somewhat greater than that found for many other liver enzymes(3).

Summary. 1. Weanling rat liver homogenate had about one-half the level of alcohol oxidation activity eventually achieved by adult rats on a chow diet (net oxygen uptake of 24 cmm per 10 minutes per 200 mg fresh liver for the adult normal rat). 85% or 75% respectively of this normal level of activity was reached by feeding chow or a purified 24% casein diet for 2 weeks. A protein-free diet removed about 85% of this enzymatic activity from the liver. 2. Adult rats responded similarly to these diets. Complete starvation for 7 days reduced this activity about 50%, while a 50% food restriction for 2 weeks gave less than a 20% decrease in the rate of alcohol oxidation.

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Occurrence of Hexose-6-phosphate Dehydrogenase in Chorioallantoic Membrane of the Chick Embryo.* (20407)

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Previous experiments on the *in vitro* metabolism of excised chorioallantoic membranes of chick embryos dealt with changes in aerobic glycolysis which occurred following inoculation with viruses(1). In connection with this problem it became necessary to investigate the metabolic pathways of this tissue in more detail. The investigation reported here was undertaken as a part of these studies, and showed that the chorioallantoic membrane contains, besides the Embden-Meyerhof-Warburg type of glycolytic enzyme system(1), a glucose-6-phosphate oxidase.

Materials and methods. The embryonated eggs of white Leghorn and Rhode Island Red hens were obtained from a local hatchery. The age of the eggs varied from 6 to 13 days. The membranes were carefully excised and thoroughly washed in ice-chilled homogenizing medium which consisted of 0.15 *M* KCl containing 0.05% ethylene-diamine tetraacetic acid. The pH of the KCl solution was brought to 7.1 with KOH, and then 2 ml of 0.5 *M* glycylglycine buffer (pH 7.3) per 500 ml were added. The membranes of 5 to 8 eggs were combined and quickly ground with ice-cold (4°C) KCl solution in a glass homogenizer. The homogenate was kept in an ice bath prior to enzyme assays. The protein content of the homogenate was determined colorimetrically according to Lowry *et al.*(2), with crystalline bovine albumen as reference standard. Triphosphopyridine nucleotide (TPN, 60% pure), diphosphopyridine nucleotide (DPN, 90% pure), and glucose-1-PO_4 K-salt were obtained from Sigma Chemical Company, Inc. The Ba-salt of glucose-6-phosphate was a purified sample received through the courtesy of Dr. D. Broida (Sigma Chemical Co., Lot 91-20) Fructose-6-phosphate (purchased as Ba-salt from Nutritional Biochemicals Corp.) and fructose-1-6-diphosphate (HDP, Ca-salt, Sigma) were purified by ion exchange(3). Both hexose monophosphates and HDP were made up to 0.1 *M* solutions as K-salts. Pyocyanine was recrystallized twice from hot water after chloroform extraction of a commercial sample (Delta Chemicals), as described by Wrede and Starck(4). Janus green B and methylene blue were commercial samples. Isatin-6-

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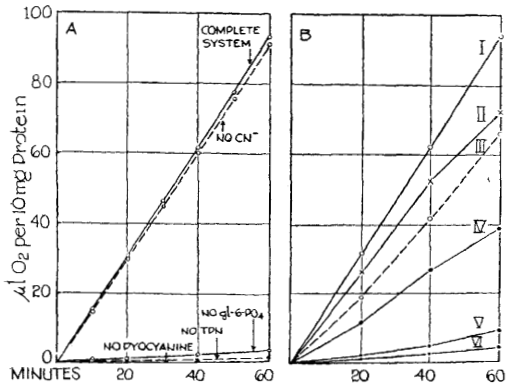

FIG. 1. Each vessel contains 1 ml homogenate. The following components are in the main compartment: A. Complete system: 10^{-2} *M* potassium glucose-6-phosphate, 10^{-2} *M* KCN(6), 1 μM TPN (dissolved in 0.2 ml 0.5 *M* glycylglycine buffer), and glass-distilled water to 2.6 ml. B. I. Complete system, containing no CN^-; II. Same as I, except with potassium iodoacetate in final concentration of 10^{-3} *M*; III. Glucose-6-phosphate replaced by equimolar fructose-6-phosphate; IV. Pyocyanine replaced by equimolar methylene blue; V., by Janus green B, and VI., by isatin-6-carboxylic acid (K-salt). The electron carriers are added as aqueous solutions in 0.3 ml volume, equivalent to 1 μM of each. 0.1 ml 20% KOH is placed in the center well with a strip of filter paper. Temp. 37°C; gas phase is air.

carboxylic acid was synthesized from m-aminobenzoic acid(5) and the final product after 2 recrystallizations made up to a 0.01 *M* solution as K-salt. Enzyme assays were carried out by conventional manometric technics. When CN^- was used in the presence of KOH in the center well the procedure of Krebs(6) was followed. The reduction of TPN was determined on the Beckman DU spectrophotometer at 3400 Å.

Results and discussion. The oxidation of hexose-6-phosphate by homogenates of chorioallantoic membranes was measured in a manometric test system. The effects of the components of the test system on the rate of O_2 absorption are summarized in Fig. 1 (A and B). The homogenizing medium as described above was chosen because in this salt solution enzyme activity is preserved for several hours. This stabilizing effect is due to the removal of traces of heavy metal impurities by the chelating agents employed. This observation is in agreement with the findings of von Euler and Adler(7), who reported enzyme inhibition by copper ions. The use of CN^- in the manometric assay of "Zwischenferment" was described by Negelein and Gerischer(8); therefore it was tested in our system. As shown in Fig. 1A, CN^- has no appreciable effect on the rate of reaction, and it was omitted in further experiments. The pH optimum of the hexose-6-phosphate oxidase activity of whole homogenates, as measured in our test system, lies between 7.0 and 7.4. The final pH of the contents of respirometer flasks was 7.2 in all experiments. The rate of O_2 absorption by the complete test system was linear over a period of 60-90 minutes and proportional to the amount of homogenate. The rate of reaction is identical in air and 100% O_2 atmosphere. No reaction occurs if TPN, glucose-6-phosphate or pyocyanine are omitted. Similarly, if glucose, HDP, or glucose-1-phosphate is substituted for glucose-6-phosphate, or DPN for TPN, no O_2 uptake occurs in this test system (Fig. 1A). Fructose-6-phosphate as substrate maintains a steady rate of O_2 consumption which is, however, lower than that with glucose-6-phosphate (Fig. 1B). Iodoacetate in concentrations which strongly inhibit the triose phosphate dehydrogenase of the chorioallantoic membrane (10^{-4} *M* iodoacetate inhibits 90%) does not alter glucose-6-phosphate oxidation, and only higher concentrations (10^{-3} *M*) produce some inhibition.

As mentioned above, no O_2 uptake occurs in the absence of pyocyanine, which was found to be the most suitable carrier. Methylene blue, Janus green B, and isatin-6-carboxylic acid are increasingly poorer catalysts (Fig. 1B). Langenbeck, Weschky, and Gödde(9) found that carboxyisatins act as potent catalysts in the nonenzymatic dehydrogenation of alanine. A study on the interaction of isatin-6-carboxylic acid with other enzyme systems, besides hexose-6-phosphate dehydrogenase, is now being pursued.

The glucose-6-phosphate oxidase activity of homogenates of chorioallantoic membranes of chick embryos between the age of 6 to 13 days remains fairly constant, Qo^2 prot. (μl O_2 per 1 mg protein per 1 hour) ranging between 7.2-9.4 (11 enzyme assays). It was found previously that the reduction of certain dyes by dehydrogenases requires a flavoprotein present in mitochondria(10). It seemed of

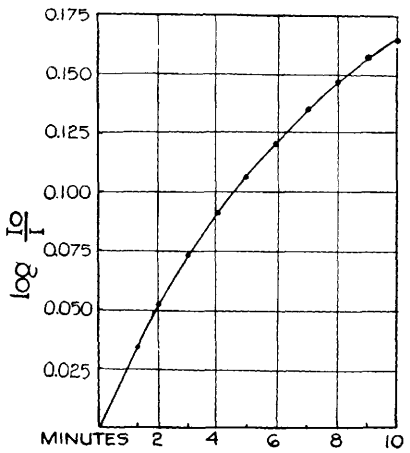

FIG. 2. The test system contains 0.3 ml centrifuged extract of chorioallantoic membrane (2.25 mg protein), 20 μM glucose-6-phosphate (0.2 ml), 1 μM TPN (in 0.2 ml glycylglycine buffer), and distilled water to a final volume of 3 ml. The reaction is started by the addition of either the enzyme, substrate, or coenzyme. Changes in optical density are recorded at 3400 Å in 1 cm cells. Temp. 24°C.

interest, therefore, to ascertain whether or not the electron transfer to O_2 occurs by means of a similar mechanism in the glucose-6-phosphate oxidase system of the chorioallantoic membrane. Homogenates were centrifuged at 25,000 x g for 60 minutes at 4°C and the sediment and clear supernatant fluid were separated. The sediment was resuspended in a volume of KCl solution equal to the original homogenate. Enzyme and protein assays were carried out in the whole homogenate, on the centrifugal supernatant fluid, and on the resuspended sediment. The following results were obtained: the total enzyme content, as expressed in μl O_2 absorbed by 1 ml of enzyme per hour, was 148.6 μl for the whole homogenate, 144.3 μl for the centrifugal supernatant fluid, and 4.2 μl for the particulate fraction. The protein content of the whole homogenate was 19.5 mg/ml, that of the supernatant fluid 12.5 mg/ml, and that of the resuspended particulate fraction 7.0 mg/ml. It is apparent that the enzyme activity of the homogenate is almost completely (96.5%) recovered in the supernatant fluid, indicating that enzyme systems of the particulate fraction do not participate in this reaction. The rate of reduction of TPN by the centrifugal supernatant fluid is determined spectrophotometrically at room temperature (Fig. 2). The rate of reduction of TPN decreases with time, an observation which can be explained on the basis that reduced TPN competes with oxidized TPN for the dehydrogenase(12). The reduction of dichlorophenolindophenol in the test system of Haas(11) can also be successfully employed if the centrifuged extracts are first dialyzed in order to minimize endogenous dye reduction.

Conclusions. It is evident that the chorioallantoic membranes of chick embryos can oxidize glucose-6-phosphate by an enzyme system similar to that discovered by Warburg and Christian(12,13). The centrifugal supernatant fluid of homogenates contains the complete catalytic system necessary for oxidation of glucose-6-phosphate as measured in our test system. The reaction is completely dependent on hexose-6-phosphate and TPN. This finding is of particular interest since it indicates that the chorioallantoic membrane contains an alternative pathway of glucose metabolism besides the glycolytic system described previously(1).

Summary. Homogenates of chorioallantoic membranes of the chick embryo oxidize glucose-6-phosphate or fructose-6-phosphate aerobically if TPN and pyocyanine are present. The enzyme activity which is recovered in the centrifugal supernatant fluid does not change appreciably with age of the embryo between 6 and 13 days.

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Chromatography of α-ε-Diaminopimelic Acid on Starch Columns.* (20408)

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In connection with studies on the amino acid composition of certain proteins it was desirable to know the behavior on starch chromatographic columns of the recently discovered amino acid, α-ε-diaminopimelic acid‡ (called in this paper DAP) isolated by Work (1,2). It was of interest also to note its mobility by means of paper electrophoresis.

Procedure. The starch columns (0.9 x 30 cm) were prepared as recommended by Moore and Stein(3), and the effluent values of the DAP and certain amino acids added to the solution as markers were followed by means of the ninhydrin reaction on fractions collected by an automatic fraction collector and drop-counting apparatus(4). Two solvents were used in this study, 1:2:1 (n-butyl-n-propyl, 0.1 N-HCl) and 2:1(n-propyl-0.5N HCl). The DAP was dissolved in 0.1 N-HCl to which was then added 4 volumes of 1:2:1 solvent, giving a final concentration of 3 mg DAP per ml. Proper aliquots were added to the columns to give the quantities designated in Table I. For paper electrophoresis§ the different mixtures of amino acids as used in the chromatography experiments were placed on strips of Whatman No. 1 filter paper and run at 130 volts and 25 milliamperes for 2 hours in barbiturate buffer pH 8.6. The buffer was made as follows. Sodium barbiturate, 29.428 g, sodium acetate 3 H_2O, 19.428 g and N/10 HCl 180 ml were dissolved in 3000 ml distilled water and then diluted with an equal amount of distilled water. The same amino acids as mentioned above were also dissolved directly in the buffer before placing them on paper strips. At the end of the experiment the strips were dried in an oven, sprayed with ninhydrin and heated 4 minutes at 90°C.

Results. In the first chromatographic experiment leucine and DAP were put on the column with the 2:1 solvent and the DAP was found to emerge at an effluent value of 80 ml, as shown in Table I. It remained on the column in experiment 2 when the 1:2:1 solvent was used and emerged only after the solvent was replaced with 2:1 solvent and then at an effluent value about 65 ml past this change. These values were suggestive of the position for lysine. Therefore, in a third experiment lysine was added to DAP and the mixture placed on a column. At the same time a mixture of proline and DAP was run in tandem. As shown in Table I, DAP emerged identically with lysine as a single sharp peak and the recovery was close to the theoretical value. (Fig. 1).

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‡ For these studies some of the amino acid was generously given to us by Dr. Work.

§ Paper strip model electrophoresis apparatus obtained from Arthur H. Thomas Co., Philadelphia, Pa.