Studies of the Metabolism *in vivo* of Cortisol by the Rat Liver

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(Received 22 July 1957)

The metabolism of cortisol (Caspi & Hechter, 1956) and cortisone (Caspi, Levy & Hechter, 1953; Caspi & Hechter, 1954; Miller & Axelrod, 1954) has been studied in the rat liver with the isolated perfused organ. In these experiments the hormones were dissolved in either homologous or heterologous blood, which was then perfused through the liver and the effluent extracted for the steroid metabolites. Since it is possible that the results obtained by this technique might be different from those in which cortisol metabolites are isolated from liver *in situ*, the metabolism of cortisol by the liver was studied after its parenteral administration in the rat.

EXPERIMENTAL

Nomenclature

Cortisol, cortisone, corticosterone, β -cortol, β -cortolone, α -cortolone and tetrahydrocortisol are used as trivial names for 11 β :17 α :21-trihydroxypregn-4-ene-3:20-dione, 17 α :21-dihydroxypregn-4-ene-3:11:20-trione, 11 β :21-dihydroxypregn-4-ene-3:20-dione, 3 α :11 β :17 α :20 β :21-pentahydroxypregnane, 3 α :17 α :20 β :21-tetrahydroxypregnan-11-one, 3 α :17 α :20 α :21-tetrahydroxypregnan-11-one, and 3 α :11 β :17 α :21-tetrahydroxypregnan-20-one respectively throughout this paper.

Materials

Radioactive cortisol. [4-¹⁴C]Cortisol (specific activity 1.467 mc/m-mole), supplied by the National Institutes of Health, U.S. Public Health Service, was purified by paper chromatography by development for 6 days in the benzene-formamide system. The area corresponding to cortisol was cut out and eluted.

Non-radioactive cortisol. Sterile solutions of 'hydrocortisone (free alcohol) intravenous' (Boots Pure Drug Co. Ltd.) in ampoules which contained 100 mg. in 20 ml. of 50% (v/v) ethanol, were diluted to 50 ml. with aq. 0.9% NaCl soln.

Reagents and solvents. Silica gel was used untreated. Ethyl acetate was washed once with one-quarter of its volume of aq. 10% Na₂CO₃ soln., twice with one-quarter of its volume of water and dried over anhydrous Na₂SO₄. Solvents for paper chromatography were used as supplied.

Animals. Male albino rats (Glaxo strain) weighing 200–350 g. were used.

Chromatography

Solvent systems. All chromatograms were developed in a descending manner on Whatman no. 1 filter paper in all-glass tanks at 23 $\pm$ 2°. Extracts were applied 4 $\frac{1}{2}$ in. from the end of the 22 $\frac{1}{2}$ in.-long paper, and after development the chromatograms were dried in an oven at less than 40°. The solvent systems used, and abbreviations used for them in the text, are listed: Benzene-formamide (Burton, Zaffaroni & Keutmann, 1951); B-5: benzene-methanol-water (2:1:1, by vol.) (Bush, 1952); B-5EtAc: benzene-methanol-water-ethyl acetate (1:1:1:0.1, by vol.) (Hübener, Fukushima & Gallagher, 1956); E₂B: 2:2:4-trimethylpentane-*tert.*-butyl alcohol-water (10:5:9, by vol.) (Eberlein & Bongiovanni, 1955); LB21/80: light petroleum (b.p. 80–100°)-benzene-methanol-water (10:5:12:3, by vol.) (I. E. Bush, personal communication); L/96: light petroleum-methanol-water (100:96:4, by vol.) (I. E. Bush, personal communication); butanol-water: aq. 86% (v/v) *m*-butanol (Markham & Smith, 1949); butanol-water-ammonia: aq. 86% (v/v) *n*-butanol with 5% (v/v) of aq. NH₃ soln. (sp.gr. 0.880) added to solvent in the bottom of the tank (Markham & Smith, 1949).

Detection of steroids on paper chromatograms. Unsaturated steroids were examined briefly under a lamp (the Chromatolite, Hanovia Lamps, Slough) emitting most of its energy at 253.7 m μ . Δ^4 -3-Ketones were detected by the soda fluorescent reaction described by Bush (1952). Steroids possessing an α -ketol side chain were detected with triphenyltetrazolium chloride (Burton *et al.* 1951). 17 α :20:21-Triol compounds were oxidized to 17-oxo compounds by dipping a paper strip once in a 4:1 (v/v) solution of absolute ethanol-4% aq. sodium metaperiodate and hanging it horizontally for 40 min. at room temperature. In order to detect the resulting 17-oxo steroid it was dipped once in a 2:1 (v/v) mixture of ethanolic 2% *m*-dinitrobenzene soln. and ethanolic 4*N*-KOH soln. (modified Zimmermann reagent), dried briefly in a draught and then heated in an oven for 2–3 min. at approx. 50°.

Elution of steroids from paper chromatograms. The area of the paper to be eluted was cut into pieces approx. 2 cm.² and these were placed into glass-stoppered test tubes. Sufficient absolute ethanol was added to cover them and the tubes were shaken for 1 hr. The ethanol was removed with a Pasteur pipette, filtered with suction through a sintered-glass funnel into a 10 ml. centrifuge tube and evaporated to dryness with a stream of air at 45–50°. This process was repeated twice.

Spectrophotometry

Absorption spectra were determined in an SP. 500 spectrophotometer (Unicam Instruments Ltd.) with silica cells with a 0.2 cm. light path. The absorption spectra of

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steroids in conc. H_2SO_4 were determined by the method of Zaffaroni (1950). Infrared spectra were determined in a model 21 double-beam infrared spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) with a KCl microplate with 150–200 μg . of the free steroid alcohol.

Oxidation and acetylation

Sodium bismuthate oxidation was carried out by the method of Brooks & Norymberski (1953). Chromic acid oxidation was carried out in the following manner: to the dry steroid, 0.3 ml. of acetic acid and 0.2 ml. of 2% (w/v) aq. chromium trioxide soln. were added, and the solution was left at room temperature for approx. 21 hr. A small volume (2 ml.) of water was added to the solution, which then was extracted four times with 2 ml. of ethyl acetate each time, and the pooled extract was evaporated to dryness with a stream of air at 45–50°. Acetates were formed by adding three drops of acetic anhydride and five drops of absolute pyridine to the dry steroid and letting it stand at room temperature for 15–18 hr. After addition of a small amount of methanol, the acetates were evaporated to dryness with a stream of air at 45–50°.

Experiments with [4- ^{14}C]cortisol

Approx. 0.8 μC of [4- ^{14}C]cortisol was dissolved in 0.9 ml. of a 20% (v/v) ethanolic 0.9% NaCl soln. and injected via the right external jugular vein into a 200 g. rat which had been anaesthetized by an intraperitoneal injection of sodium pentobarbitone (5 mg./100 g. body wt.). Three minutes after the end of the injection, the rat was exsanguinated by withdrawing blood from the inferior vena cava into a heparinized syringe. A second rat was exsanguinated 10 min. after the end of the injection. Immediately thereafter the animal was perfused with 60–70 ml. of 0.9% NaCl soln., first via the inferior vena cava at a point just below the entrance of the renal veins and then via the abdominal aorta. The right external jugular vein was cut after the start of the perfusion. When the fluid coming out of the jugular vein was colourless and the liver was pale pink the perfusion was stopped, and the liver removed and stored at –20° until it was extracted.

The liver was thawed, put into a 10 ml. glass cup which was surrounded by cracked ice, and homogenized (MSE homogenizer) with 2 ml. of 0.9% NaCl soln. Immediately after the liver was homogenized it was extracted four times with 25 ml. of ethyl acetate each time, and the pooled extract distilled *in vacuo* at 40–45° to a volume of approx. 2 ml. The concentrated extract was dissolved in 25 ml. of light petroleum (b.p. 40–60°)–ethyl acetate (1:1, v/v) and chromatographed on 2 g. of silica gel, as described by Bush & Sandberg (1953). The eluate from the silica-gel column was evaporated to dryness with a stream of air at 45–50°. To the dried eluate was added 0.1 ml. of absolute ethanol, which then was applied to the paper together with three 0.05 ml. washings. The paper was developed for 6 days in the benzene–formamide system and the overflow after being evaporated to dryness and redissolved in ethanol was chromatographed for 3 hr. in the same system. Radioautographs were obtained by placing the paper chromatograms in contact with Blue-brand X-ray film (Kodak Ltd.) for 14 days and developing the films by standard photographic procedures.

Experiments with non-labelled cortisol

The object of the experiments with ^{14}C -labelled cortisol was to obtain some information on (1) the number of cortisol metabolites which were present in liver after parenteral administration of the hormone, (2) the relative polarity of these metabolites, so as to get some idea of their structure, and (3) the relative amounts of these metabolites. Since most of the radioactivity was concentrated in two or more very polar compounds, an attempt was made to identify them by injecting rats with large amounts of non-radioactive cortisol. The following experiments were designed with this purpose in mind.

Rats anaesthetized with sodium pentobarbitone were injected with 8 mg. of cortisol in 20% ethanolic 0.9% NaCl soln. via the right external jugular vein, at a rate of approx. 1 mg./min. Twenty minutes after the start of the injection, the rats were exsanguinated and perfused as described in the previous section. In the first of these experiments ethyl acetate extracts of the livers were chromatographed on silica gel, as described above, to remove large amounts of fatty non-steroidal material. When it was found that washing the extracts with hexane was quicker and achieved similar results to those obtained by partition on silica gel, the following procedure was adopted.

The combined ethyl acetate extracts from the livers of six to eight rats were concentrated to a volume of 0.5–1 ml. by distillation *in vacuo* at 40–45°. The extract then was dissolved in 50 ml. of aq. 70% methanol soln., and washed six times with 100 ml. of hexane. Methanol was removed by distillation *in vacuo* and the resulting solution extracted six times with 15 ml. of ethyl acetate. The combined extract was concentrated as before and transferred to a 10 ml. centrifuge tube with several washings of methanol. After the solution was evaporated to dryness with a stream of air at 45–50°, it was ready to be chromatographed on paper.

RESULTS

Experiments with 4- ^{14}C -labelled cortisol

Radioautographs from the chromatograms developed for 6 days showed that most of the radioactivity was concentrated just in front of the origin in two poorly resolved spots whose *R* relative to cortisol was 0.07 and 0.21. Two very weak radioactive areas were also seen whose *R* relative to cortisol was 0.37 and 0.60. With the exception of a radioactive area with the same mobility as a standard spot of cortisol, the only other radioactive spot seen was a barely visible one with an *R* relative to cortisol of 1.8.

No radioactive areas could be detected in the radioautograph from the benzene–formamide chromatogram developed for 3 hr.

Experiments with non-labelled cortisol

In two radioactive-hormone experiments, examination of the benzene–formamide chromatograms developed for 6 days with a lamp emitting most of its energy at 253.7 $\text{m}\mu$ showed a red-fluorescing

area just in front of the origin, which corresponded exactly to the two poorly resolved strongly radioactive spots seen on the radioautograms. When this area was rechromatographed for 18 hr. in the B-5 system, the radioactive and fluorescent areas still were superimposed. Chromatography in the B-5 system for 72 hr. resolved the u.v. light-fluorescing area into two spots, one of which (compound NP) had moved 5.75 cm. from the origin, whereas the other (compound P), which appeared to be present in a much smaller quantity as judged by the intensity of the fluorescence, was located 2.5 cm. from the origin.

The absorption spectrum of compound NP in absolute ethanol showed a maximum at $262\text{ m}\mu$ with a shoulder between 256 and $258\text{ m}\mu$ and a minimum between 245 and $246\text{ m}\mu$, whereas the absorption spectrum of compound P in absolute ethanol revealed a maximum between 257 and $258\text{ m}\mu$ and a minimum at $238\text{ m}\mu$. When the solution of compound NP was evaporated to dryness with a stream of air and the compound dissolved in 0.1N-HCl, the absorption maximum and minimum had shifted to 260 and $238\text{ m}\mu$ respectively and the shoulder had disappeared. The compound could not be extracted into ethyl acetate from the 0.1N-HCl solution. The absorption spectrum of compound NP in N-NaOH was identical with that in absolute ethanol, and the compound could be extracted into ethyl acetate from the alkaline solution.

Since these experiments indicated that compound NP might be a nucleotide, it was chromatographed on paper for 22 hr. in butanol-water-aq. NH_3 soln. and its R_f found to be 0.67 (R_f values of adenine, cortisol, corticosterone and $11\beta:17\alpha:20\beta:21$ -tetrahydroxypregn-4-en-3-one were 0.37, 0.91, 0.94 and 0.87 respectively). After hydrolysis in N-HCl at 110° for 2 hr., the R_f of compound NP in the same system shifted to 0.22 (R_f values of adenine, xanthine and hypoxanthine were 0.37, 0.05 and 0.13 respectively), but its absorption spectrum in absolute ethanol was unchanged.

In order to determine whether the radioactive material could be separated from the u.v. light-fluorescing material by chromatography in a solvent system not generally used for adrenocortical steroids, the very polar area present in the benzene-formamide chromatogram developed for 6 days was eluted and rechromatographed for 16 hr. in butanol-water. A radioautogram showed a single radioactive spot with R_f 0.91, and examination of the chromatogram with a u.v. light lamp ($253.7\text{ m}\mu$) revealed a single fluorescing spot with R_f 0.67. A compound was also found in the livers of rats not given cortisol which had properties identical with those of the compound isolated from the livers of rats injected with cortisol. Since this compound

appeared to be a normal constituent in rat liver and not to be a metabolite of cortisol, no further attempt was made to identify it.

Paper-chromatographic separation of cortisol metabolites

The complete separation of the strongly u.v. light-fluorescing compound from the cortisol metabolites appeared to be possible by chromatographing the liver extracts in butanol-water. However, it was found that when the extracts from six to eight livers were chromatographed on a single sheet of paper in this system, such a large quantity of this u.v. light-fluorescing compound was extracted that even though the steroids moved virtually with the solvent front, some of this compound overlapped the area on the chromatogram where the steroids were located. Therefore after the very polar metabolites of cortisol were separated from each other, the u.v. light-fluorescent compound which was present as a contaminant in each of these polar steroids was separated from the latter by chromatography in the E_2B system. The sequence of paper chromatography which was finally used was the following.

The hexane-washed liver extracts were chromatographed in the B-5 system for 24 hr. and the chromatogram was divided into four zones: zone I (from the origin up to and including the area corresponding to $11\beta:17\alpha:20\beta:21$ -tetrahydroxypregn-4-en-3-one; zone II (the area just in front of $11\beta:17\alpha:20\beta:21$ -tetrahydroxypregn-4-en-3-one up to but not including the cortisol area); zone III (the area corresponding to cortisol); zone IV (the area in front of cortisol).

Zone I was eluted and rechromatographed for 48 hr. in the B-5EtAc system. In this system, $11\beta:17\alpha:20\beta:21$ -tetrahydroxypregn-4-en-3-one, β -cortolone, $3\beta:11\beta:17\alpha:20\beta:21$ -pentahydroxy-5 α -pregnane and β -cortol (in order of increasing polarity in B-5EtAc) were completely separated from each other. Each of the metabolites found in zone I (compounds 1-4, see below) was eluted and rechromatographed in the E_2B system for 72 hr.

Metabolites in zone I

Identification of compound 1 as β -cortol ($3\alpha:11\beta:17\alpha:20\beta:21$ -pentahydroxypregnane). When this substance was oxidized on paper with periodic acid and then caused to react with the modified Zimmermann reagent, it gave a positive test indicating the possible presence of a $17\alpha:20:21$ -triol-type of side chain. The compound had the same mobility as an authentic sample of β -cortol when it was chromatographed for 72 hr. in both the B-5EtAc and the E_2B systems, and a mixed chromatogram developed for 72 hr. in the B-5EtAc system gave a single spot. Oxidation with sodium bismuthate

resulted in a compound which had the same mobility on paper as an authentic sample of 3 α :11 β -dihydroxy-5 β -androstan-17-one when chromatographed for 24 hr. in the LB 21/80 system. When the compound oxidized with sodium bismuthate was acetylated and chromatographed for 13 hr. in the L/96 system, its mobility was the same as 3 α -acetoxy-11 β -hydroxy-5 β -androstan-17-one. When the acetylated compound oxidized with sodium bismuthate was further oxidized with chromic acid, a compound was formed which when chromatographed for 7.5 hr. in the L/96 system had the same mobility as an authentic sample of 3 α -acetoxy-5 β -androstan-11:17-dione. The sulphuric acid and infrared spectra of compound 1 were identical with those of an authentic sample of β -cortol except for a maximum at 1675 cm.⁻¹ in the infrared-absorption spectrum of compound 1, the presence of which is difficult to explain.

Identification of compound 2 as 3 β :11 β :17 α :20 β :21-pentahydroxy-5 α -pregnane. The chromatographic behaviour on paper of this compound in the B-5EtAc and E₂B systems was identical with that of an authentic sample of 3 β :11 β :17 α :20 β :21-pentahydroxy-5 α -pregnane. After oxidation with sodium bismuthate its mobility in the LB21/80 system was the same as that of 3 β :11 β -dihydroxy-5 α -androstan-17-one, and acetylation of the compound oxidized with bismuthate resulted in a substance which had the same mobility in the L/96 system as 3 β -acetoxy-11 β -hydroxy-5 α -androstan-17-one. When the acetylated compound oxidized with sodium bismuthate was further oxidized with chromic acid, a compound was formed which when chromatographed for 7.5 hr. in the L/96 system had the same mobility as an authentic sample of 3 β -acetoxy-5 α -androstan-11:17-dione. In the L/96 system, although 3 β -acetoxy-5 α -androstan-11:17-dione has the same mobility as 3 α -acetoxy-5 α -androstan-11:17-dione, these two compounds run behind 3 α -acetoxy-5 β -androstan-11:17-dione. Similarly, 3 β -acetoxy-11 β -hydroxy-5 α -androstan-17-one and 3 α -acetoxy-11 β -hydroxy-5 α -androstan-17-one have the same mobility in the L/96 system but they run behind 3 α -acetoxy-11 β -hydroxy-5 β -androstan-17-one. The sulphuric acid and infrared spectra of compound 2 were identical with those of an authentic sample of 3 β :11 β :17 α :20 β :21-pentahydroxy-5 α -pregnane.

Partial identification of compound 3 as 3 α :17 α :20 β :21-tetrahydroxy-5 α -pregnan-11-one. This compound gave a positive test when it was oxidized on paper with periodic acid and then caused to react with the modified Zimmermann reagent, indicating the possible presence of a 17 α :20:21-triol-type of side chain. Its mobility in the B-5EtAc system was the same as that of 3 α :17 α :20 β :21-tetrahydroxy-pregnan-11-one and a mixed chromatogram with

an authentic sample of the latter compound gave a single spot in the B-5EtAc system. The absorption spectrum in sulphuric acid of compound 3 was identical with that of 3 α :17 α :20 β :21-tetrahydroxy-pregnan-11-one. However, when compound 3 was oxidized with sodium bismuthate and chromatographed on paper for 24 hr. in the LB21/80 system, its mobility was the same as that of 3 α -hydroxy-5 α -androstan-11:17-dione, and acetylation of the compound oxidized with bismuthate resulted in a substance with the same mobility as 3 α -acetoxy-5 α -androstan-11:17-dione in the L/96 system. Unfortunately an authentic sample of 3 α :17 α :20 β :21-tetrahydroxy-5 α -pregnan-11-one could not be obtained, and the amount of compound 3 isolated from liver was not sufficient for an infrared absorption spectrum.

Partial identification of compound 4 as 11 β :17 α :20 β :21-tetrahydroxypregn-4-en-3-one. On paper this compound gave a positive soda-fluorescence reaction (Bush, 1952) indicating the presence of the Δ^4 -3-ketone grouping, and it gave a positive test when it was oxidized on paper with periodic acid and then caused to react with the modified Zimmermann reagent. It exhibited a u.v. light maximum of 242–243 m μ when its absorption spectrum was determined in absolute ethanol. When compound 4 was chromatographed in the B-5EtAc system for 48 hr. and in the E₂B system for 72 hr., its mobility was the same as that of an authentic sample of 11 β :17 α :20 β :21-tetrahydroxypregn-4-en-3-one, and mixed chromatograms in both systems gave single spots. The absorption spectrum in sulphuric acid of compound 4 was similar to that of 11 β :17 α :20 β :21-tetrahydroxypregn-4-en-3-one. An infrared-absorption spectrum of compound 4 was not made because not enough of the compound could be isolated from liver.

Metabolites in zone II

Two compounds (compounds 5 and 6), which reacted with triphenyltetrazolium chloride to give a red colour and which gave a negative soda-fluorescence reaction, were found in the area corresponding to tetrahydrocortisol. One of these, compound 5, had a mobility which was slightly less than that of tetrahydrocortisol when chromatographed for 24 hr. in the B-5 system and also when chromatographed for the same length of time in the B-5EtAc system. When compound 5 was oxidized with sodium bismuthate and chromatographed for 24 hr. in the LB 21/80 system, its mobility was slightly greater than that of 3 β :11 β -dihydroxy-5 α -androstan-17-one and it ran behind 3 α :11 β -dihydroxy-5 β -androstan-17-one. Acetylation of the compound oxidized with sodium bismuthate resulted in a substance which, when developed for 4 hr. in

the L/96 system, ran just behind 3 α -acetoxy-11 β -hydroxy-5 α -androstane-17-one. The sulphuric acid spectrum of compound 5 showed maxima at 338–342, 412–413, 505–508 m μ and minima at 264–266, 381–382 and 475 m μ .

Compound 6 had a mobility which was slightly greater than that of tetrahydrocortisol when chromatographed for 24 hr. in the B-5 system. After oxidation with sodium bismuthate, compound 6 ran just in front of 3 α :11 β -dihydroxy-5 β -androstane-17-one in the LB 21/80 system and acetylation of the compound oxidized with sodium bismuthate resulted in a substance with a mobility in the L/96 system which was slightly greater than that of 3 α -acetoxy-11 β -hydroxy-5 β -androstane-17-one.

Metabolites in zone III

Partial identification of compound 7 as cortisol (11 β :17 α :21-trihydroxypregn-4-ene-3-20-dione). On the paper chromatogram this compound gave a positive soda-fluorescence reaction, and in both the B-5 and the benzene-formamide systems its mobility was the same as that of an authentic sample of cortisol. It exhibited a u.v. light maximum at 240 m μ when its absorption spectrum was determined in absolute ethanol.

Metabolites in zone IV

Traces of a compound which gave a positive soda-fluorescence reaction, and which had the same mobility as cortisone in the B-5 system, were found in this zone.

Quantities of metabolites identified

The relative quantities of steroid metabolites were estimated by visual comparison on paper with known amounts of reference compounds, periodate oxidation followed by the Zimmermann reaction being used for the 17 α :20:21-triol type of compounds and the triphenyltetrazolium chloride reaction for the tetrahydro type of compounds. Visual comparison with β -cortolone was used for estimating the quantity of the compound partially identified as 3 α :17 α :20 β :21-tetrahydroxy-5 α -pregnan-11-one and comparison with tetrahydrocortisol was used for estimating the two tetrahydrocortisol-like metabolites. Metabolites with the α - β unsaturated ketone group were estimated after determining in absolute ethanol the optical density at the u.v. light maximum by the use of the known molar-extinction coefficients of the compounds.

It was estimated that from each rat liver were isolated 150–200 μ g. of β -cortol, 100–150 μ g. of 3 β :11 β :17 α :20 β :21-pentahydroxy-5 α -pregnane, 5–8 μ g. of 3 α :17 α :20 β :21-tetrahydroxy-5 α -pregnan-

11-one, and 1–5 μ g. each of 11 β :17 α :20 β :21-tetrahydroxypregn-4-en-3-one, cortisol and the two tetrahydrocortisol-like compounds.

DISCUSSION

It should be emphasized that the cortisol metabolites from liver which have been detected and identified in this study do not necessarily give a complete picture of the metabolic fate of this hormone, conjugation excluded. Caspi & Hechter (1956), who perfused [4-¹⁴C]cortisol through isolated rat livers, were able to extract only 55 % of the initially added radioactivity from the effluent, and Cope & Sewell (1956), studying the metabolism of [4-¹⁴C]cortisol in the mouse, found that up to 60 % of the radioactivity in the whole animal had been converted into products which could not be extracted into chloroform even after acid hydrolysis.

The data obtained in the present study confirm, at least in part, the perfusion experiments of Caspi & Hechter (1956), who showed that rat liver could reduce the Δ^4 -3-ketone group of cortisol to 3 β -hydroxylated metabolites, and also reduce the carbonyl group at C-20 to the 20 β -hydroxy grouping. However, whereas the three major transformation products in Caspi & Hechter's study were 3 β :11 β :17 α :20 β :21-pentahydroxy-5 α -pregnane, 3 β :11 β :17 α :21-tetrahydroxy-5 α -pregnan-20-one and 11 β :17 α :20 β :21-tetrahydroxypregnan-4-en-3-one (39, 19 and 15 % respectively of the metabolites isolated) in the experiments described in this paper, it is estimated that 80–90 % of the cortisol metabolites were accounted for by 3 β :11 β :17 α :20 β :21-pentahydroxy-5 α -pregnane and β -cortol. Only very small amounts of metabolites were found with the Δ^4 -3-ketone grouping and reduction of the C-20 carbonyl group (11 β :17 α :20 β :21-tetrahydroxypregn-4-en-3-one), or reduction of the Δ^4 -3-ketone group but with the C-20 carbonyl group intact ('tetrahydro' group of metabolites).

Of the two principal cortisol metabolites identified in the present study *in vivo*, one was found to be a 3 β -alcohol and the other a 3 α -alcohol. Caspi *et al.* (1953), Caspi & Hechter (1954) and Miller & Axelrod (1954) also identified 3 β - and 3 α -metabolites in the effluent from rat livers perfused with cortisone. However, in their perfusion of cortisol through rat liver, Caspi & Hechter (1956) were able to isolate only metabolites with the 3 β -configuration. This is difficult to understand in view of the work of Tomkins (1956), who was able to isolate and partially purify a 3 α -hydroxysteroid dehydrogenase from rat liver.

In the liver-perfusion experiments of Caspi *et al.* (1953), Caspi & Hechter (1954, 1956) and Miller & Axelrod (1954) with cortisone and cortisol the

metabolites in which the Δ^4 -3-ketone group had been reduced were all of the 5α -configuration. This was not found to be so in the present experiments in which metabolites with a saturated A ring had both the 5α - and 5β -configuration. The data obtained in this study accord better with the finding of Forchielli & Dorfman (1956), who reported that rat liver contains both the Δ^4 - 5α -hydrogenase and the Δ^4 - 5β -hydrogenase systems.

One further point of difference between the data obtained from liver-perfusion experiments and the data presented in this paper is that Caspi & Hechter (1956) identified two 17-oxosteroids in their effluents: 3β : 11β -dihydroxyandrostane-17-one and 11β :hydroxyandrostene-4-ene-3:17-dione, whereas in the present study no 17-oxosteroids were found. If any 17-oxosteroids had been present they would have been detected as radioactive spots when the overflow from the benzene-formamide chromatogram developed for 6 days was chromatographed for 3 hr. in the same system. This discrepancy, together with the others which have been described above, possibly may be due to the differences in the experimental methods employed, one of which is essentially a technique *in vitro*, whereas the other is *in vivo*. In a recent discussion on the possible mechanisms of hormone action (Hechter, 1955) it was pointed out that many of the effects of adrenocortical hormones observed *in vivo* could not be obtained *in vitro*, and Hechter, Solomon & Caspi (1953) emphasized that liver perfusion was 'not necessarily a system for studying liver action upon corticosteroids under physiological conditions'.

Although only small amounts of what is probably 3α : 17α : 20β : 21 -tetrahydroxy- 5α -pregnan-11-one were partially identified in the livers of rats injected with cortisol, it would appear that rat liver *in vivo* is capable of oxidizing, possibly only to a limited extent, the 11β -hydroxy group of cortisol to an 11-oxo group. That such a reaction undoubtedly also takes place in humans was shown by Fukushima *et al.* (1955), who isolated 3α : 17α : 20α : 21 -tetrahydroxypregnan-11-one and its 20β -epimer from the urine of human patients after administration of adrenocorticotrophic hormone and [4 - ^{14}C]cortisol. Gray & Lunnon (1956) also showed that in patients with Addison's disease or after adrenalectomy cortisone appears in the urine after administration of cortisol.

In this study, as well as with liver perfusion, it has been shown that two of the principal transformations occurring in the cortisone and cortisol molecules after passage through the liver are reduction of the Δ^4 -3-ketone group or reduction of the C-20 carbonyl group or both. It has also been found that reduction at one or both of these positions markedly reduces the biological activity of

these hormones when they are administered parenterally (Abelson, Ulrich & Long, 1955; Hechter, 1955). Therefore since cortisone and cortisol disappear from the blood (Tomizawa, Narahara, Gibbons & Williams, 1954; Ulrich & Long, 1956) and liver (Hechter, 1955) at such a rapid rate and are transformed into physiologically less active or inactive metabolites, it is difficult to understand how an adrenalectomized rat can be maintained on a single daily dose of cortisol. It has been suggested that adrenocortical hormones 'are not active at the cellular level unless they are first transformed by some other organ system into the active agent' and some evidence for this has been obtained (Hechter, 1955). It is possible that such physiologically active metabolite(s) may be found in that fraction which Caspi & Hechter (1956) and Cope & Sewell (1956) were not able to extract with organic solvents (see beginning of Discussion).

SUMMARY

1. The metabolism of cortisol in the liver of the rat was studied by intravenously administering [4 - ^{14}C]cortisol and non-labelled cortisol and identifying the unconjugated metabolites in the organ.

2. 3α : 11β : 17α : 20β : 21 -Pentahydroxypregnane and 3β : 11β : 17α : 20β : 21 -pentahydroxy- 5α -pregnane were found to be the two principal metabolites occurring in the liver, and they were estimated to account for 80–90% of the metabolites found. Small amounts of cortisol, 11β : 17α : 20β : 21 -tetrahydroxypregn-4-en-3-one, 3α : 17α : 20β : 21 -tetrahydroxy- 5α -pregnan-11-one and two tetrahydrocortisol-like compounds were also detected but the quantities were too small for definite identification.

3. The results obtained in this study are compared with those obtained from liver-perfusion experiments, and the differences in the metabolites identified by the two techniques are discussed.

I am greatly indebted to Professor A. Neuburger, F.R.S., for his helpful advice and for providing the facilities of his department in which this study was carried out. I would also like to thank Dr R. K. Callow for recording and interpreting the infrared spectra and for his generous gifts of non-labelled cortisol. Dr D. K. Fukushima, Dr W. K. Klyne, Dr O. Mancera, Dr M. Tishler and Dr J. J. Schneider generously provided reference steroids, and the Endocrinology Study Section, National Institutes of Health, U.S.A., supplied the 4 - ^{14}C -labelled cortisol.

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Hormones and Liver Cytoplasm

5. ENZYMES CONCERNED IN NUCLEIC-ACID CATABOLISM, AS AFFECTED BY HYPOPHYSECTOMY OR ADRENALECTOMY*

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(Received 24 June 1957)

Stevens & Reid (1956) found that hypophysectomy or adrenalectomy increased the activities, assayed at pH 5, of deoxyribonuclease (DNA-ase) and ribonuclease (RNA-ase) in rat-liver cytoplasm. There were no corresponding increases in the activity of a second ribonuclease with optimum activity at about pH 8. Hypophysectomy also induced an increase in xanthine oxidase activity, whereas adrenalectomy decreased this activity (Reid, O'Neal & Lewin, 1956*a*). These effects of hypophysectomy were not altered by administration of growth hormone.

These studies have now been extended to include the related enzymes, nucleotidase, adenosine deaminase, purine nucleoside phosphorylase and uricase. To assess the bearing of changes in enzymic activity on ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) metabolism, kidney tissue has now been studied with respect to possible changes in nuclease activities, and liver tissue has been studied (as briefly reported by Reid & Stevens, 1956) with respect to the rapidity with which nucleases and other constituents change after adrenalectomy. Since the rises in liver-nuclease activities after hypophysectomy, and especially after adrenalectomy, were due in part to rises in the normally low activity of the supernatant fraction

separated from the cytoplasm by differential centrifuging (Stevens & Reid, 1956), the supernatant fraction has been studied as well as the whole cytoplasmic fraction.

EXPERIMENTAL

Animals and tissue-fractionation technique

Reference should be made to Reid, O'Neal, Stevens & Burnop, 1956*b* for a full description of the general procedures. The rats were males of about 250 g. body wt. which had been given, in restricted amount, a '20% protein diet' (Elson, 1952) enriched by the addition (per 100 g. dry wt.) of 3.5 µg. of aneurin, 2.4 µg. of riboflavin, 8.2 µg. of calcium pantothenate, 3 µg. of pyridoxine hydrochloride and 588 µg. of choline chloride. The rats were fasted overnight before autopsy. The hypophysectomized rats were killed 2-3 weeks after operation, and the adrenalectomized rats (which had been maintained with saline) were, unless otherwise stated, killed 10-21 days after operation. To minimize the effect of possible day-to-day variations in environment or technique, the experimental rats were always compared with control rats (intact or sham-operated) investigated simultaneously. At least five untreated hypophysectomized rats, four hypophysectomized rats treated with growth hormone and six adrenalectomized rats were used in the study of each enzyme. Each point on Fig. 1 represents data for two to six adrenalectomized rats.

Samples of perfused liver were homogenized in 0.25M-sucrose soln. with a plastic pestle rotating at 2000 rev./min.,

* Part 4: Reid & Stevens (1957).

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