The Metabolism of Sodium Cortisone 21-[³⁵S]Sulphate in the Rat

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(Received 24 November 1971)

1. The metabolism of sodium cortisone 21-[³⁵S]sulphate was investigated in rats. 2. Quantitative and qualitative experiments showed that substantial amounts of ³⁵SO₄²⁻ appeared in the urine of free-ranging rats receiving the ester. 3. Whole-body radioautograms indicated considerable biliary elimination of ³⁵S and also pointed to the liver as the site of metabolism. 4. When female rats with bile-duct cannulae received sodium cortisone 21-[³⁵S]sulphate approx. 70% of the dose appeared in the bile as a doubly conjugated steroid (metabolite I). This metabolite was identified as 3α-(β-D-glucopyranuronosido)-17α-hydroxy-21-[³⁵S]sulpho-oxy-5α-pregnane-11,20-dione. 5. When metabolite I was administered to a rat with a bile-duct cannula 90% of the dose appeared in the bile unchanged. After the administration (intraperitoneally or orally) of metabolite I to free-ranging rats considerable amounts of ³⁵SO₄²⁻ appeared in the urine. 6. The route by which ³⁵SO₄²⁻ might be produced from cortisone [³⁵S]sulphate in free-ranging animals is discussed.

Cortisone sulphate is readily hydrolysed by crude preparations of sulphatases from molluscs (Roy, 1956; Dodgson, 1961; Jarrige *et al.*, 1963), but it is apparently resistant to hydrolysis by any enzyme yet isolated from mammalian tissues (Roy, 1957). Corticosterone 21-sulphate is likewise resistant to hydrolysis during perfusion through human placenta under conditions where extensive hydrolysis of dehydroepiandrosterone sulphate (17-oxoandrost-5-en-3β-yl sulphate) occurs (Pasqualini *et al.*, 1967). In view of the formation of corticosteroid 21-sulphates *in vivo* (Pasqualini & Jayle, 1961) and *in vitro* (Wengle & Bostrom, 1963) it was decided to re-investigate the apparent inability of preparations from mammalian tissues to hydrolyse cortisone 21-sulphate, an inability which was confirmed by preliminary experiments in this laboratory (Gatehouse, 1964). During the latter work it was noted, however, that the administration of cortisone 21-[³⁵S]sulphate to rats leads to the urinary excretion of ³⁵SO₄²⁻. The present paper gives these findings in more detail and describes the identification of metabolite I, a metabolite produced from cortisone 21-[³⁵S]sulphate in bile in the rat (Dodgson *et al.*, 1965).

Materials and Methods

Sodium cortisone 21-[³⁵S]sulphate

To a well-stirred solution of cortisone (1g) in pyridine (6.6ml) at 2°C was added dropwise 0.2ml

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of chloro[³⁵S]sulphonic acid (sp.gr. 1.76; specific radioactivity 3.2mCi/mmol; obtained from The Radiochemical Centre, Amersham, Bucks., U.K.). Care was taken to prevent the temperature rising above 4°C during the addition of the acid, and when the addition was complete the mixture was stirred at 2°C for 4h. The amber-coloured solution was added to an aqueous solution of Na₂CO₃ (0.56g in 6ml). Precipitated solids were filtered and pyridine extracted from the filtrate with four successive portions (12.5ml) of ether. The resulting aqueous layer was then concentrated by rotary evaporation under reduced pressure at 37°C to give a thick oil which was dissolved in the minimum amount of dry methanol at room temperature. Insoluble material was removed by filtration and crystallization was induced by the cautious addition of ether and cooling to 5°C. The product was collected by filtration, recrystallized three times and dissolved in methanol (50ml). The solution was applied as streaks to thick-layer chromatography plates (1.25mm layers of Whatman Silica Gel SG41) which had been activated at 110°C for 16h. Chromatograms were run for 2.5h in benzene-ethyl methyl ketone-ethanol-water (3:3:3:1, by vol.). Cortisone 21-[³⁵S]sulphate was located by radioautography and the appropriate area of silica gel was removed and extracted with 3 × 80ml of methanol. After centrifuging to remove silica gel, the eluate was concentrated to approx. 10ml by rotary evaporation. Crystallization was induced by the cautious addition of ether and cooling to 5°C. The final micro-crystalline product was collected by centrifuging and dried over P₂O₅ *in vacuo* at 40°C. The yield was 610mg and the product gave analysis results for Na and SO₄²⁻ agreeing with a structure of anhydrous sodium cortisone 21-[³⁵S]sulphate

(Found: Na, 5.1; SO_4^{2-} , 20.4. $\text{C}_{21}\text{H}_{25}\text{O}_5\text{SNa}$ requires Na, 5.1; SO_4^{2-} , 21.4%). The product had m.p. 185–186°C (decomp.), $[\alpha]_D^{20} + 181^\circ$ (1%, w/v, in water) and ϵ_{245} 15875. Sodium cortisone 21- ^{35}S]sulphate was stored as a frozen aqueous solution (5mg/ml) to minimize any breakdown by self-irradiation processes.

Experimental animals

Adult male and female MRC hooded rats were used throughout. Intraperitoneal and intravenous (tail vein) injections to free-ranging rats were carried out while the animals were under ether anaesthesia. Animals were placed in metabolism cages that permitted separate collection of urine and faeces, they were allowed water without restriction and were removed from the cages for 20 min in each 24 h when they were allowed to feed. After each urine collection the cages were rinsed with water and the washings added to the appropriate sample. Urine and washings were filtered through a glass-wool plug before being made up to a suitable volume (usually 250 ml).

In experiments on rats with bile-duct and bladder cannulae, animals were anaesthetized with ether, the jugular vein was cannulated and Nembutal (10–15 mg/kg body wt.) was administered via the cannula. The bile duct and bladder were cannulated and bile and urine samples collected at suitable times after administration (by the jugular-vein cannula) of substances under investigation. Animals were maintained under Nembutal anaesthesia throughout the experiments.

Whole-body radioautography

Young MRC hooded rats (30–50 g body wt.) under light ether anaesthesia each received an intraperitoneal injection of sodium cortisone 21- ^{35}S]sulphate (0.5 mg dissolved in 0.2 ml of water). Rats were killed at suitable time-intervals (up to 6 h) and whole-body radioautograms were prepared by the method of Powell *et al.* (1967).

Measurement of radioactivity in biological materials

Determination of inorganic ^{35}S]sulphate (as $\text{Ba}^{35}\text{SO}_4$) and total ^{35}S]sulphate (as $\text{Ba}^{35}\text{SO}_4$ after acid hydrolysis) in urine and bile was as described by Lloyd (1961). The total ^{35}S contents of faeces and the whole rat carcass were precipitated and counted as $\text{Ba}^{35}\text{SO}_4$ after oxidation with fuming HNO_3 by the method of Young *et al.* (1949).

Chromatography and paper electrophoresis

Paper chromatograms were run on Whatman no. 1 paper and developed by descending irrigation. Analytical thin-layer chromatograms were run on

250 μm layers of Merck Silica Gel G, and preparative chromatograms were run on 1.25 mm layers of Whatman Silica Gel SG41. All plates were activated by heating at 110°C for 16 h before use. The solvents are specified in the appropriate sections below.

Horizontal paper electrophoresis was carried out on Whatman no. 1 paper in 0.1 M-ammonium acetate for 2.5 or 5 h at a potential gradient of 8 V/cm.

Radioactive areas on chromatograms and electrophoretograms were detected by radioautography, for suitable periods, on Ilford Industrial B X-ray film.

Determination of reducing steroids

Steroids containing the α -ketol side chain were determined by the Tetrazolium Blue reaction as described by Carstensen (1962).

Experimental and Results

In preliminary experiments, four male rats were injected intraperitoneally with sodium cortisone 21- ^{35}S]sulphate (1 mg dissolved in 0.4 ml of water). Urine was collected after 24 and 48 h and analysed for inorganic ^{35}S]sulphate and total ^{35}S]sulphate. About 70% of the recovered radioactivity appeared in the first urine sample as inorganic ^{35}S]sulphate. However, only 34–61% of the radioactive dose was recovered in the urine over the total 48 h period, which suggested that other pathways of excretion were involved in the disposal of ^{35}S . Further experiments (see Table 1) were therefore carried out in which rats received sodium cortisone 21- ^{35}S]sulphate, and urine was collected after various times ranging from 5 to 48 h and faeces were collected over the 48 h period. The animals were then killed and carcasses were taken for the determination of residual ^{35}S . The major proportion of the radioactive dose was eliminated in the urine within 24 h. Only small amounts of ^{35}S were found in the urine after this period and only a relatively small percentage of the injected dose remained in the carcass at 48 h. Substantial amounts of radioactivity appeared in the faeces, indicating a significant biliary excretion. As in the previous experiments the inorganic ^{35}S]sulphate content of the urine indicated significant breakdown of the injected ester, the inorganic ^{35}S]sulphate content of the urine being greater than the ester ^{35}S]sulphate content in the large majority of the individual urine samples.

Sodium cortisone 21- ^{35}S]sulphate (1 mg dissolved in 0.4 ml of water) was also injected intravenously into male and female rats. Urine was collected after 6, 12, 24 and 48 h and faeces were collected over the 48 h period. Table 2 records the results, which were substantially the same as those obtained after intraperitoneal administration of the ester. The major portion of the radioactivity was eliminated in the

Table 1. *Distribution of ^{35}S in the urine, faeces and carcasses of male rats injected intraperitoneally with sodium cortisone 21- ^{35}S sulphate (1 mg dissolved in 0.4 ml of water)*

		Distribution of ^{35}S (% of ^{35}S injected)*											
		Rat 1				Rat 2				Rat 3			
		Inorganic [^{35}S]sulphate		Ester [^{35}S]sulphate		Inorganic [^{35}S]sulphate		Ester [^{35}S]sulphate		Inorganic [^{35}S]sulphate		Ester [^{35}S]sulphate	
Time (h)													
^{35}S content of urine samples	5	—	—	—	—	—	—	—	—	—	0.3	—	9.7
	7.5	—	—	—	—	—	—	—	—	—	—	—	—
	10	27.5	23.9	—	—	42.5	12.2	—	—	17.1	7.2	—	—
	24	14.3	1.1	—	—	16.5	0	—	—	0.2	2.1	6.5	3.2
	36	—	—	—	—	—	—	—	—	33.7	6.6	3.2	1.0
Total ^{35}S content of faeces	48	3.9	0.4	—	—	5.1	0.2	—	—	4.3	0.5	—	—
		45.7	25.4	—	—	64.1	12.4	—	—	2.6	0.7	—	—
										57.9	17.1	—	—
Residual ^{35}S in carcass after 48 h % of injected dose recovered		22.3	15.8	—	—	—	—	—	—	16.8	22.8	—	—
		5.3	5.2	—	—	—	—	—	—	4.5	4.2	—	—
		98.7	97.5	—	—	—	—	—	—	96.3	97.0	—	—

* Where values are not recorded, urine collections were not made.

urine within 24h as inorganic [^{35}S]sulphate, and significant amounts (18–28%) of the radioactive dose appeared in the faeces.

Qualitative examination of ^{35}S in urine of animals receiving sodium cortisone 21-[^{35}S]sulphate

Free-ranging rats received intraperitoneal injections of sodium cortisone 21-[^{35}S]sulphate as described above and the urine was collected over 12h. The urine was concentrated by freeze-drying and portions were subjected to paper electrophoresis, paper chromatography (butan-1-ol-acetic acid-water, 50:12:25, by vol.) and t.l.c. (benzene-ethyl methyl ketone-ethanol-water, 3:3:3:1, by vol.). The following controls were run on the same chromatograms and electrophoretograms as for the test urine: normal rat urine to which sodium cortisone 21-[^{35}S]sulphate had been added and normal rat urine to which inorganic [^{35}S]sulphate had been added. Radioautograms showed the presence of two major radioactive spots in the test urine, which corresponded in mobility with marker inorganic [^{35}S]sulphate and authentic cortisone 21-[^{35}S]sulphate.

Whole-body radioautography

The most striking features of the whole-body radioautograms were the cellular accumulation of radioactivity in the liver and the accumulation of radioactivity in the gastrointestinal tract. In all the radioautograms only traces of radioactivity were associated with the circulating blood, and apart from the liver and gastrointestinal tract, the kidney was the only other organ in which radioactivity was present to any significant extent. The appearance of radioactivity in the kidney reflects the urinary excretion of ^{35}S , whereas the proportion of radioactivity associated with the gut strongly indicates the biliary excretion of ^{35}S . The ability of the cells of the liver to accumulate radioactivity points to this organ as the major site of metabolism of cortisone 21-[^{35}S]sulphate.

Biliary excretion of ^{35}S after administration of sodium cortisone 21-[^{35}S]sulphate

Anaesthetized female rats, with bile-duct and bladder cannulae, were each injected intravenously with sodium cortisone 21-[^{35}S]sulphate. Urine and bile were collected over a 6h period and analysed for inorganic [^{35}S]sulphate and ester [^{35}S]sulphate content. The results (Table 3) show that 85–92% of the radioactive dose was recovered within 6h and that the major portion of it appeared as ester sulphate in the bile. Other experiments in which bile samples were collected at 30min intervals showed that

Table 2. Distribution of ^{35}S in the urine and faeces of male and female rats injected intravenously with sodium cortisone 21-[^{35}S]sulphate (1 mg dissolved in 0.4 ml of water)

		Distribution of ^{35}S (% of ^{35}S injected)											
		Male rats						Female rats					
Time (h)	^{35}S Content of urine samples	Ester sulphate		Inorganic sulphate		Ester sulphate		Inorganic sulphate		Ester sulphate		Inorganic sulphate	
		Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate	Inorganic sulphate	Ester sulphate
6		1.9	1.4	1.1	8.8	0.7	10.9	0.5	4.9	13.4	4.9	2.0	22.1
12		12.5	1.4	1.5	0.8	5.4	2.1	8.8	2.1	23.3	2.9	37.0	3.8
24		27.6	1.4	42.0	5.3	30.6	5.6	19.1	8.2	13.4	4.4	11.8	2.7
48		11.7	0	7.5	1.0	11.0	2.2	6.3	0.8	3.9	0.4	2.9	0.6
Total		53.7	4.2	52.1	15.9	47.7	20.8	34.7	15.6	54.0	12.6	53.7	29.2
Total ^{35}S content of faeces		20.2	12.3	18.0	86.5	28.4	78.7	21.0	16.0	21.0	87.6	16.0	98.9
% of injected dose recovered		78.1	80.3	86.5	86.5	78.7	78.7	87.6	98.9	87.6	98.9	98.9	98.9

For experimental details see the text.

Table 3. *Excretion of ³⁵S in the urine and bile in a 6h period after the intravenous injection of 1 mg of sodium cortisone 21-[³⁵S]sulphate into anaesthetized female rats with bile-duct and bladder cannulae*

For experimental details see the text.

	Distribution of ³⁵ S (% of ³⁵ S injected)	
	Urine	Bile
Animal 1		
Ester sulphate	5.5	77.3
Inorganic sulphate	0.6	1.3
Animal 2		
Ester sulphate	17.2	72.4
Inorganic sulphate	0.9	1.3
Animal 3		
Ester sulphate	24.0	64.1
Inorganic sulphate	1.1	1.9

virtually all the biliary excretion of ³⁵S occurred during the first hour.

The nature of the ester sulphate excreted in the bile was investigated by chromatography. Bile was collected, via a bile-duct cannula, for 30 min from a female rat that had received 1 mg of sodium cortisone 21-[³⁵S]sulphate intravenously. Portions of bile were subjected to t.l.c. (benzene-ethyl methyl ketone-ethanol-water, 3:3:3:1, by vol.). Normal rat bile to which sodium cortisone 21-[³⁵S]sulphate or inorganic [³⁵S]sulphate had been added was run on the same chromatograms. Radioautograms showed the presence of three radioactive components in the test bile; one of these (the minor component, *R_F* 0.46) had the same mobility as sodium cortisone 21-[³⁵S]sulphate, whereas the major component (metabolite I, *R_F* 0.07) was chromatographically distinct from the parent ester. A third trace component (*R_F* 0.17) could be detected only after prolonged radioautographic exposure.

Metabolite I occurred in a region of the chromatogram containing glucuronic acid, detected by the naphtharesorcinol-phosphoric acid reaction (Randerath, 1968) and the obviously highly polar nature of the metabolite suggested that it might be a double conjugate containing both glucuronic acid and sulphate. This was also suggested by the destruction of metabolite I that occurred on incubation with a crude preparation of β -glucuronidase (Sigma bacterial glucuronidase, type I).

In all routine experiments, female rats were used as experimental animals. The pattern of metabolism of cortisone 21-sulphate in male rats was different, in that only relatively small amounts of metabolite I were formed and the major material in the bile had a

mobility close to that of cortisone sulphate. The total biliary excretion of ³⁵S was similar in both sexes.

Preparation of metabolite I

Bile (about 4 ml) collected over a period of 1 h from eight female rats, each of which had received 1 mg of cortisone 21-[³⁵S]sulphate, was applied to eight thick-layer plates of Whatman Silica Gel SG41, which were developed in benzene-ethyl methyl ketone-ethanol-water (3:3:3:1, by vol.). Areas of gel corresponding to metabolite I were located by radioautography and scraped off the plates. Metabolite I was extracted from the gel by stirring for 1 h at room temperature with 50 ml of water, the gel removed by filtration on a Buchner funnel and was washed with 5 $\times$ 5 ml of water, and the combined filtrate and washings were evaporated to dryness in a rotary evaporator at a bath temperature of 40–45°C. Material obtained in this way was used in subsequent investigations of the chemistry and metabolism of metabolite I.

Preparation of the steroid component of metabolite I

Metabolite I from 8 mg of cortisone sulphate was dissolved in 2 ml of 0.1 M-sodium acetate-acetic acid buffer, pH 5.0, and to this was added 50 μ l (about 10⁵ Fishman units) of rat preputial-gland β -glucuronidase [stage 3 of Levvy *et al.* (1958)]. After incubation at 37°C for 20 h the mixture was applied to four thicklayer plates and chromatographed as described above. The radioactivity had disappeared from the region corresponding to metabolite I and now had a mobility similar to that of cortisone sulphate. The appropriate areas were removed from the plates and the steroid sulphate corresponding to metabolite I was extracted with water as described above. In a typical preparation the recovered radioactivity, expressed as a percentage of that administered, was 61% in the bile, 39% in metabolite I and 31% in the steroid sulphate obtained from the second chromatogram.

The steroid sulphate was then solvolysed by the method of Drayer & Giroud (1965). It was dissolved in 5 ml of water and the aqueous solution washed twice with 5 ml portions of chloroform before the addition of 1 g of NaCl and 0.25 ml of 4M-HCl: the solution was then immediately extracted with one 5 ml and one 2 ml portion of tetrahydrofuran. The tetrahydrofuran extract was filtered to remove water droplets, acidified with 15 μ l of 60% (w/v) HClO₄ and kept for 18 h at 37°C. After the addition of 3 ml of 0.5M-NaHCO₃ the tetrahydrofuran was removed in a stream of N₂ at 40°C and the aqueous solution extracted with 4 $\times$ 2 ml of ethyl acetate. The extract was dried over Na₂SO₄ and evaporated to dryness in a stream of N₂ at 40°C. The residue was dissolved

in 1 ml of methanol, filtered, and the solution again evaporated to dryness to give, in a typical experiment, 2.7 mg of solid, of which 1.5 mg contained the α -ketol side chain (calculated as cortisone).

Examination of 15 μ g samples of this material by t.l.c. on silica gel in benzene-acetone (1:1, by vol.) showed that it contained only traces of u.v.-absorbing material (which remained at the origin) and one major and one minor component detectable by phosphomolybdic acid (Randerath, 1968) or by Tetrazolium Blue (Bush, 1961). The crude steroid (1.3 mg, calculated as cortisone) was therefore dissolved in 0.2 ml of methanol and applied as a streak to a t.l.c. plate of silica gel and developed in benzene-acetone (1:1, by vol.). Marker lanes were stained with Tetrazolium Blue and the area corresponding to the major α -ketol was removed, extracted with methanol, filtered and evaporated to dryness as described above. About 40% of the α -ketol was recovered. As well as showing the major component, the t.l.c. showed three minor components, one more polar and two less polar than the major component. The three minor components were present in extremely small amounts and were not investigated further.

Characterization of the steroid from metabolite I

Thin-layer chromatography. The steroid, prepared as described above, gave only a single spot, either with Tetrazolium Blue or phosphomolybdic acid, when chromatographed on silica gel in benzene-acetone (1:1, by vol.). No u.v.-absorbing material was present. The material was clearly not identical with the tetrahydro-reduction product of cortisone $3\alpha,17\alpha$,

21-trihydroxy- 5β -pregnane-11,20-dione but could have been either the 3α - or 3β -hydroxy derivative of the corresponding 5α -pregnanes, which were not resolvable in this system.

Paper chromatography. The steroid was chromatographed on paper by using as the mobile phase the upper layer of the system toluene-methanol-water (4:3:1, by vol.). In this solvent the steroid from metabolite I had R_F 0.11 and was indistinguishable from $3\alpha,17\alpha,21$ -trihydroxy- 5α -pregnane-11,20-dione. It was clearly separable from $3\beta,17\alpha,21$ -trihydroxy- 5α -pregnane-11,20-dione, which had R_F 0.06.

Oxidation to a 17-oxosteroid. A sample (0.4 mg) of the steroid was oxidized with sodium bismuthate as described by Appleby *et al.* (1955). Chromatography of the product on paper in Bush system B₃ (Neher, 1958) showed it to be indistinguishable from 3α -hydroxy- 5α -androsterone-11,17-dione.

Infrared spectrum. A sample (1.4 mg) of the steroid was purified by paper chromatography followed by t.l.c. under the conditions described above. The resulting 0.7 mg of white solid was incorporated into a KBr disc for spectrophotometry in a Perkin-Elmer 621 spectrophotometer. The resulting spectrum was not absolutely identical with that of authentic $3\alpha,17\alpha,21$ -trihydroxy- 5α -pregnane-11,20-dione but was consistent with a large proportion of the isolated steroid being identical with the standard.

Metabolic fate of metabolite I

The solution of metabolite I prepared as described above (500 μ l containing 3100 c.p.m.) was injected intravenously into an anaesthetized female rat. Bile

Table 4. Distribution of ^{35}S in urine, faeces and carcasses of male rats receiving a solution of metabolite I either intraperitoneally or orally

For experimental details see the text. The results of the experiment in which metabolite I was administered intraperitoneally are expressed as % of ^{35}S injected. The results obtained on oral administration are expressed as % of ^{35}S recovered.

		Distribution of ^{35}S			Carcass
		Urine		Faeces (48 h collection)	
		Urine sample (h)	Inorganic sulphate	Ester sulphate	
Animal 1	Intraperitoneal administration	12	29.9	0.7	
		24	24.9	3.6	32.2
		48	3.6	0.4	4.2
Animal 2	Oral administration	12	43.8	3.3	
		24	19.7	3.9	35.8
		48	4.3	0	0

was collected 1, 2 and 2.5 h after injection and the inorganic [³⁵S]sulphate and ester [³⁵S]sulphate contents of the samples were determined. Of the injected radioactivity, 92% appeared in the bile during the first hour and 90% of the dose was present as ester [³⁵S]sulphate. At the end of the experiment, analysis of the bladder contents for ³⁵S revealed that a small amount (3.1%) of the injected radioactivity was present in urine as ester [³⁵S]sulphate. Inorganic [³⁵S]sulphate was not detected.

In a further experiment, bile was collected 10, 20, 30 and 40 min after intravenous administration of a solution containing metabolite I as described above. When bile samples were subjected to t.l.c. in butan-1-ol-acetic acid-water (3:1:1, by vol.) only one radioactive spot (*R_F* 0.43), identical in mobility with metabolite I, was detected in all bile samples.

The fate *in vivo* of intraperitoneally administered metabolite I was investigated in an experiment in which a male rat received the solution (550 μl containing 5400 c.p.m.) containing metabolite I by intraperitoneal injection. Urine was collected after 12, 24 and 48 h and the inorganic [³⁵S]sulphate and ester [³⁵S]sulphate content determined. Faeces were also collected over 48 h and at the end of the experiment, the animal was killed and the carcass examined for radioactivity. The results (Table 4) show that considerable amounts of ³⁵SO₄²⁻ were produced, 58.4% of the radioactive dose appearing as ³⁵SO₄²⁻ in the urine over 48 h. Similar results were obtained (see Table 4) when an aqueous solution (440 μl) of metabolite I was introduced into the stomach of a lightly anaesthetized rat.

Discussion

Quantitative and qualitative experiments show that when sodium cortisone 21-[³⁵S]sulphate is administered to free-ranging rats substantial amounts of ³⁵SO₄²⁻ appear in the urine. Whole-body radioautograms strongly indicate the liver as the site of metabolism of cortisone 21-[³⁵S]sulphate and provide evidence of considerable biliary elimination of ³⁵S. When the ³⁵S-labelled ester is administered to female rats with bile-duct cannulae about 70% of it is rapidly eliminated in the bile as a steroid doubly conjugated with [³⁵S]sulphate and glucuronic acid (metabolite I). The steroid component of this has been identified as 3α,17α,21-trihydroxy-5α-pregnane-11,20-dione, a compound previously shown to be a metabolite of cortisone in rat liver (Caspi *et al.*, 1953; Miller & Axelrod, 1954). Metabolite I is therefore 3α-(β-D-glucopyranuronosido)-17α-hydroxy-21-[³⁵S]sulpho-oxy-5α-pregnane-11,20-dione. Other metabolites of cortisone 21-[³⁵S]sulphate are undoubtedly formed but after removal of the [³⁵S]sulphate group the steroids derived from these cannot be identified with certainty because they

are indistinguishable from the endogenous steroids of the bile.

The trace metabolite (*R_F* 0.17 on t.l.c.) of cortisone 21-[³⁵S]sulphate that is excreted in the bile (see the Experimental and Results section) has not been identified. It differed strikingly from metabolite I in that its chromatographic mobility was not altered by treatment with β-glucuronidase, so that it may be a disulphate.

The rapid formation of metabolite I from cortisone sulphate by the female rat is consistent with what is known of the metabolism of corticosteroids in this animal, the predominating reactions being the saturation of the A ring followed by reduction of the 3-oxo group to give a 3α- or a 3β-hydroxy steroid. The reduction of the 4,5 double bond is catalysed by steroid 5α-reductase (McGuire *et al.*, 1959), an enzyme that equally well reduces testosterone and testosterone 17-sulphate (Wu & Mason, 1965) so that the reduction of cortisone 21-sulphate is not unexpected. The action of the 3-hydroxy steroid dehydrogenases, which catalyse the reduction of a 3-oxo group, on steroid sulphates does not appear to have been studied but the related 17β-dehydrogenase can certainly reduce steroid 3-sulphates (Milgrom & Baulieu, 1970) so that again reduction of the 3-oxo group of a sulphate ester is not unlikely. Once the 3α-hydroxy-5α-pregnane derivative has been formed then conjugation with glucuronic acid at the 3-position would follow (Jayle & Pasqualini, 1966). The sex difference in the formation of metabolite I is also consistent with previous observations, because steroid 5α-reductase seems to play a very much less important role in steroid metabolism in the male than in the female rat (Forchielli *et al.*, 1958; Leybold & Staudinger, 1959).

As shown in Tables 1-3, the administration of cortisone 21-[³⁵S]sulphate to rats is followed by the elimination of ester [³⁵S]sulphate in the urine. It must not be presumed that the latter consists only of cortisone 21-[³⁵S]sulphate. Metabolites of this ester, in particular 3α,17α-dihydroxy-21-[³⁵S]sulpho-oxy-5α-pregnane-11,20-dione, will be present although they would not be separable from cortisone 21-[³⁵S]sulphate by the techniques used in the present study. These sulphate esters could arise directly from cortisone sulphate or indirectly from metabolite I (and related conjugates), which is likely to undergo hydrolysis by β-glucuronidase during its enterohepatic circulation. Certainly metabolite I can be converted into a steroid sulphate by incubation with homogenates of rat intestine (Dodgson *et al.*, 1965). Such an enterohepatic circulation should perhaps be considered in studies of the metabolism of corticosterone 21-[³⁵S]sulphate in man (Pasqualini, 1965, 1967), although the short half-life of this ester (45 min) in man (Kielmann *et al.*, 1966) may suggest that such a circulation is unimportant in this case.

The route by which $^{35}\text{SO}_4^{2-}$ is produced from cortisone 21- ^{35}S sulphate is still unknown. It should be noted, however, that the rate of production is low. Table 1 shows that approx. 50% of a single dose of 1 mg of sodium cortisone 21-sulphate is converted into SO_4^{2-} in 24 h: this corresponds to the formation of approx. 45 nmol of SO_4^{2-} /h. As discussed above, the metabolism of cortisone 21-sulphate probably occurs principally in the liver and, accepting the results of Roy (1958), this organ can hydrolyse, in the female rat, approx. 2 μmol of dehydroepiandrosterone sulphate/h. Therefore, if the steroid sulphatase of the rat hydrolysed cortisone 21-sulphate at only 2% of the rate at which it hydrolyses dehydroepiandrosterone sulphate, the liberation *in vivo* of SO_4^{2-} from the former could be explained on the basis of the steroid sulphatase activity of liver alone. Certainly the hydrolysis of cortisone 21-sulphate at such a low rate would not have been detected by the insensitive method used by Roy (1957). The possibility of the SO_4^{2-} being formed from cortisone 21-sulphate by the action of a known sulphatase cannot therefore be discounted.

Another route that might lead to the formation of SO_4^{2-} could involve the conversion of cortisone, and corticosteroids in general, into 17-oxo steroids (Caspi *et al.*, 1953; Caspi & Hechter, 1954). If such a conversion could take place with cortisone 21-sulphate the side chain would presumably appear as the sulphate ester of glycollic acid or glycollic aldehyde. So far as is known there exists in the rat no sulphatase which will hydrolyse such alkyl sulphates, but it must be stressed that SO_4^{2-} can be liberated from sulphate esters *in vivo* by mechanisms not involving sulphatases, either enzymically, as in the desulphation of serine *O*-sulphate (Thomas & Tudball, 1967), or non-enzymically, as in the desulphation of butyric acid 4-sulphate (Ottery *et al.*, 1970).

We thank Dr. E. Spinner, Department of Medical Chemistry, Australian National University, for determining the i.r. spectra. This work was supported by U.S. Public Health Service Grant no. AMO-1982 and by the Wellcome Trust.

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