respectively. Both factors are also heat labile, and their effects can be counteracted by crystalline insulin. The pars distalis of the hypophysis receives unmyelinated fibers from the carotid plexus(10); it is barely possible that they constitute the source of the anterior pituitary factor. An effort is being made at present to further purify and identify the inhibitor of hexokinase found in nervous tissue.

Summary. 1. Nerve homogenates, but not those of muscle or liver, inhibit brain glycolysis, the inhibition being a linear function of the amount of nerve. 2. Two thirds of the inhibitor activity is in a protein and lipid fraction precipitated at pH 5.3 to pH 5.5. From 200 mg of fresh nerve, 3 mg of protein and a like amount of lipid are obtained. Even this semi-purified fraction loses activity slowly in the cold, almost at once on heating. 3. The inhibition is specific for brain hexokinase. The enzyme from yeast or liver is not inhibited; glycolysis of hexose phosphates by brain is not inhibited. Fructose loss is inhibited half as strongly as that of glucose. 4. Crystalline insulin can counteract the nerve inhibitor. This and its other properties are similar to those of the inhibitor from the anterior pituitary.

10. Maximow, A. A., and Bloom, W., Textbook of Histology, W. B. Saunders, Philadelphia, Pa., 1948.

Received June 7, 1951. P.S.E.B.M., 1951, v77.

Properties of a Chromoprotein Containing Extract of the Particulate Fraction of Rat Liver. (18807)

ERNEST KUN.†

*From the Department of Medicine, The University of Chicago.**

It is generally recognized that certain reactions of terminal electron transfer to molecular oxygen are catalyzed by enzymes structurally associated with the particulate fraction of tissue suspensions. One of the difficulties in studying these reactions is the inaccessibility of tissue granules to enzyme isolation procedures. Organic solvents have been used (1,2) for the separation of catalytically active chromoproteins, associated with insoluble cell particles. It was attempted in this laboratory to use alcohols and a protective colloid simultaneously for the extraction of enzymes bound to tissue particles. The following report contains a procedure for the extraction of a catalytic system from rat liver particles, which can transfer electrons from reduced pyridine nucleotides to cytochrome C. Some physical properties of this optically homogenous system are also discussed. While this work was in progress Morton(3) reported that he could successfully employ n-butanol for the extraction of enzymes hitherto considered to be inseparable from tissue particles.

Experimental. Preparation of extract. 50 g of rat liver were homogenized in a Waring blendor for 30 seconds in ice cold solution of 0.15 *M* KCl, brought to pH 7.6 with bicarbonate. The volume of KCl solution was 100 ml. The temperature of the suspension during blending was kept at 0°C by the addition of crushed ice particles (made of distilled H_2O). The liver suspension was centrifuged between 0-4°C on the Servall angle centrifuge (type SS-1A) at 15.000 x g for 30 minutes. The supernate was decanted and the sedimented particulate fraction removed with a porcelain spoon, leaving the layer of agglutinated erythrocytes on the bottom of the centrifuge tube. The particulate fraction, or

* Present address: The Tulane University of Louisiana, Department of Medicine and Biochemistry.

† This work was supported by a grant of the U. S. Public Health Service.

1. Straub, F. B., *Biochem. J.*, 1939, v33, 787.

2. Yakushiji, E., and Mori, T., *Acta. phytochim.* (Japan), 1937, v10, 113.

3. Morton, R. K., *Nature*, 1950, v166, 1092.

sediment, was resuspended in about 120 ml KCl-bicarbonate, transferred to ice-chilled centrifuge tubes, and recentrifuged for 15′ at the same speed as previously indicated. The removal and washing of the sediment was repeated three times. After the third washing the sediment was suspended in 75 ml 0.15 *M* KCl (containing no bicarbonate) and the suspension rehomogenized. The following operations were all carried out with reagents chilled in ice bath, until their temperature was below 5°C. To the 75 ml suspension of liver particles 100 ml ice chilled isobutanol (Merck C.P.), 75 ml 0.1 *M* K-phosphate buffer (pH 7.6) and 3 ml of carboxymethylcellulose solution were added. The carboxymethylcellulose solution was prepared as follows: 0.5 g carboxymethocel-S (Viscosity X-Hi, The Dow Chemical Company, Midland, Mich.) was added with stirring to 100 ml distilled H_2O at 40°C, then heating was continued until the solution started to boil and kept at this temperature for 1 hour. The volume was made up to 100 ml with H_2O. The viscous solution was slowly cooled to room temperature and thereafter centrifuged at 4000 R.P.M. on an International centrifuge for 1 hour. This procedure removed a slightly turbid sediment, leaving a water clear supernatant, which was poured off and stored in the ice box. The mixture of liver particles, isobutanol, buffer and carboxymethylcellulose solution was vigorously shaken for a few seconds and immersed into the ice bath. A viscous light yellow paste was formed which was kept for 2 hours in the ice bath with occasional stirring, thereafter centrifuged on the Servall at 0-4°C in glass centrifuge tubes at top speed for 30 minutes. Three layers were formed, the top consisting of isobutanol, then a gummy protein cake and a reddish yellow clear bottom layer. The isobutanol was decanted, and the bottom layers were collected in an ice chilled flask. This solution, which still contained gross particles, was recentrifuged on the angle centrifuge for 60′ top speed. In some instances the centrifugation was carried out at 100,000 x g on the Spinco ultracentrifuge. In each case the resulting supernatant was a strongly fluorescent, optically clear reddish yellow solution.

Physical properties of the extract. The solution which remained clear below 10°C was inspected in a spectroscope and showed a strong band upon reduction with hydrosulfite at 560 mμ. This band diminished upon standing in air. When the isobutanol containing extract was warmed up to 14°C a dense precipitate appeared, forming a ring at the top of the solution. The precipitate disappeared rapidly upon chilling the solution in ice bath. This reversible thermoprecipitation could be repeated many times with the same solution, kept in ice bath for even a week. This dissolution of the precipitate did not occur when the temperature rose to 16-20°C. After removal of the precipitate the reduced band at 560 mμ disappeared, and the remaining strong yellow solution showed a typical flavoprotein absorption spectrum, with maxima at 450 and 469 mμ. These bands faded upon reduction with hydrosulfite. Upon removal of the isobutanol by extensive dialysis against KCl-PO_4 buffer (pH 7.6 1:1) at 4°C the thermoprecipitation no longer occurred, indicating that isobutanol precipitated, and above 16°C irreversibly denatured, a protein component of the extract. Isobutanol was most effectively removed by freeze-drying the extract. The resulting reddish yellow powder was dissolved in H_2O, and some insoluble residue centrifuged off. This solution was stable in the ice box for at least 2 weeks. The absorption spectrum of this extract was measured in the Beckman D. U. spectrophotometer (Fig. 1). Before addition of hydrosulfite a typical Soret band was found at 415 mμ, which shifted to 430 mμ after reduction, with a simultaneous appearance of a small band at 530 mμ and a marked maximum at 560 mμ. There was no spectrophotometric evidence indicating that the extract contained other compounds with heme protein like spectrum besides the one closely resembling cytochrome B.

Catalytic properties of the extract. The extract had a powerful peroxidase activity, as measured with the benzidine test, which seemed to parallel the amount of heme protein content, as measured by the degree of

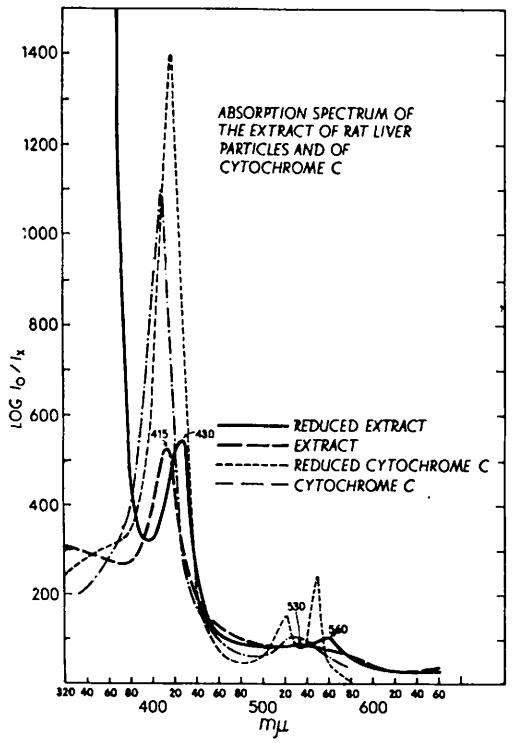

FIG. 1. Absorption spectrum of extract of rat liver particles in oxidized and reduced form compared with the spectrum of cytochrome C (3.10^{-2} micromoles). The extract is equivalent to approximately 0.5 g fresh liver. Volume of the optical system 3 ml, pH = 7.6 (0.1 M K-phosphate buffer). Optical density is plotted in terms of log $I_0/I_x \times 10^3$.

light absorption at 560 mμ upon reduction with hydrosulfite. Attempts were made to reduce the heme protein component of the extract by the yeast alcohol dehydrogenase-DPN and the Zwischenferment-TPN system. Alcohol dehydrogenase was prepared according to Racker(4), Zwischenferment as described by Warburg and Christian(5). In preliminary experiments the reduction of pyridin nucleotides by alcohol dehydrogenase or hexose 6-phosphate dehydrogenase was determined by measuring the change in optical density at 340 mμ. The reduction of 0.1 ml DPN (106 γ) by the alcohol enzyme was determined at pH 8.7, in presence of 0.1 ml glycine buffer (0.3 *M*) as heavy metal binding agent, that of TPN (120 γ 32% pure) by Zwischenferment at pH 7.4 in a final volume of 3 ml. The pH of each test system was maintained constant at 8.7 or 7.4 respectively with 0.4 ml 0.5 *M* trishydroxymethyl-aminomethane buffer(6). The dehydrogenases (0.1 ml) and substrates were in excess in each case. Carboxymethylcellulose in amounts present in the extract had no effect on the rate of reduction of the pyridine nucleotides. The enzymatic reduction of the coenzymes was complete in 30-50 seconds. The amount of extract of liver particles per cuvette was equivalent to approximately 0.4 g fresh liver. The enzymatic reduction of the extract was started by addition of either the substrate (0.1 ml 95% ethanol, or 0.1 *M* Na-glucose-6-phosphate), coenzyme, or dehydrogenases. A corresponding system, where the same component was replaced by water, served as blank. There was no change in light absorption at 430 and 560 mμ unless all the components of the reducing system (rat liver particle extract, substrate, dehydrogenase, coenzyme) were present, while a slow increase of optical density at 430 and 560 mμ occurred with both complete dehydrogenase systems. The cytochrome B-like component was under these aerobic conditions reduced by 40% only in 15 minutes. The addition of 0.1 ml cytochrome C ($3.4.10^{-2}$ micromoles) to these systems resulted in an instantaneous and complete reduction of the C component (at 550 mμ), and a subsequent increase of the reduction of the cytochrome B-like component (at 560 mμ) in 2-3 minutes. The reduction of component B in the presence of cytochrome C was about 70% with alcohol dehydrogenase-DPN, while it was complete with the Zwischenferment-TPN-reducing system. The cytochrome C reductase activity of the extract of rat liver particles was also tested by using smaller amounts of enzyme (equivalent to 50-100 mg of fresh liver). The components of the optical system in a final volume of 3 ml, were identical with the ones described previously. In each case immediate reduction of cytochrome C occurred when either chemically or enzymatically reduced coenzymes were added. Cytochrome C could

4. Racker, E., *J. Biol. Chem.*, 1950, v184, 313.

5. Warburg, O., and Christian, W., *Biochem. Z.*, 1932, v254, 438.

6. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 33.

not be replaced by methylene blue or triphenyltetrazolium chloride(7) indicating the absence of diaphorase(1) activity. In fact, the extract inhibited dye reduction. It is believed that this inhibition is partly due to the enzymatic inactivation of pyridine nucleotides by the extract. Incubation of 0.2 ml extract (equivalent with approximately 80 mg fresh liver) with 2.35 micromoles of DPN in glycylglycine buffer (0.15 *M*, pH 7.5) for 60 minutes at 37°C resulted in the appearance of 0.53 micromole of nicotinamide (determined chemically)(8), and the disappearance of 0.76 micromole of DPN (determined enzymatically)(4). No change in light absorption at 550 or 560 mμ occurred if in presence of cytochrome C and rat liver particle extract succinate, acetaldehyde, or choline-chloride were added.

Discussion. The catalytic activity of the extract of rat liver particles can be defined as an electron mediating system between reduced coenzymes and cytochrome C. The cytochrome C reductase of Haas, Horecker, and Hogness(9) isolated from yeast, links reduced TPN to cytochrome C. The DPN linked dehydrogenases of mammalian tissues also reduce cytochrome C via a reductase(10,11). The separation of these systems from mammalian tissue particles has hitherto not been reported. The circumstance that the rat liver preparation can mediate electrons both from DPN • H_2 and TPN • H_2 to cytochrome C would suggest that this enzyme has no specificity toward coenzymes, or that 2 separate reductases may be present in this preparation. Hogeboom and Schneider(11,12) showed that mitochondria can catalyse the reduction of cytochrome C by both TPN • H_2 and DPN • H_2. The participation of a component of our system, which has a cytochrome-B-like spectrum, is as yet not quite clear. The possible identity of this component with cytochrome B is based at present merely on spectroscopic evidence. Keilin and Hartree(13) suggested that attempts to "solubilize" cytochrome B may modify the heme protein. This problem can only be solved by isolation of cytochrome B, since it is difficult to see what the criterion of modification of a substance is which is defined almost exclusively by its absorption spectrum. Spectrophotometric properties of the B-like compound indicate that it can be slowly reduced enzymatically. The slow rate of aerobic reduction of component B may be due to its low E_o' (–0.040 v) (Ball)(14) and its autooxidizibility. There was some indication that the Zwischenferment-TPN system reduced the B component more readily. Addition of cytochrome C greatly accelerated the reduction of B by the coenzyme linked dehydrogenases. The sequence of the appearance of the reduced bands (first C, then B) may be explained in such a way that traces of reduced B are sufficient to maintain a steady flow of electrons to C, and B becomes visibly reduced only after C is completely in reduced form. If this explanation is valid it substantiates the view of Potter and Lockhart(15), who suggested that cytochrome B is necessary to link pyridine nucleotide dependent dehydrogenases through a flavoprotein component to cytochrome C. Slater(16) recently showed that a factor is necessary between DPN • H_2, flavoprotein and cytochrome C. It cannot be excluded that the B component of the extract of liver particles is related to, or identical with this compound. It is of interest that variations in the procedure of extraction of liver particles altered primarily the heme protein content of the extract. Prolonged extraction (for 5-6 hours)

7. Kun, E., and Abood, L. G., *Science,* 1949, v109, 144.

8. McIlwain, H., and Rodnight, R., *Biochem. J.,* 1949, v44, 470.

9. Haas, E., Horecker, B. L., and Hogness, T. R., *J. Biol. Chem.,* 1939, v130, 425; *ibid.,* 1940, v136, 747.

10. Potter, V. R., *J. Biol. Chem.,* 1946, v165, 311.

11. Hogeboom, G. H., and Schneider, W. C., *J. Nat. Cancer Inst.,* 1950, v10, 983.

12. Hogeboom, G. H., and Schneider, W. C., *J. Biol. Chem.,* 1950, v186, 417.

13. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Ser. B,* 1939, v127, 167; *ibid.,* 1940, v129, 277.

14. Ball, E. C., *Biochem. Z.,* 1938, v295, 262.

15. Potter, V. R., and Lockhart, E. E., *Nature,* 1939, v143, 942.

16. Slater, E. C., *Biochem. J.,* 1949, v44, XLVIII.

or the use of less isobutanol or replacement of phosphate by bicarbonate resulted in low heme protein content of the extract. When the extraction was carried out at 30-37°C for 1 hour no heme protein could be detected spectrophotometrically. This yellow extract had diaphorase activity. The fractionation of the extract on a larger scale and characterization of the components is now being attempted.

Summary. Washed rat liver particles were subjected to treatment with isobutanol in the presence of dilute solution of carboxymethylcellulose sodium salt and phosphate buffer at ice bath temperature. The resulting extract, after being freed from isobutanol, contained a water soluble catalytic system capable of transferring electrons from DPN • H_2 or TPN • H_2 to cytochrome C. The possible identity of a heme protein-like component, with cytochrome B and other enzyme properties of the extract (peroxidase, DPN-ase) are discussed.

I am deeply indebted to Dr. Brigit Vennesland for her continuous interest and criticism in this work. The generous gift of samples of DPN, TPN, alcoholdehydrogenase, Zwischenferment and cytochrome C from Dr. Eric Conn is greatly appreciated. My thanks are due to Dr. H. G. Williams-Ashman, who determined the enzymatic breakdown at DPN.

Received June 14, 1951. P.S.E.B.M., 1951, v77.

A Method for Determining Blood Volume of the Rat Using Radioactive Phosphorus.* (18808)

PHILIP O'B. MONTGOMERY. (Introduced by Shields Warren.)

From the Cancer Research Institute, New England Deaconess Hospital, Boston, Mass.

With the advent of radioactive phosphorus, methods for the determination of the blood volume using the P^{32} labeled red cells have been worked out for a variety of mammals including man. The purpose of this paper is to present a method for measuring the blood volume of the rat using P^{32} labeled red cells, and to report the results obtained.

Method. The method used is a modification of the method employed by Reid and Orr(1) for human blood volume determinations using radioactive phosphorus labeled red cells. A normal adult ether-anesthetized rat is bled to death by either aortic or cardiac puncture and the blood heparinized and centrifuged. The serum and buffy coat are pipetted off, an equal volume of physiologic saline solution containing 12 μc of P^{32}† (2) is added and gently mixed. (Either carrier or carrier-free radioactive phosphorus may be used. If carrier P^{32} is used, care must be taken to prevent the K salt concentration from becoming too high, as hemolysis will result.) The cells are incubated for 45 minutes at 37°C; then are centrifuged and washed 3 times with normal saline. The final standard suspension of cells should be approximately equal to the original volume of blood—ordinarily about 8 ml. When the determination is to be made, the rat is weighed to the nearest half-gram and lightly anesthetized with ether. The jugular bulb is exposed and a half ml of the standard labeled cell suspension injected intravenously. (For this purpose we use 27 gauge needles and half ml tuberculin syringes.) Ten minutes is allowed to elapse and one-half ml of blood is withdrawn by cardiac puncture. This unknown rat sample is laked in 2 ml of tap water in a counting

* This work was done under U. S. Atomic Energy Commission Contract AT (30-1)-901 with the New England Deaconess Hospital.

1. Reid, A., and Orr, M. K., *J. Clin. Invest.*, 1950, v29, 313.

†The P^{32} used was supplied by the Oak Ridge National Laboratories, Oak Ridge, Tenn.

2. Cartland, G. F., and Kock, F. C., *Am. J. Physiol.*, 1928, v85, 540.