comparable with that of liver tissue in guinea pigs (Dutton, 1959). To account for the large quantities of conjugated oestrogens excreted in the urine during pregnancy it will be necessary to know the rates of glucuronidation of these compounds by various organs.

SUMMARY

1. Oestrone 3-glucuronide has been identified as the reaction product after incubation of oestrone with a rabbit-liver microsomal preparation, uridine diphosphate glucuronic acid being used as the glucuronic acid donor. The formation of this compound was studied under various experimental conditions.

2. The reaction showed a maximum at pH 8.0–8.2, and K_m for oestrone was found to be $9.7 \times 10^{-5} M$.

3. The results are discussed in relation to the general problem of the physiological production of oestrogen glucuronides.

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The Binding of Pyridoxal 5-Phosphate to Aspartate Aminotransferase of Pig Heart

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In previous studies of the effects of sulphate and phosphate esters of oestrogens on the reconstitution of aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) prepared by the method of O'Kane & Gunsalus (1947), the enzyme was freed from the coenzyme by incubation

at 60° for 1 min. in the presence of 20 % (w/v) ammonium sulphate (Scardi, Magno & Scarano, 1960; Scardi, Iaccarino & Scarano, 1962). In later work the enzyme was prepared by the method of Jenkins, Yphantis & Sizer (1959) that permits the preparation in a relatively simple way of large

amounts of the highly purified enzyme. But, unexpectedly, with this enzyme preparation the resolution procedure of Scardi *et al.* (1960) was not effective even when temperature, incubation time and ammonium sulphate concentration were varied. It was also impossible to obtain, with the same enzyme preparation, a resolution higher than about 30 % by the procedure described by Banks & Vernon (1961). Because of these differences in the ease of resolution, we have studied the resolution of the enzyme in the pyridoxamine and the pyridoxal forms and we have compared the effects of various ions in the resolution of the enzyme.

A preliminary communication was made at the International Symposium on the Chemical and Biological Aspects of Pyridoxal Catalysis, held in Rome, 25–31 October 1962.

MATERIALS AND METHODS

Chemicals. Pyridoxal 5-phosphate and pyridoxamine 5-phosphate were products from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; L-aspartic acid and α -oxoglutaric acid were from Hoffmann-La Roche, Basle, Switzerland. All other chemicals were pure products obtained from different sources.

Preparation and assay of aspartate aminotransferase. Aspartate aminotransferase was prepared from pig heart by the method of Jenkins *et al.* (1959) and had a specific activity of 27, expressed as μ moles of oxaloacetic acid formed/mg. of protein/min. at 37°. The activity was measured spectrophotometrically as described by Cammarata & Cohen (1951) in a Unicam SP. 500 spectrophotometer equipped with a temperature-controlled cell-holder. The temperature variation did not exceed 0.1°.

Protein concentrations were determined spectrophotometrically as described by Kalckar (1947).

To obtain the enzyme in the pyridoxamine form an excess of L-aspartate or L-glutamate was added as follows: 20 μ l. of enzyme (20.5 mg./ml.) was incubated at room temperature (about 18°) for 15 min. with 40 μ moles of aspartate or of glutamate adjusted to pH 7.4 (with N-NaOH) in a final volume of 2 ml.

Evaluation of the degree of resolution. With each portion of the incubation mixture containing enzyme and reagents, two separate activity determinations were performed, with and without the addition of an excess of pyridoxal 5-phosphate (about 3 μ g./ μ g. of enzyme protein). The percentage resolution is given by: $100(A - a)/A$, where A and a are the activities measured in the presence and absence respectively of pyridoxal 5-phosphate. In general A was equal or almost equal to the activity before resolution.

RESULTS AND DISCUSSION

Aspartate aminotransferase, prepared by the method of Jenkins *et al.* (1959), was incubated at 60° or at 65° for 2 min. in the presence of ammonium sulphate at concentrations of 20–30 % (w/v) without any resolution being observed. The same result was obtained by prolonging the incubation

time up to 80 min. in the presence of 15 % (w/v) ammonium sulphate.

No resolution was observed in similar experiments performed with a partially purified aspartate-aminotransferase preparation (first step of the purification method of Jenkins *et al.* 1959), before and after dialysis. This excludes the possibility that the negative results were due either to the higher degree of purification or to the protective action of maleate buffer used during the purification procedure.

A partial resolution (about 30 %) was observed by following the procedure described by Banks & Vernon (1961).

Since the enzyme prepared by the method of Jenkins *et al.* (1959) is in the pyridoxal form, the working hypothesis was made that it was not possible to resolve this form of aspartate aminotransferase. Indeed, the binding of coenzyme to apoenzyme should be markedly weakened in the pyridoxamine form of the enzyme because of the absence of the azomethine linkage whose existence was indicated by Bonavita & Scardi (1958). Jenkins *et al.* (1959) found that the pyridoxamine form of the enzyme is more labile than the pyridoxal form to heat-inactivation, but their results do not decide whether the higher lability is due to resolution or to denaturation.

Resolving action of sulphate. After incubation with 20 % (w/v) ammonium sulphate at 60° or at 65° for 0–120 min., almost complete resolution of aspartate aminotransferase (pyridoxamine form) was obtained, but no resolution of the enzyme in the pyridoxal form occurred (Fig. 1). Experiments

Fig. 1. Action of 20 % $(\text{NH}_4)_2\text{SO}_4$ on aspartate aminotransferase (pyridoxamine form) at 60° (○) and at 65° (●), and in the pyridoxal form at 60° (◐). Experimental details are given in the text. Arrows indicate the addition of an excess of pyridoxal 5-phosphate. The 100% activity corresponds to 27 μ moles of oxaloacetic acid formed/min./mg. of protein, as specified in the Materials and Methods section.

with sodium sulphate in place of ammonium sulphate gave similar results.

Resolving action of phosphate. In preliminary experiments the conditions described by Banks & Vernon (1961) were used, namely incubation at 60° for 50 min. in *m*-potassium phosphate buffer, pH 6.0. However, after 10 min. of incubation only a 60% recovery of the inactivated enzyme mixture was obtained, thus showing a partial denaturation together with the resolution. Then the incubation temperature was decreased and a standard temperature of 30° was chosen for all other experiments reported in the present paper, unless otherwise stated. In Fig. 2 the percentage resolution is plotted against the inverse of the concentration. A linear relationship exists between the residual activity after 30 min. and the reciprocal of the phosphate concentration at least in the range 0.1–0.5 *M*. This may be ascribed probably to a competition between phosphate anion and the phosphate group of the coenzyme for the cationic site of the apoenzyme. A linear relationship appears to exist also between percentage resolution and pH over the range of pH 4.7–6.8 in 0.15 *M*-potassium phosphate at 30°; below pH 4.7 irreversible inactivation of the enzyme occurs. These results indicate probably that the resolution attains an equilibrium whose value depends on the pH and on the phosphate concentration.

Resolving action of other anions. Figs. 3 and 4 show the influence of acetate and arsenate, as compared with sulphate and phosphate, on as-

partate aminotransferase (pyridoxamine form). (With arsenate at pH values below 6.0 an irreversible inactivation of the enzyme occurs.) Under the same conditions chloride, borate, formate and citrate were without resolving effect. Quinolate and phthalate caused irreversible inactivation. On the basis of these experiments, phosphate is the most active resolving anion.

Method of resolution. The standard procedure arrived at may be summarized as follows: (a) incubation of the enzyme at room temperature for 15 min. with *L*-aspartate or *L*-glutamate (final concn. 5–10 *mM*) in the proportion of 15 μ moles of

Fig. 3. Effects of arsenate (Δ), sulphate ($\circ$), acetate ($\bullet$) and phosphate ($\bullet$) at pH 6.0 on aspartate aminotransferase (pyridoxamine form). Experimental details are given in the text.

Fig. 2. Effect of phosphate concentration on the resolution of aspartate aminotransferase (pyridoxamine form). The enzyme activities after (●) 10 min., (◐) 20 min. and (○) 30 min. of incubation at 30° are shown. Experimental details are given in the text. The 100% activity corresponds to 27 μ moles of oxaloacetic acid formed/min./mg. of protein, as specified in the Materials and Methods section.

Fig. 4. Effects of sulphate ($\circ$), acetate ($\bullet$) and phosphate ($\bullet$) at pH 4.75 on aspartate aminotransferase (pyridoxamine form). Experimental details are given in the text.

amino acid/mg. of protein; (b) incubation with potassium phosphate (final concn. 0.5 M) at pH 4.75 and 30° for 30 min.

L-Aspartate can be replaced only by L-glutamate, the D-forms of these amino acids being ineffective. A twofold concentration of the racemates is necessary to obtain comparable results. DL-Alanine, α -oxoglutaric acid, oxaloacetic acid, γ -aminobutyric acid, maleic acid and succinic acid have no resolving effect under the same conditions. So it seems likely that only the amino acids that are substrates can effect the resolution in this procedure.

To confirm that the resolution of aspartate aminotransferase is possible only if the enzyme is in the pyridoxamine form and that the presence of the amino acid substrate during the resolution procedure is not necessary, the pyridoxamine and pyridoxal forms of the enzyme were prepared by addition of the two forms of the coenzyme to resolved apo-(aspartate aminotransferase) as follows: original unresolved enzyme was first converted into the pyridoxamine form by incubation with aspartate, and the mixture was then passed through a column of Dowex 1 (X 8; 100–200 mesh; formate form) to eliminate any possible traces of keto acid that could rapidly transform the pyridoxamine form back into the pyridoxal form (Jenkins &Sizer, 1960). After dialysis the absorption spectrum was identical with that reported by Jenkins &Sizer (1960), thus confirming that the enzyme was in the pyridoxamine form. The enzyme was then resolved by treatment with phosphate and dialysed. Part of this material was reconstituted by the addition of pyridoxamine 5-phosphate and then it was dialysed. This enzyme was readily resolved by the addition of phosphate (step b above). The reconstituted pyridoxal form of the enzyme, obtained by the addition of pyridoxal 5-phosphate to the apoenzyme, needed preincubation with either aspartate or glutamate before the addition of phosphate to obtain resolution.

The present results strengthen the view that an azomethine linkage occurs between the 4-formyl group of the pyridoxal 5-phosphate and an amino group of the enzyme protein, and that this linkage is rather strong, being broken only by a specific enzymic interaction between the 4-formyl group and L-aspartic acid or L-glutamic acid, i.e. the amino acid substrate. When the azomethine linkage

disappears, the remaining binding can be easily loosened by the intervention of anions, such as phosphate. This conclusion agrees with the findings of Wada & Snell (1962) that the pyridoxamine 5-phosphate can be removed more easily from aspartate aminotransferase than the pyridoxal 5-phosphate.

The conditions under which phosphate is effective in resolving aspartate aminotransferase suggest that this phenomenon may be of physiological significance and conceivably be a mechanism by which the cell can regulate its pool of amino acids. Indeed, in some definite region of the cell, conditions may arise that cause a reversible dissociation of the pyridoxamine form of the enzyme.

SUMMARY

1. Conditions are described under which aspartate aminotransferase can be dissociated into coenzyme and apoenzyme.

2. Only the pyridoxamine form of the enzyme can be resolved. The resolution can be performed by incubation with several anions, of which phosphate is the most effective.

3. The conditions under which phosphate is effective in resolving aspartate aminotransferase suggest the possibility that this phenomenon has physiological significance.

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