SUMMARY

1. The seminal plasma of the domestic cock has been shown to contain a very active acid phosphatase(s); the enzyme is especially active against 4-nitrophenyl phosphate, and less so against the 2- and 1-phosphate of glycerol. It shows two broad activity peaks about pH 4.9 and pH 5.2.

2. An alkaline phosphatase is also present; its activity is considerably lower than (less than one-hundredth of) that of the acid enzyme and is of the same order as found in the blood plasma.

3. High acid-phosphatase activity (13 000 units) is shown by the secretions of the vas deferens. The activity of the fluids from the erectile cloacal tissues is relatively weak (1620–5800 units).

4. The nature of the acid phosphatase differs from that present in human seminal plasma or prostate as it is not inhibited by dextrorotatory tartrate. Its behaviour towards Cu^{2+} , Ca^{2+} and F^{-} ions is similar.

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Inactivation of Anaerobic Glycolysis in Fractions of Rat-Brain Homogenates

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It has been shown that under anaerobic conditions the rate of production of lactic acid from glucose by a brain supernatant fraction is markedly stimulated by the addition of brain mitochondria, although the mitochondrial fraction itself has quite a low glycolytic activity (Rossiter, 1957; Abood, Brunngraber & Taylor, 1959; Cremer, 1960; Hochstein & Cohen, 1960; Johnson, 1960). Stimulation is here defined as the excess of activity of a mixture of mitochondrial and supernatant fractions over the summed activities of the separate components. A reasonable explanation is that a relative deficiency of hexokinase in the supernatant is made good by the addition of the mitochondria with which most of rat-brain hexokinase is associated (Johnson, 1960). If fructose 1,6-diphosphate were the substrate instead of glucose in a medium containing an adequate adenosine diphosphate-regenerating system, the

lactate production by a mixture of mitochondrial and supernatant fractions would be expected to represent the summed activities of the mixture components with little or no stimulation, since all the enzymes involved are distributed similarly among the fractions of a rat-brain homogenate [75–90% of each being present in a crude supernatant fraction (Johnson, 1960)]. However, Brunngraber & Abood (1960) have reported that a large stimulation is obtained when a brain mitochondrial fraction is mixed with supernatant and assayed in a medium containing fructose 1,6-diphosphate and an adenosine diphosphate-regenerating system. Under the same conditions glycolysis of both the supernatant fraction and the mixture, but not the mitochondria, was stimulated by addition of purified muscle aldolase. They concluded that aldolase was rate-limiting in the supernatant and in the mixture, and that a dis-

proportionately large amount of this enzyme was present in the mitochondrial fraction. The reported rates of lactate production in $\mu\text{mole/min.}$ were 0.034, 0.168 and 0.307 respectively for the mitochondria, supernatant and mixture. If aldolase is rate-limiting in the mixture and supernatant fractions, then calculation shows that 55% of total aldolase must be in the mitochondrial fraction and 45% in the supernatant; also not more than 11% of whichever enzyme is rate-limiting in the mitochondrial fraction can be present in that fraction. This deduction concerning the distribution of aldolase contradicts earlier reports based on direct measurements (Abood, Gerard, Banks & Tschirgi, 1952; Johnson, 1960).

I have extended my previous study of the intracellular distribution of aldolase and have also shown that there are certain defects in the experimental procedure used by Brunngraber & Abood (1960). When these are corrected, no stimulation of anaerobic glycolysis is obtained when mitochondrial and supernatant fractions from rat brain are mixed.

A preliminary report of these findings has been published (Johnson, 1961).

METHODS

Abbreviation. BAL, 2,3-dimercaptopropanol (British anti-lewisite).

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: lithium lactate, tetrasodium salt of fructose 1,6-diphosphate and triethanolamine (British Drug Houses Ltd.) (the triethanolamine was fractionally distilled before being partially neutralized with HCl for use as a buffer); EDTA, potassium salt (Hopkin and Williams Ltd.); sodium pyruvate, cysteine hydrochloride and nicotinamide (Roche Products Ltd.); GSH (Schwarz Laboratories Inc., U.S.A.); disodium salt of NADH₂, trisodium salt of ADP and a mixture of purified α -glycerophosphate dehydrogenase and triose phosphate isomerase (C. F. Boehringer und Soehne GmbH., Mannheim, Germany); disodium salt of ATP (Sigma Chemical Co., U.S.A.); the succinic acid ester of BAL (Boots Pure Drug Co. Ltd.). NAD was supplied by Mr C. J. Threlfall and was about 80% pure; hexokinase was supplied by Mr V. H. Parker and had been prepared from baker's yeast by an unpublished modification of the method of Berger, Slein, Colowick & Cori (1946); when assayed by their procedure, but at 37°, the preparation had an activity of 4600 units/ml.

Fractionation procedure. Brains from two decapitated rats (mature females of the Porton strain) were homogenized in 0.3M-sucrose with a smooth glass tube and a Perspex pestle having an all-round clearance of 0.005 in. (Aldridge, Emery & Street, 1960) and fractions were prepared by two methods. The method of isolating and washing fractions by the first method and the nomenclature applied to them have been described in detail (Aldridge & Johnson, 1959), except that an increased speed was used to separate the nuclei. Thus the sediment obtained by centrifuging the homogenate at 1500g for

10 min. contained nearly all the nuclei plus cell debris and some mitochondria: this sediment was washed by resuspending in 0.3M-sucrose and resedimenting under the same conditions. The washed fraction is designated 1.5.N.10. The nuclear washings were used to wash the single mitochondrial fraction (13.M.15): the wash-fluid so obtained was combined with the supernatant from the first sedimentation of mitochondria and centrifuged to sediment any remaining mitochondria plus some smaller particles (20.P.30) leaving a crude supernatant (S). When the method of Brunngraber & Abood (1960) was followed, the supernatant was prepared directly by centrifuging a homogenate in 0.25M-sucrose containing 0.1 mM-EDTA at 100 000g for 20 min.; washed mitochondrial preparations were made by centrifuging the homogenate at 12 000g for 15 min. after removing and washing two nuclear fractions at 600 and 1500g each for 10 min. For studies of glycolysis, the mitochondrial pellet was suspended in water, and for spectrophotometric assay it was suspended in sucrose and diluted with water at the time of the assay. Mixtures of mitochondrial and supernatant fractions were made up in the same proportion as in the original homogenate.

Enzyme assays. The manometric method for succinic dehydrogenase, the colorimetric method for hexokinase and spectrophotometric methods for lactic dehydrogenase and aldolase have been reported previously (Johnson, 1960). For aldolase, but not lactic dehydrogenase, higher activities were found when tissues were diluted with water rather than with 0.3M-sucrose: this had not been observed previously.

Measurement of anaerobic glycolysis. Glycolysis was carried out in Warburg flasks at 37° in the medium of Brunngraber & Abood (1960), which was at pH 7.5 and contained (in 3 ml.) potassium phosphate (8 mM), KHCO₃ (17 mM), MgCl₂ (7 mM), NAD (0.36 mM), ATP (0.5 mM), fructose 1,6-diphosphate (3.3 mM), nicotinamide (27 mM), glucose (10 mM), sodium pyruvate (10 mM) and yeast hexokinase (230 Berger units); the gas phase was CO₂ + N₂ (5:95). Additions of sulphhydryl compounds to the medium were made as described in the text. Whether sulphhydryl compounds were added or not, lactate production was linear with respect to time whenever tested, as had been reported by Brunngraber & Abood (1960). Measurements were therefore usually made at one time only, the reaction being stopped at 30 min. by addition of ice-cold HClO₄ (7% final concentration). Lactate was measured in the acid-quenched medium by the method of Barker & Summerson (1941). Since certain glycolytic intermediates may break down to lactate during the alkaline copper treatment (R. Balázs, personal communication) the time of treatment was kept at 30–40 min. and the temperature below 5°: this treatment does not remove all pyruvate from the original medium and a moderate blank was therefore obtained in the lactate assay. Lactate production was negligible when the yeast hexokinase was incubated in the medium without tissue.

RESULTS AND DISCUSSION

Distribution of aldolase

The distribution of this enzyme among washed fractions of rat-brain homogenate prepared in 0.3M-sucrose is shown in Table 1. This confirms

Table 1. *Distribution of aldolase and other enzymes among washed fractions of rat-brain homogenates*

Fraction designations and assay conditions were as described under Methods. Assays were performed in duplicate and the mean of at least two experiments is reported.

Enzyme	Total recovered activity in fraction (%)			
	1-5.N.10	13.M.15	20.P.30	S
Aldolase	7, 9	21, 22	9, 11	63, 58
Lactic dehydrogenase*	6	19	5	70
Hexokinase*	13	72	15	
Succinic dehydrogenase	13	79	8	

* The distribution of these enzymes is derived from studies with similar but not identical fractions (Johnson, 1960).

Fig. 1. Loss of glycolytic activity on dilution of brain supernatant. Fraction was prepared by the second method and activity measured in standard medium without sulphhydryl compounds.

that the majority is in the supernatant fraction and that the distribution is similar to that of lactic dehydrogenase, but quite different from that of hexokinase and succinic dehydrogenase: the distributions of these other enzymes are shown for comparison. Neither the use of 0.25 M-sucrose containing 0.1 mM-EDTA as used by Brunngraber & Aboud (1960), nor reduction of the pH of homogenizing medium to 6.5 altered the distribution of aldolase, although Roodyn (1956) reported that pH alterations changed the adsorption of liver aldolase on liver nuclei.

Inactivation of anaerobic glycolysis

Since the deduction by Brunngraber & Aboud (1960) that aldolase is present in excess in the mitochondrial fraction was based on glycolysis measurements, an examination was made of their experimental procedure and it was found that, in the defined medium, lactate production fell drastically as the tissue concentration was de-

Fig. 2. Linearity of glycolytic activity with respect to concentration of brain fractions in presence of glutathione. Fractions were prepared by the first method and activity was measured in standard medium fortified with 2 mM-glutathione. ●, Mixture; ○, supernatant; ■, mitochondria.

creased. The relation of lactate production to quantity of brain supernatant incubated is shown in Fig. 1. Although the curve becomes linear at moderate tissue concentrations, there is a large zero error on the lactate-production co-ordinate (about 300 $\mu\text{g./30 min.}$) Non-linear relationships were also shown in the assay of a mixture of mitochondria and supernatant and, less markedly, with the mitochondrial fraction. There was no difference between the fractions prepared by either of the procedures described in the Methods section. Serious error would be incurred if the activity of a fraction were calculated from measurements made with only one tissue concentration in this medium on the assumption that the curve is linear through the origin, and it appears that Brunngraber & Aboud made this error.

Fortification of the medium with 2 mM-glutathione or BAL succinate led to a rectilinear relation of lactate production to fraction concentration being obtained whichever way fractions were prepared (Fig. 2). It might be argued that this is fortuitous, and represents the summation of the tissue activities shown in Fig. 1 and some non-

enzymic interaction of glutathione with the added pyruvate of the medium: this proposition must include the unlikely requirement that the non-enzymic reaction declines in the presence of larger amounts of tissue. However, the non-rectilinear relationship also obtains with a medium which lacks pyruvate, and rectilinearity is established by addition of glutathione to the medium in this case also.

There is some evidence that glutathione is a fairly tightly bound coenzyme of muscle glyceraldehyde phosphate dehydrogenase (Krimsky & Racker, 1954), and it seems possible that in the brain enzyme the coenzyme is present but is less tightly bound, being partially lost on dilution. Cysteine hydrochloride was ineffective as a preservative, whereas it is known to restore the activity of the muscle enzyme after inactivation by oxidation (Rapkine, 1938). It is strange that BAL succinate should be an effective preservative although it was needed in slightly higher concentrations than was glutathione. Geiger (1940) and Muntz & Hurwitz (1951) found that glutathione had a variable stimulating effect on the glycolytic capacity of acetone extracts of rat brain and that this was not due to protection against heavy-metal ions. Krimsky & Racker (1949) showed glutathione to have a potent protective effect against the inactivation of mouse-brain glyceraldehyde phosphate dehydrogenase in homogenates.

Stimulation of glycolysis

If the activity of only one concentration of each brain fraction is measured in the medium without glutathione, it is possible to demonstrate summation of the activities of the mixture components, or an apparent stimulation (as obtained by Brunngraber & Abood, 1960), or an apparent inhibition. Thus when mitochondrial and supernatant fractions having apparent activities of 3.1 and 8.4 mg. of lactate/g. of brain/30 min. respectively were mixed in the original homogenate proportion, then values of the apparent activity of the mixture varied from 5.9 to 15.2 mg./g. of brain/30 min. depending on the choice of tissue concentration (within a twofold range) used for the assay. It appears that the experiments reported by Brunngraber & Abood (1960) were performed under unsatisfactory conditions and that the stimulation found was an artifact. Deductions concerning the identity and distribution of a rate-limiting enzyme based upon apparent activities observed under such conditions cannot therefore be regarded as valid.

In three experiments with the glutathione-fortified medium and fractions prepared by the first method, the proportion of lactate-producing activity in the mitochondrial fraction was $21 \pm 1\%$,

in the supernatant $79 \pm 1\%$, and in the mixture $106 \pm 5\%$. The mixture activity was 25.6 ± 5.9 mg. of lactate/g. of brain/30 min. (mean of 4 experiments $\pm$ S.D.).

A check was made to see if the mode of homogenization affected the possibility of stimulation. A triple-washed mitochondrial fraction was prepared by Dr Balázs from brain homogenized by vigorous use of a blade homogenizer: its activity was 12% of the total and no stimulation was found when this fraction was mixed with the supernatant. The lower proportion of activity in this fraction than in one as usually prepared is in accord with the observation that a lower proportion of lactic dehydrogenase is associated with particles in a homogenate prepared with a blade homogenizer (Johnson, 1960).

SUMMARY

1. A study is reported of the anaerobic conversion of fructose 1,6-diphosphate into lactate by fractions of rat-brain homogenate. A partial inactivation occurs when the fractions are assayed in a medium lacking sulphhydryl compounds.

2. Glutathione preserved the activity; cysteine was ineffective but the succinic acid ester of British anti-lewisite was nearly as effective as glutathione.

3. Little or no stimulation of lactate production occurs when the mitochondrial and supernatant fractions are mixed and assayed in the glutathione-fortified medium. Published reports of a large stimulation were based on determinations in an inadequate medium.

4. Mitochondrial preparations from homogenates made with a high-speed smooth pestle homogenizer have about 21% of the summed fructose 1,6-diphosphate-glycolytic activity of mitochondria and supernatant. This proportion is decreased to 12% if a vigorous blade homogenizer is used and the fraction is more thoroughly washed.

5. The distribution of aldolase in rat-brain fractions has been further studied and its predominant location (60%) in the supernatant fraction confirmed.

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Comparative Studies of 'Bile Salts'

14. ISOLATION FROM SHARK BILE AND PARTIAL SYNTHESIS OF SCYMNOL*

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The name 'scymnol' was given by Hammarsten (1898) to an alcohol believed by him to occur as a sulphate in the bile of the shark *Scymnus borealis* (*Somniosus microcephalus* Schn.). 'Scymnol' has been isolated after hydrolysis with alkali of the bile salts of about ten species of selachians. It has been shown (Fieser & Fieser, 1959; Cross, 1960) that this substance has the formula (III*a*). Haslewood (1951) suggested that the compound now recognized as (III*a*) might be an artifact, the oxide ring arising by elimination of SO_4^{2-} ion between an $-\text{OH}$ group and $-\text{O}\cdot\text{SO}_3^-$ during alkaline hydrolysis. This suggestion was shown (Briggs & Haslewood, 1961) to be correct, for direct oxidation of the sulphate from shark bile gave dehydrocholic acid (IV*b*); this work is here described in full. It follows that the native bile salt (III*b*) is the sulphate of a hexahydric alcohol which, in agreement with Dr A. D. Cross, we call 'scymnol' (III*c*), the substance (III*a*) being renamed 'anhydroscymnol'.

Scymnol, $\text{C}_{27}\text{H}_{48}\text{O}_6$, has now been isolated from a sample of shark bile salt sulphate and has also been made by partial synthesis from cholic acid.

RESULTS

The shark bile sulphate used was a sample, the gift of Professor S. Bergström, from Professor O. Hammarsten's collection: it was labelled 'scymnol-sulphuric acid sodium salt', and showed a single spot on paper chromatograms.

Cold chromic acid oxidation of this material gave dehydrocholic acid (IV*b*) in a yield of about

26%. When anhydroscymnol (III*a*) was treated in the same way, the product (yield, about 40%) was the known 'scymnol acid' (anhydroscymnol acid, $\text{C}_{27}\text{H}_{38}\text{O}_6$), in which the oxide ring is retained (Bergmann & Pace, 1943) and which must have the structure (IV*a*).

For removal of the sulphate ester group, scymnol sulphate was first partially acetylated and the crude product was treated with dry dioxan-trichloroacetic acid (Cohen & Oneson, 1953). The water-insoluble material obtained was hydrolysed with alkali and purified by partition chromatography. The product, crystallized from moist solvents, was much more 'polar' on paper chromatograms than anhydroscymnol: elementary analysis suggested that it was a dihydrate, $\text{C}_{27}\text{H}_{48}\text{O}_6 \cdot 2\text{H}_2\text{O}$, of scymnol (III*c*).

In the partial synthesis, trimethylcholoyl chloride (I) was condensed with diethyl sodiomalonate. The partially purified product, containing (II), was reduced, first with NaBH_4 in dimethylformamide and then with LiAlH_4 in tetrahydrofuran. Saponification followed; the neutral fraction was separated and four crystalline substances and a gum were obtained from it, as described below.

A principal product was 3 α ,7 α ,12 α ,24-tetrahydroxycholane (III*d*), which could easily be isolated by direct crystallization. Paper chromatography showed that at least four other compounds were present, and three of these were isolated after chromatography on alumina. A sample obtained by this process, and showing a single spot on paper chromatograms corresponding to one given by the scymnol described above, was finally purified by partition chromatography. Crystalline material,

* Part 13: Haslewood (1961).