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Purification and Properties of the 3α -Hydroxy Steroid-Dependent Nicotinamide-Adenine Dinucleotide Transhydrogenase of Rat Liver

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The occurrence in rat liver of a 3α -hydroxy steroid dehydrogenase [3α -hydroxy steroid-NAD(P) oxidoreductase, EC 1.1.1.50], active in the presence of both NAD and NADP, was established by Tomkins (1956). Hurlock & Talalay (1958) showed that the enzyme from rat liver could catalyse hydrogen transfer between NADPH₂ and NAD only in presence of steroid. Stein & Kaplan (1959), however, failed to demonstrate any increase in the rate of transhydrogenation on addition of androsterone to an appropriate reaction mixture containing either the soluble or the mitochondrial fraction from rat-liver homogenate. Bloom (1960) measured the incorporation of tritium (from [2-³H]-lactate) into either hexose via NADH₂ or into fatty acid via NADPH₂ in rat-liver slices. In the presence of androstanedione there was no alteration in the relative distribution of ³H into these products, from which Bloom concluded that this steroid did not significantly influence transhydrogenation. Hurlock & Talalay (1958) and Koide, Chen & Freeman (1960) noted that the 3α -hydroxy steroid-mediated transhydrogenase activity of the soluble fraction of a rat-liver homogenate was not demonstrable in the presence of large amounts of NADPH₂; in the presence of a NADPH₂-generating system and a minute amount of NADP, however, significant hydrogen transfer could be detected.

The question whether steroid-dependent transhydrogenation between NADPH₂ and NAD is a function of a single transhydrogenase or of a dehydrogenase with dual nucleotide specificity has not yet been settled (Hagerman & Villee, 1959; Jarabak, Adams, Williams-Ashman & Talalay, 1962) and the answer may be different for different tissues and different species. For the purposes of this work the 3α -hydroxy steroid-dependent transhydrogenating system from rat liver is referred to below as transhydrogenase. This has been studied in rat liver by a coupled transhydrogenase method with glucose 6-phosphate, or isocitrate, and the appropriate dehydrogenase, as generator of NADPH₂ (Scheme 1).

Using partially purified rat-liver enzyme, we observed that transhydrogenase activity was increased by bivalent anions (Pietruszko, Gore & Baron, 1962). Some of the properties of the transhydrogenase have been studied in the light of this anion activation.

EXPERIMENTAL

Materials

NAD, NADP, disodium glucose 6-phosphate and glucose 6-phosphate dehydrogenase (5 mg./ml. in 3.3M-ammonium sulphate solution; specific activity, 70 μ moles of substrate oxidized/mg./min.) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. The 3-acetylpyridine analogue of NAD was obtained from Pabst Laboratories,

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Scheme 1

Wis., U.S.A. Isocitric acid lactone and isocitrate dehydrogenase (specific activity, 15 μ moles of substrate oxidized/mg./min.) were obtained from Sigma Chemical Co., Mo., U.S.A. Androsterone, androstenedione and aetiocholanolone were obtained from Organon Laboratories Ltd. Ammonium sulphate (Hopkin and Williams Ltd., A.R. grade) was purified by two recrystallizations from water containing 0.2% of EDTA. Tris buffer, brought to the appropriate pH with hydrochloric acid, was used throughout. The diethylaminoethylcellulose (DEAE-cellulose) was Whatman DE50.

Methods

Enzyme assays. Transhydrogenase, steroid-dehydrogenase and glucose 6-phosphate-dehydrogenase activities were assayed by following the reduction of either NAD or NADP at 340 $m\mu$, or the 3-acetylpyridine analogue of NAD at 363 $m\mu$, at room temperature (20–25°), with cuvettes of 1 cm. light-path in a Hilger Uvispek spectrophotometer. Enzyme activities are expressed as changes in extinction (ΔE), or as specific activities calculated from the molar extinction coefficient, 6220 at 340 $m\mu$, for NADH₂ and NADPH₂.

(a) Transhydrogenase. The reaction system, in a micro-cuvette, consisted of the following components in a final volume of 0.27 ml. (the quoted molarities are final concentrations): tris buffer, pH 7.5 (or as specified), 55 mM; nicotinamide, 74 mM; NAD, 1.4 mM; NADP, 22 μ M; glucose 6-phosphate, 5.5 mM, or isocitrate, 3.0 mM; glucose 6-phosphate dehydrogenase (0.5–25 μ g./0.27 ml.) or isocitrate dehydrogenase (10 μ g./0.27 ml.), which gave instantaneous conversion of all NADP present into NADPH₂. Manganese chloride (0.2 mM) was present in the reaction system utilizing isocitrate/isocitrate dehydrogenase. The steroid (12 μ M) was initially added as a suspension in water-acetone (49:1, v/v; final concentration of acetone 0.04%), and in later experiments as a solution in water-acetone (1:1, v/v; final concentration of acetone 1%), the appropriate water-acetone mixture being added to the controls. The rate of transhydrogenation was unaffected by these concentrations of acetone. The reaction was started by addition of a suitable amount of a liver preparation. The following controls were set up: (1) control containing all appropriate components except steroid; (2) control with all appropriate components except NADP. The rate of reaction under these conditions was constant for 1 hr.

Readings were taken at 1 min. intervals for 15–30 min. Steroid-dependent transhydrogenase activity was calculated from the difference in the rate of NADH₂ formation in the complete system and that occurring in the absence of steroid (control 1). Control 2 gave a measure of NAD-linked non-specific dehydrogenase activity of a preparation. After partial purification this activity was negligible.

(b) Steroid dehydrogenase. This was assayed in a final volume of 2.1 ml. by measuring the rate of reduction of NAD or NADP with androsterone. The reaction system finally contained: tris buffer, pH 8.5, 0.1 M; NAD or NADP, 0.36 mM; steroid, 65 μ M (40 μ g./2.1 ml., added in 0.02 ml. of dioxan). The reaction was initiated by addition of rat-liver enzyme and readings were taken at 15 sec. intervals for 2–3 min. Enzyme activity was calculated from graphically determined initial velocities.

(c) Glucose 6-phosphate dehydrogenase. This was measured, in a total volume of 0.27 ml., in a system that finally contained: tris buffer (at required pH), 55 mM; nicotinamide, 74 mM; glucose 6-phosphate, 5.5 mM; NADP, 0.16 mM; glucose 6-phosphate dehydrogenase, 0.02 μ g./cuvette. Enzyme activity was calculated from the initial rates of reduction of NADP.

Protein concentrations. These were calculated from the difference in E at 215 and 225 $m\mu$ of a suitable dilution in glass-distilled water (Waddell, 1956).

Preparation of DEAE-cellulose columns. DEAE-cellulose (100 g.) was sedimented with glass-distilled water containing 0.2% of EDTA. The slurry was transferred to a separating funnel and washed successively with 2 l. of sodium hydroxide (0.5 N), 3 l. of hydrochloric acid (0.5 N) and 3 l. of tris buffer (0.1 M, pH 7.5). Between each operation the slurry was washed with 1 l. of glass-distilled water. A portion of the slurry of DEAE-cellulose in 0.1 M-tris buffer was packed into an ice-cold water-jacketed glass column, 2 cm. diam., to a length of 10 cm. and the column washed with tris buffer (50 ml., 0.1 M, pH 7.5) before use.

Preparation of enzyme. Male or female rats (various strains) were killed by spinal dislocation and each liver was rapidly perfused with 0.9% sodium chloride solution at room temperature. The livers were removed, cooled in ice, weighed and homogenized for 30–60 sec. in a microhomogenizer (Measuring and Scientific Equipment Ltd.) at maximum velocity in 2 vol. (w/v) of an ice-cold solution containing 200 ml. of glycerol, 1.22 g. of nicotinamide, 0.12 g. of cysteine and 0.37 g. of EDTA/l., adjusted to

pH 7.5. The homogenate was ultracentrifuged for 30 min. at 66 000g in a Spinco model E preparative ultracentrifuge and the clear supernatant solution collected. The remaining operations were carried out at 0–2°. Saturated ammonium sulphate solution was added dropwise with constant stirring to the tissue extract. The fraction that precipitated between 50 and 80% saturation with ammonium sulphate was collected by centrifuging. The precipitated protein was resuspended in water and dialysed in washed cellophan tubing for 15–18 hr. against 0.01M-tris buffer, pH 7.5, containing 0.5 μ M-EDTA. A sample of the dialysed protein solution was applied to the DEAE-cellulose column and the column eluted with 30–40 ml. of 0.1M-tris buffer, pH 7.5. The eluate was collected in 2 ml. portions and tested for protein and transhydrogenase activity. The column eluates containing the transhydrogenase activity were pooled and the protein was reprecipitated at 75% ammonium sulphate saturation. The precipitated protein was separated by centrifuging and dialysed as described above. This partially purified enzyme retained its full activity for at least 2 weeks when stored at 4°.

RESULTS

Purification of transhydrogenase on a DEAE-cellulose column. Fig. 1 shows the results of elution of a DEAE-cellulose column with 0.1M-tris after 2 ml. of enzyme solution containing 56 mg. of protein had been applied. The specific transhydrogenase activity of this solution, with optimum concentrations of steroid and activating anion, was 1.4 μ m-moles of NADH₂/mg./min. A pigmented protein eluted in fractions 6–8, comprising 43% of total protein, was inactive. Androsterone-dependent transhydrogenase activity (81% of the initial activity) was recovered in fractions 10–15, which comprised 13.2% of the total protein. The specific activity of this enzyme was 10.2 μ m-moles of NADH₂/mg./min. This represents a sevenfold increase over the activity of the material applied to the column. The transhydrogenase preparation was contaminated with a small amount of an enzyme that oxidized NADH₂.

Effect of ammonium sulphate on the coupled transhydrogenase system. When steroid-dependent hydrogen transfer from NADPH₂ to NAD by the partially purified enzyme was coupled with glucose 6-phosphate dehydrogenase, the rate of transhydrogenation, under conditions in which NADPH₂ production was instantaneous, increased with increasing concentrations of commercial glucose 6-phosphate dehydrogenase. If dialysed commercial enzyme preparation was used, the rate of hydrogen transfer from NADPH₂ to NAD was low and did not increase with increasing amounts of glucose 6-phosphate dehydrogenase. When, however, heat-inactivated undialysed commercial glucose 6-phosphate dehydrogenase was added to the reaction system containing the dialysed glucose 6-phosphate dehydrogenase, the rate of hydrogen transfer from NADPH₂ to NAD increased 25-fold. The same in-

creased rate could be produced by adding ammonium sulphate (Fig. 2). Further experiments showed that the rate increased with the concentration of ammonium sulphate, becoming constant at between 0.1 and 0.2M. The rate of transhydrogenation in the absence of added ammonium sulphate was 3–5% of that in the presence of 0.1M salt. Because of these findings, glucose 6-phosphate-dehydrogenase activity was assayed at pH 7.5 in the absence of ammonium sulphate and in the presence of 0.025, 0.05, 0.1 and 0.2M-ammonium sulphate. The rates were respectively 57, 59, 59, 48 and 22 μ m-moles of NADPH₂/mg./min. Thus glucose 6-phosphate dehydrogenase is inhibited at ammonium sulphate concentrations that give maximal stimulation of the coupled transhydrogenase system.

Effect of other salts on the coupled transhydrogenase system. The following sodium salts increased the rate of hydrogen transfer from NADPH₂ to NAD: selenate, phosphate, arsenate, tungstate, oxalate, malonate, succinate and citrate. In Fig. 3 the activation by these salts is compared with that obtained with ammonium, sodium and potassium sulphate. The effect produced by selenate, arsenate or oxalate was quantitatively similar to that of sulphate. Phosphate, tungstate, malonate, succinate and citrate were less effective. The activation of the system by ammonium, sodium and potassium sulphate slightly decreased with increasing molecular weight of the cation. The significance of this is not clear as ammonium, sodium and potassium chloride, sodium nitrate and sodium hydrogen carbonate had no effect on the rate of hydrogen transfer from NADPH₂ to NAD.

Fig. 1. Chromatography on a DEAE-cellulose column. Dialysed enzyme solution (56 mg. of protein in 2 ml.; specific activity, 1.5 μ m-moles of NADH₂/mg./min.) was added to the column and eluted with 0.1M-tris buffer, pH 7.5. The eluate was collected in 2 ml. fractions. ●, Total protein/fraction (mg.); ×, specific activity/fraction (μ m-moles of NADH₂ formed/mg./min.); ○, total activity/fraction (specific activity $\times$ protein).

Magnesium, manganese, cobalt and zinc, added as chlorides, were tested at a final concentration of 1 mM. None of these increased the initial rate, and zinc functioned as a powerful inhibitor. Thus the anion of the activating salts was responsible for stimulating the transhydrogenase.

Possible relation between metal-binding and the anion-activation effect. The anions that activate transhydrogenation have a general property of forming sparingly soluble metal salts, especially with group IIA (alkaline-earth metals). To investigate the possible effect of Ca^{2+} ions on the system, use was made of transhydrogenase activated by selenate, as calcium selenate is relatively soluble. Hydrogen transfer in the presence of androsterone and sodium selenate (0.1M) was unaffected by calcium chloride (final concentration 0.22 mM). On the other hand the metal-binding agents cyanide (3 mM), EDTA (15 mM) or 8-hydroxy-quinoline (0.1 mM), added to the test system in the absence of activating salt, did not stimulate transhydrogenation. This suggests that removal of an inhibiting metal was not responsible for activation.

Effect of ammonium sulphate and pH on transhydrogenase activity. The effect of pH on the rate of the transhydrogenase reaction at five different

Fig. 2. Effect of ammonium sulphate on transhydrogenase. The test was made in 0.27 ml. of a system containing: 15 μ moles of tris buffer, pH 7.5; 1.5 μ moles of glucose 6-phosphate; 0.38 μ mole of NAD; 7 μ m-moles of NADP; 20 μ moles of nicotinamide; 1 μ g. of androsterone added in 0.005 ml. of 50% (v/v) acetone; 25 μ g. of dialysed glucose 6-phosphate dehydrogenase; and 0.01 ml. of rat-liver enzyme. The arrow indicates the point of addition of ammonium sulphate (final concn. 0.1M).

concentrations of ammonium sulphate is shown in Fig. 4. The rate increases with the increase of ammonium sulphate at all pH values tested, the optimum pH being in the region of 7.5 in each case. Thus the activation by sulphate is not due to a shift in the pH optimum of the enzyme (Dixon & Webb, 1958). Glucose 6-phosphate dehydrogenase, which was used for generation of NADPH_2 , is inhibited by concentrations of ammonium sulphate of 0.1M or more. The effect of pH on glucose

Fig. 3. Effect of various salts on the rate of transhydrogenation. Experimental conditions were similar to those described in Fig. 2 except that 0.5 μ g. of glucose 6-phosphate dehydrogenase was used and the androsterone was added as a suspension in 0.005 ml. of 2% (v/v) acetone. Rates of transhydrogenation are corrected for non-specific NADH_2 formation that occurred in the absence of androsterone. ●, Ammonium sulphate; ○, sodium sulphate; ▲, potassium sulphate; △, sodium selenate; ■, sodium oxalate; □, sodium arsenate; ▽, sodium malonate; ∇, sodium phosphate; ×, sodium citrate; +, sodium tungstate or sodium succinate.

Fig. 4. Effect of pH and ammonium sulphate concentration on the rate of transhydrogenation. Experimental conditions were as in Fig. 2 except that 2.5 μ g. of glucose 6-phosphate dehydrogenase was used. Final concn. of ammonium sulphate (M): ●, 0.006; ○, 0.025; △, 0.05; ×, 0.1; ▲, 0.2.

6-phosphate-dehydrogenase activity in 0.2M-ammonium sulphate is shown in Table 1. The inhibition of glucose 6-phosphate dehydrogenase is more pronounced at low pH values. In these experiments the NADPH₂-generating enzyme was in such excess that it gave instant reduction of NADP under all conditions except when the concentration of SO₄²⁻ ion was 0.2M and the pH was less than 7.5. The relatively steep fall in activity with pH from 7.5 to 6.5 in 0.2M-ammonium sulphate may be due to a fall in the NADPH₂ concentration to a rate-limiting level.

Effect of ammonium sulphate on transhydrogenase activity in the presence of different concentrations of androsterone. The results obtained by varying concentrations of androsterone and ammonium sulphate are shown in Fig. 5. In the presence of 2.5 and 25 mM-ammonium sulphate the rate of transhydrogenation increases with androsterone concentration over the range tested (0–32 μM). At higher sulphate concentrations, however, the rate increased to a maximum at approximately 10 μM-androsterone, further increase resulting in a slight inhibition.

Effect of ammonium sulphate on transhydrogenase activity in the presence of different concentrations of NADPH₂. The effect of varying NADPH₂ concentration on the rate of NAD reduction in the presence of five concentrations of ammonium sulphate is shown in Fig. 6. This experiment was carried out at NADPH₂ concentrations between 5 and 50 μM, and at pH 8.5 to avoid using a large excess of glucose 6-phosphate dehydrogenase. At final ammonium sulphate concentrations of 6 mM, 25 mM, 0.05M and 0.1M, the maximum observed rate of transhydrogenation was with 5 μM-NADPH₂. The fall in the rate of transhydrogenation from this maximum with higher amounts of NADPH₂ diminished as the concentration of SO₄²⁻ ion rose. In 0.2M-SO₄²⁻ ion, 15 μM-NADPH₂ gives the maximum rate and there is very little inhibition with higher concentrations of NADPH₂. Fig. 6 suggests that the optimum conditions for transhydrogenation lie at concentrations of SO₄²⁻ ion

of about 0.05M and NADPH₂ concentrations less than 5 μM.

Effect of ammonium sulphate on steroid-dehydrogenase activity. During this work enzyme preparations in which androsterone-activated transhydrogenation occurred always contained NAD- and NADP-linked androsterone dehydrogenases. In view of the hypothesis that steroid-activated transhydrogenation is a function of a dehydrogenase with dual nucleotide specificity (Talalay &

Fig. 5. Effect of ammonium sulphate and androsterone concentrations on the rate of transhydrogenation. Experimental conditions were as in Fig. 2 except that androsterone was added in 0.005 ml. of 50% (v/v) acetone and 1 μg. of glucose 6-phosphate dehydrogenase was used. Final concn. of ammonium sulphate (M): ●, 0.0025; ○, 0.005; ▲, 0.01; ×, 0.02; △, 0.05.

Fig. 6. Effect of ammonium sulphate and NADPH₂ concentrations on the rate of transhydrogenation. Experimental conditions were as in Fig. 2 except that pH of tris buffer was 8.5 and 1 μg. of glucose 6-phosphate dehydrogenase was used. Final concn. of ammonium sulphate (M): ●, 0.006; ○, 0.0025; △, 0.05; ×, 0.01; □, 0.02.

Table 1. *Effect of ammonium sulphate on glucose 6-phosphate dehydrogenase*

pH	Glucose 6-phosphate-dehydrogenase activity (μmoles of NADPH ₂ /mg./min.)		Ratio of activity 0.2M-ammonium sulphate present ammonium sulphate absent
	Rate without ammonium sulphate	Rate with 0.2M-ammonium sulphate	
6.8	42.4	10.9	0.26
7.3	54.3	23.9	0.44
7.8	50.0	37.2	0.75
8.3	57.8	44.5	0.77

Williams-Ashman, 1958), it was relevant to examine the effect of ammonium sulphate on NAD- and NADP-linked dehydrogenation of androsterone. The rate of NADP-linked dehydrogenation was found to be unaffected by 0.1M-ammonium sulphate. The rate of NAD-linked dehydrogenation was approximately doubled in the presence of 0.1M-ammonium sulphate. The effect of 0.1M-ammonium sulphate on the rate of androsterone dehydrogenation is therefore of a much lower order of magnitude than the effect on the rate of steroid-dependent transhydrogenation.

Effect of ammonium sulphate on transhydrogenase in modified assay systems. The usual assay system was altered by: (a) replacement of androsterone by androstanedione or aetiocholanolone or androsterone sulphate; (b) replacement of NAD by the 3-acetylpyridine analogue of NAD; (c) replacement of glucose 6-phosphate/glucose 6-phosphate dehydrogenase by isocitrate/isocitrate dehydrogenase; (d) reversal of transhydrogenase reaction. The rate of steroid-dependent transhydrogenation was determined under each of these conditions in the presence and absence of ammonium sulphate. The rates of the reaction in the absence of sulphate in conditions (a) and (b), except when androsterone sulphate was used, were 4–6% of the rate in the presence of 0.1M-ammonium sulphate. Transhydrogenation did not proceed at all when androsterone sulphate replaced androsterone. In condition (c) the rate in the absence of sulphate was 15%

of the rate in the presence of 0.1M-ammonium sulphate. Under condition (d) hydrogen transfer from NADH₂ to NADP could not be effected directly owing to the inhibiting effect of NADPH₂ on the enzyme system. The reaction was therefore performed in the presence of a relatively high concentration of NADH₂ with a small amount of NADP present. This was coupled with the glutathione-reductase system (present in our partially purified enzyme), which oxidized NADPH₂ as it was formed. A requirement of this reverse transhydrogenation for SO₄²⁻ ions is shown in Fig. 7.

DISCUSSION

The anion activation of steroid-dependent transhydrogenase of rat liver, which has been noted in this study, resembles previously observed anion stimulation of enzymes in that it is induced by a fairly wide range of anions and requires the presence of a high concentration of salt for maximal effect (Dixon & Webb, 1958). The anions do not appear to activate transhydrogenase by removing inhibitory metal ion. Anion activation has been previously observed to be associated with a shift in enzyme pH optimum (Dixon & Webb, 1958); but the optimum pH of the transhydrogenase reaction is not altered with increased activation by SO₄²⁻ ions.

Theorell, Nygaard & Bonnichsen (1955), using liver alcohol dehydrogenase, observed an alteration in the velocity constants in the enzyme–NAD(H₂) complex in the presence of certain anions including sulphate, and they attempted to explain this in terms of altered stability of the enzyme–nucleotide or enzyme–alcohol linkages. In our system increase in ammonium sulphate concentration appears to increase the sensitivity of the system to androsterone. The results obtained by varying the concentrations of both SO₄²⁻ ion and NADPH₂ indicate an increased tolerance for NADPH₂ at the concentrations of SO₄²⁻ ion that produced maximal stimulation of transhydrogenation. How SO₄²⁻ ions alter the affinity of the transhydrogenase for NADPH₂ or androsterone is a matter for further study. The theory of Theorell *et al.* (1955) that anions affect the stability of enzyme–substrate linkages might be applicable. An ion that weakened the NADPH₂–enzyme linkage would diminish NADPH₂ inhibition of transhydrogenation and produce an increase in the rate of NADH₂ formation if the concentration of NADPH₂ exceeded the optimum value. Anion activation may also be related to an increase in the affinity of the enzyme for androsterone. The exact mechanism of steroid-dependent transhydrogenation, based on a study of the 17 β -hydroxy steroid-dependent transhydrogenase of human placenta, is still disputed (Hager-

Fig. 7. Transfer of hydrogen from NADH₂ to NADP. The complete test system contained, in 0.27 ml.: 18 μ moles of tris buffer, pH 7.5; 0.05 μ mole of NADH₂; 7 μ m-moles of NADP; 1 μ mole of glutathione; 24 μ moles of ammonium sulphate; 3.5 μ m-moles of androsterone (1 μ g.). Ammonium sulphate, NADP and androsterone were omitted as indicated. ●, All components; ○, all components except ammonium sulphate; ×, all components except NADP; ▲, all components except androsterone.

man & Vilee, 1959; Jarabak, Adams, Williams-Ashman & Talalay, 1962). Talalay & Williams-Ashman (1958) suggested that a steroid dehydrogenase with dual nucleotide specificity is responsible for steroid-dependent transhydrogenation, with one site on the enzyme for which both nucleotides compete. At the stage of enzyme purification reached in the present work it has not been possible to separate NAD- or NADP-linked 3α -hydroxy steroid-dehydrogenase activities from the transhydrogenase activity. It has been found, however, that ammonium sulphate stimulated the NAD-linked dehydrogenase but not the NADP-linked dehydrogenase activity.

Until the mechanism of 3α -hydroxy steroid-dependent transhydrogenation in rat liver is more fully explained by work with purer enzyme preparations, the role of anions in these reactions will be difficult to define.

SUMMARY

1. 3α -Hydroxy steroid-dependent nicotinamide-adenine dinucleotide transhydrogenase from rat liver, partially purified by ammonium sulphate fractionation and column chromatography on diethylaminoethylcellulose, is activated by some bivalent anions.

2. The effect of SO_4^{2-} ions on the optimum H^+ ion, steroid and NADP concentrations of the

system, and on the reversibility of the reaction, have been examined.

3. Some effects of the SO_4^{2-} ions on NAD- and NADP-linked androsterone-dehydrogenase activity have also been studied.

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Tissue Concentrations of Coenzyme Q, Ubichromenol and Tocopherol in Relation to Protein Status in the Rat

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Aromatic biosynthesis, except for partial synthesis of the steroid nucleus in the oestrogens, is known not to occur in animal tissues. Therefore it was considered that coenzyme Q (ubiquinone), a benzoquinone, may in some measure be a vitamin (Morton, 1958). However, a state of deficiency in coenzyme Q could not be produced in rats by any of the known synthetic diets (Lawson, Threlfall, Glover & Morton, 1961; Olson, Dialameh & Bentley, 1961). Further, several lines of evidence (summarized by Ramasarma, 1961) rule out the possibility of intestinal bacteria being the source of coenzyme Q. Olson *et al.* (1961) showed that uniformly labelled phenylalanine was incorporated

into coenzyme Q and established that phenylalanine serves as the precursor of the quinone nucleus. It is logical therefore to expect that changes in the concentration of dietary protein, and consequently of phenylalanine, will affect the amounts of coenzyme Q in the tissues.

EXPERIMENTAL

The diet contained 5% of groundnut oil, 5% of salt mixture (U.S. Pharmacopeia, 1947), casein in the percentage mentioned in the text, 10% of cane sugar and starch to 100%. Each rat received 0.5 ml. of vitamin B-complex solution (U.S. Pharmacopeia, 1947)/day. Vitamin A