nuclei prepared at a low pH lost activity on reisolation at a higher pH and (c) nuclei prepared at the higher pH gained activity on reisolation at a lower pH.

Redistribution of enzyme during fractionation procedure

In some of the fractionations satisfactory recovery of enzyme (98.0–100%) made it possible to observe that alterations in nuclear activity occurred

in parallel with equal and opposite alterations in activity in the supernatants. Thus for four pairs of fractionations the loss in activity in the nuclear fractions was 9.3, 9.7, 4.8 and 5.3% of the total activity in the homogenate, and the rise in activities in the supernatants was 10.0, 10.4, 3.2 and 5.1%, respectively. Unfortunately such analyses could not always be made when the most striking effects were observed, since very high activities in the nuclear fraction were often associated with poor recovery of enzyme.

There is other evidence to suggest that the total amount of enzyme in the system remains constant. In nearly forty fractionations by Method 1 a total recovery of enzyme greater than 103% was never found, indicating that high activities in the nuclear fraction were not caused by activation of the nuclei after they had been isolated. In one fractionation it was found that the aldolase activity of the homogenate, supernatants and nuclear fraction were virtually stable at 0° for up to 6 hr. after they were obtained. (In Method 1 all fractions were estimated at the same time, within 1–2 hr. after the death of the rat.) It is also unlikely that the total activity in the system increases during the time involved in filtering and layering the homogenate, since the latter had a constant activity at 0° between 6 and 30 min. after the death of the rat.

Variation in nitrogen content of nuclear fractions

There are indications that other nitrogen-containing material has followed the observed alteration in nuclear aldolase activity. Parallel but smaller changes were observed in the nitrogen content of the nuclear fractions on several occasions that changes in aldolase occurred. Table 2 shows that a delay of fractionation of 55 min. (fractionations 1–4) resulted in a decrease in nuclear aldolase activity of 3.71 units, and in parallel with this the nitrogen content of the nuclear fraction fell by 0.0185 mg. Hence the specific activity of the material lost was 201 μ moles of hexose diphosphate/hr./mg. of N. Liver aldolase has not yet been purified but purified muscle aldolase had a specific activity of about 100 times this value in the assay system used. Also, it was calculated that if the changes in nitrogen content of the nuclei were due to changes in aldolase alone, and if the activity per mg. of N of aldolase left in the nuclei was the same as that lost, then 27% of the nitrogen in the first nuclear fraction (fractionation no. 1 in Table 2) would be aldolase nitrogen. This seems unlikely.

DISCUSSION

The first paper (Roodyn, 1956) demonstrated some of the necessary precautions to be taken before it could be said that an observed activity in a cell fraction was associated with the major component of that fraction. The work presented in this paper illustrates that this association is by no means sufficient evidence to claim that the activity of an isolated component is a measure of its true activity. The most disturbing observation was the alteration in activity caused by delaying the fractionation for relatively short times. In particular, the large redistribution of enzyme occurring in a 12-min. delay at 0° (Table 2) makes it very difficult to say what was the initial distribution of enzyme. The

results suggest that the pH fluctuations in the homogenate are associated with the enzyme redistribution, and it is clear that valid enzyme studies on isolated nuclei require the use of buffered isolation media. [Such media have been described by Wilbur & Anderson (1951), Maver, Greco, Lovtrup & Dalton (1952) and Philpot & Stanier (1956).] Although acid formation in homogenates is well known (Duve & Berthet, 1954), the author has not seen any report of the observed secondary rise in pH, and its origin is not obvious.

The study of different conditions of cell breakage in the Potter–Elvehjem apparatus suggests that the state of suspension of the liver is more important than the actual time of treatment. The effect is confirmed by the observation of Palade (1951) that cell rupture is more efficient with more concentrated homogenates. The optimum concentration of suspension observed may be that required to break the maximum number of cells without causing excessive damage to the nuclei. The effect of the Waring Blendor may have been directly on the nuclei, causing them to release aldolase, or may have been indirect. In this respect Dounce, Witter, Monty, Pate & Cottone (1955) have recently reported that mitochondria contain an enzyme which is released when they are damaged and which prevents nuclei from forming gels in alkali, the latter property being regarded by these authors as an index of integrity of the nucleus. Similarly, Philpot & Stanier (1956) have shown that protection of the lysosomes (Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) present in the homogenate prevents damage to the nuclei.

The results reported above bear some relation to the problem of whether nuclei lose a soluble protein component if they are isolated in aqueous, rather than organic, media (cf. Allfrey, Stern, Mirsky & Saetren 1952; Stern & Mirsky, 1953; Kay, Smellie, Humphrey & Davidson, 1956). First, with aldolase aqueous media do not *per se* result in the complete washing out of enzyme; on some occasions nuclei with a relative concentration of about 3 were obtained in the sucrose media used. Secondly, the influence of the mechanical methods of cell breakage discussed above suggests that, if the nuclei are isolated from liver broken in the mildest manner, it might be possible to obtain quite high aldolase activities. The results with aldolase may, therefore, be analogous to the findings of Hogeboom & Schneider (1952) that mechanical damage to the nuclei can cause a release of the diphosphopyridine nucleotide-synthesizing enzyme from them. It would be of great interest to know whether the variable component of liver nuclei studied above included the diphosphopyridine nucleotide-synthesizing enzyme and whether it corresponded to a protein fraction that could be extracted from iso-

lated nuclei. The author has observed that 90–95 % of the total nuclear aldolase could be extracted with 0.15 M-NaCl, and Kirkham & Thomas (1953) have reported a globulin fraction readily extracted with 0.14 M-NaCl from nuclei isolated in organic solvents, the fraction comprising 30–50 % of the dry weight of the nuclei.

Until more is known of the nature of the binding between aldolase and nuclei, and until protein redistribution can be prevented (at least after the homogenization process) by the use of better isolation media, it cannot be categorically stated that aldolase is a true component of liver nuclei.

SUMMARY

1. A study has been made of factors affecting the aldolase activity of rat-liver nuclei.

2. The method of homogenization affected nuclear aldolase. Treatment of the homogenate with a Waring Blendor for 30 sec. resulted in a 66 % fall in the total enzyme recovered in the nuclear fraction.

3. Delay in isolating the nuclei after the preparation of the homogenate resulted in alterations in nuclear activity. In one fractionation a delay of 12 min. resulted in the loss of 47 % of the nuclear enzyme. The fall in activity was coincident with a rise in pH of the homogenate.

4. Isolation of nuclei from weakly buffered homogenates showed that the aldolase activity of the nuclear fraction was greatly affected by the pH of the homogenate, a nuclear fraction obtained at pH 6.0 having 3.3 times the activity of one isolated at pH 6.75.

5. Alterations in nuclear activity were due to a

redistribution of enzyme between nuclei and supernatants and not to activation or inactivation.

6. Indirect evidence suggested that the effects observed with aldolase applied to other nitrogen-containing materials.

7. It is concluded that the aldolase activity of isolated nuclei is greatly affected by the method of isolation of the nuclei.

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REFERENCES

- Allfrey, V., Stern, H., Mirsky, A. E. & Saetren, H. (1952). *J. gen. Physiol.* **35**, 529, 559.
 Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 139.
 Duve, C. de & Berthet, J. (1954). *Int. Rev. Cytol.* **III**, p. 225. New York: Academic Press.
 Duve, C. de, Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
 Hogeboom, G. H. & Schneider, W. C. (1952). *J. biol. Chem.* **197**, 611.
 Hogeboom, G. H., Schneider, W. C. & Striebich, M. J. (1952). *J. biol. Chem.* **196**, 111.
 Kay, E. R. M., Smellie, R. M. S., Humphrey, G. F. & Davidson, J. N. (1956). *Biochem. J.* **62**, 160.
 Kirkham, W. R. & Thomas, L. E. (1953). *J. biol. Chem.* **200**, 53.
 Maver, M. E., Greco, A. E., Lovtrup, E. & Dalton, A. J. (1952). *J. nat. Cancer Inst.* **13**, 687.
 Palade, G. E. (1951). *Arch. Biochem.* **30**, 144.
 Philpot, J. St L. & Stanier, J. E. (1956). *Biochem. J.* **63**, 214.
 Roodyn, D. B. (1956). *Biochem. J.* **64**, 361.
 Stern, H. & Mirsky, A. E. (1953). *J. gen. Physiol.* **37**, 177.
 Wilbur, K. M. & Anderson, N. G. (1951). *Exp. Cell Res.* **2**, 47.

The Use of Radioactive Carbon Dioxide in the Measurement of Carbon Dioxide Fixation in *Rhodospirillum rubrum*

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The Athiorhodaceae utilize a wide variety of organic compounds for growth. Light is essential for anaerobic growth, but most of these organisms will grow in the dark in the presence of oxygen. Growth in the light is dependent on chlorophyll. According to the hypothesis of van Niel (1941, 1944, 1949) the primary light reaction in the photosynthetic bacteria is the same as that in green plant

photosynthesis and involves the splitting of water with the formation of a reduced component and an oxidized component. The former is thought to reduce carbon dioxide, presumably in the form of 1:3-diphosphoglyceric acid (Bassham *et al.* 1954), whereas the oxidized component is the terminal acceptor in the oxidation of the substrate. In the light most of the organic substrate is assimilated as was shown by van Niel (1944). Gaffron (1933, 1935) was the first to use manometric methods for the

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