and mercuric chloride (0.1 mM) but not by sulphhydryl reactants.

3. The reaction is characterized by a marked increase in iodine-staining power and β -amylolysis limit but α -(1 $\rightarrow$ 4)-glucosidic linkages in glycogen are not attacked. Enzyme action is incomplete, only the outer α -(1 $\rightarrow$ 6)-glucosidic linkages being hydrolysed.

4. Isoamylase differs from the plant and animal debranching enzymes [R-enzyme and amylo-(1 $\rightarrow$ 6)-glucosidase] since both amylopectin and glycogen are substrates.

5. Purified isoamylase has no action on maltose, isomaltose or maltotriose.

6. Isoamylase is also present in baker's yeast.

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REFERENCES

- Chanda, N. B., Hirst, E. L. & Manners, D. J. (1957). *J. chem. Soc.* p. 1951.
- Conchie, J. & Levvy, G. A. (1957). *Biochem. J.* **65**, 389.
- Cori, G. T., Colowick, S. P. & Cori, C. F. (1938). *J. biol. Chem.* **123**, 375.
- Cowie, J. M. G., Fleming, I. D., Greenwood, C. T. & Manners, D. J. (1957). *J. chem. Soc.* p. 4430.
- Fleming, I. D. & Manners, D. J. (1958). *Chem. & Ind.* p. 831.
- Gunja, Z. H. (1959). Ph.D. Thesis: University of Edinburgh.
- Gunja, Z. H., Manners, D. J. & Khin Maung (1960). *Biochem. J.* **75**, 441.
- Hobson, P. N., Whelan, W. J. & Peat, S. (1951). *J. chem. Soc.* p. 1451.
- Hopkins, R. H. (1955). *Proc. European Brewery Convention Congress*, p. 52.
- Hopkins, R. H. (1958). *Wallerstein Lab. Commun.* **21**, 309.
- Hopkins, R. H. & Kulka, D. H. (1957a). *Proc. European Brewery Convention Congress*, p. 182.
- Hopkins, R. H. & Kulka, D. H. (1957b). *Arch. Biochem. Biophys.* **69**, 45.
- Kobayashi, T. (1956). *Chem. Abstr.* **50**, 16,898.
- Kobayashi, T. (1958). *Chem. Abstr.* **52**, 5505.
- Liddle, A. M., Manners, D. J. & Wright, A. (1961). *Biochem. J.* **80**, 304.
- Lindberg, B. & McPherson, J. (1954). *Acta chem. scand.* **8**, 985.
- MacWilliam, I. C. & Harris, G. (1959). *Arch. Biochem. Biophys.* **84**, 442.
- Manners, D. J. (1953). *Rep. Progr. Chem.* **50**, 288.
- Manners, D. J. (1957). *Advanc. Carbohydr. Chem.* **12**, 261.
- Manners, D. J. & Khin Maung (1955). *Chem. & Ind.* p. 950.
- Maruo, B. & Kobayashi, T. (1951). *Nature, Lond.*, **167**, 606.
- Meyer, K. H. & Bernfeld, P. (1942). *Helv. chim. acta*, **25**, 399.
- Minagawa, T. (1932). *Chem. Abstr.* **26**, 5583.
- Nishimura, S. (1931). *Chem. Abstr.* **25**, 2449.
- Nishimura, S. & Minagawa, T. (1931). *Chem. Abstr.* **25**, 5905.
- Peat, S., Whelan, W. J., Hobson, P. N. & Thomas, G. J. (1954). *J. chem. Soc.* p. 4440.
- Peat, S., Whelan, W. J. & Thomas, G. J. (1952). *J. chem. Soc.* p. 4546.
- Phillips, L. L. & Caldwell, M. L. (1951). *Analyt. Chem.* **23**, 1172.
- Posternak, T. (1951). *J. biol. Chem.* **188**, 317.
- Reese, E. T. & Mandels, M. (1959). *Research Report: Microbiology Series* no. 18. Pioneering Research Division, Quartermaster Research and Engineering Center, Natick, Mass., U.S.A.
- Roberts, P. J. P. & Whelan, W. J. (1960). *Biochem. J.* **76**, 246.
- Schaffner, A. & Specht, H. (1938). *Naturwissenschaften*, **26**, 494.
- Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
- Ueda, S. (1957). *Bull. agric. chem. Soc. Japan*, **21**, 379.
- Whelan, W. J. (1958). In *Encyclopedia of Plant Physiology*, vol. 6, p. 154. Ed. by Ruhland, W. Berlin: Springer Verlag.

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The Metabolism of [4-¹⁴C]Progesterone in the Cat: Biliary and Urinary Excretion of Conjugated Metabolites

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Marrian (1949, 1954) stressed the need for a systematic study of progesterone metabolism in order to account for the large proportion of that hormone which is not excreted in the urine, by man and some animals, as pregnanediol (5 β -pregnane-

3 α :20 α -diol) and closely related substances. The availability of steroid hormones labelled with ¹⁴C and ³H now permits such systematic studies to be carried out, and some investigations with labelled progesterone have been reported. When [21-¹⁴C]-

progesterone was administered to rats and mice the major part of the excreted radioactivity was present in the bile or faeces, but no attempt was made to characterize the metabolites (Riegel, Hartop & Kittinger, 1950; Grady, Elliott, Doisy, Bocklage & Doisy, 1952; Shen, Elliott, Doisy & Doisy, 1954). In contrast, after administration of [4-¹⁴C]progesterone to human subjects, more than 50% of the excreted radioactivity was in the urine and up to 30% in the bile or faeces (Davis & Plotz, 1957; Sandberg & Slaunwhite, 1958). A post-menopausal woman investigated by Wiest, Fujimoto & Sandberg (1958) excreted a larger proportion of radioactive metabolites in the bile than in the urine: pregnanediol, pregnanolone (3 α -hydroxy-5 β -pregnan-20-one) and more 'polar' material were found in the β -glucuronidase-hydrolysed fractions of the bile and urine. Pearlman (1957) studied the metabolism of [16-³H]progesterone in human females but confined himself to investigating metabolites present in neutral extracts after hot-acid hydrolysis of the urine. In the light of the results reported above it was felt that further investigation of progesterone metabolism *in vivo* might prove of value.

Because of the difficulties and risks associated with the use of labelled steroids in human subjects, and because of the difference between rodents and man described above, it was decided to investigate the metabolism of [4-¹⁴C]progesterone in cats. These animals are of sufficient size to make the necessary surgical procedures relatively easy, and to permit the administration of a reasonably large dose of the steroid. In this investigation male cats were used to eliminate any possible effects on progesterone metabolism of the ovarian status of the animals. Labelled progesterone has been administered intravenously to three pairs of anaesthetized animals: the first pair received a single rapid injection in 10 sec. and in the second and third pairs the steroid was given as a continuous infusion over 1 and 3 hr. respectively. Bile and urine were collected and treated by standard hydrolytic methods. The neutral, ether-extractable radioactivity in each fraction was determined. A preliminary account of part of this work has been published (Taylor & Scratcherd, 1960).

MATERIALS AND METHODS

[4-¹⁴C]Progesterone. [4-¹⁴C]Progesterone (20.8 mc/mole) was obtained in benzene solution from The Radiochemical Centre, Amersham, Bucks. Approximately ten times the weight of unlabelled progesterone, which had been crystallized twice from hexane and once from aqueous ethanol and had m.p. 121–121.5°, was added. The benzene was evaporated in a stream of air at 45° and the progesterone was crystallized once from hexane. The crystals were stored

in the dark at –15° and, although a slightly yellowish colour developed after a few days, no detectable deterioration occurred in the several weeks of storage. Progesterone solutions were prepared by dissolving the steroid in 0.5 ml. of warm ethanol and adding the required amount of 0.154 M-NaCl immediately before injection (0.5 ml. for rapid single injections, 25 ml. for the more prolonged infusions).

Operative procedure. Male cats, which had been starved of solid food overnight but allowed access to water and milk, were anaesthetized by intravenous injection of a mixture of 2% (w/v) of chloralose (75 mg./kg.) and 20% (w/v) of urethan (450 mg./kg.) after preliminary induction with ether. The splanchnic nerves were cut extraperitoneally. To collect bile a glass cannula (5 cm. long, 0.2 cm. bore) was inserted through the fundus of the gall bladder. One end of the cannula was covered with soft rubber and was placed as close as possible to the opening of the cystic duct. The common bile duct was tied off. Urine was collected by passing a cannula through the apex of the bladder, the cannula being tied in place so that its tip was adjacent to the openings of the ureters. All solutions were injected into a cannula in the saphenous vein. Rectal temperature was maintained at 38.5°. After the operation the animal was left for 2 hr. before injection of the steroid to ensure that the bile and urine flows were steady. Generally the early bile and urine samples were contaminated with small amounts of blood, but no detectable contamination occurred during the collection period proper.

At the end of each experiment the liver and kidneys of each animal were removed. No abnormality was detected on gross or histological examination.

Injections of radioactive solutions were made through a three-way adapter which allowed the original syringe to be rinsed out with 0.9% NaCl solution without removing it from the cannula. Prolonged infusions were carried out by connecting the syringe to a calibrated, electrically driven plunger which gave a constant rate of infusion.

Treatment of bile and urine samples. Immediately after collection the bile and urine samples were weighed and then dried to constant weight in an evacuated desiccator containing conc. H₂SO₄. The dry residues were dissolved in a known volume of water. Portions of the solutions were plated in duplicate and the total corrected radioactivity was determined. Volumes of the solutions equivalent to 5000–10 000 counts/min. were then subjected to extractions and hydrolyses to provide the following fractions:

(1) Non-conjugated. Radioactivity extractable before any form of hydrolysis.

(2) Glucuronides. After the initial extraction the sample was taken to pH 4–5 with 2N-acetic acid, 0.5 vol. of 0.2M-acetate buffer, pH 4.5, containing 5000 units of β -glucuronidase, and 0.1 vol. of 0.2M-KH₂PO₄ were added, and the mixture was incubated at 45° for 18 hr. and then extracted.

(3) Cold-acid-hydrolysed metabolites. The enzyme-hydrolysed solution was then made 2N with respect to H₂SO₄ by the addition of 2N-H₂SO₄. The acid was added drop by drop while the tubes were immersed in ice-water. A volume of wet ether equal to the total volume of aqueous solution was added, and the tubes were left at room temperature with occasional shaking (Burstein & Lieberman, 1958). After 72 hr. the ether was removed and the aqueous phase was extracted twice more with ether.

(4) Hot-acid-hydrolysed metabolites. The acidified aqueous solution remaining after the previous hydrolysis

was now less than 2N with respect to H_2SO_4 because of the addition of the alkali and water washings of the ether extract, but the pH was still less than 1. Ether dissolved in the water was removed by placing the tubes in a water bath at 45° and playing a stream of air over the surface of the liquid. The tubes were then heated in a boiling-water bath for 30 min., cooled and extracted.

(5) Water-soluble metabolites. After all the above methods of hydrolysis the aqueous solution was neutralized with 5N-NaOH. When sufficient radioactivity was present portions of the solution could be plated directly and self-absorption corrections applied. In most cases, however, the concentration of radioactivity was low, and the ^{14}C content was therefore determined by the differential counting method of Heard, Thompson & Yates (1952).

Extraction procedure. AnalaR ether was washed with acidified FeSO_4 and then with water until neutral, dried over anhydrous Na_2SO_4 and distilled. Wet ether used for extractions was prepared by saturating the distilled ether with water.

Aqueous solutions were extracted three times with 2 vol. of wet ether. The combined ether extracts were washed twice with 0.1 vol. of 0.1N-NaOH and then with 0.1 vol. of water until neutral, the alkali and water washings being added to the original aqueous solution. The washed extracts were dried over anhydrous Na_2SO_4 for at least 2 hr. and then filtered. The Na_2SO_4 was washed three times with small amounts of dry ether, and the combined washings and filtrates were evaporated to dryness on a water bath at 45°. The dry residues were dissolved in chloroform-methanol (1:1, v/v), the volume used depending on the amount of radioactivity in the extracts. Portions of the solution were plated in duplicate and their radioactivities determined.

β -Glucuronidase. Powder B obtained from acetone-dried limpet viscera as described by Dodgson & Spencer (1953) was used as a source of β -glucuronidase. It contained 10⁶ Fishman units/g. Since this preparation also contains sulphatases, hydrolyses were carried out in the presence of 0.02M- KH_2PO_4 , a potent inhibitor of these enzymes (Dodgson & Spencer, 1953; Roy, 1956). The dry powder was homogenized in buffer solution before addition to incubation vessels. In β -glucuronidase-inhibition experiments 0.16M-potassium hydrogen saccharate was boiled for 30 min., cooled, taken to pH 4.5 and added to incubation vessels to give a final concentration of 0.03M (Levy, 1952).

Assay of radioactivity. This was carried out in a window-less gas-flow proportional counter (SC-16P; Tracerlab Inc., Boston, Mass., U.S.A.); argon-methane (90:10, v/v) was supplied by British Oxygen Co. Ltd. Samples were plated on duralumin planchets (1 in. diam. and scoured with concentric grooves to ensure uniform distribution of material) and dried under an infrared lamp. All assays of extracts were performed at infinite thinness (<100 μg . of total solids/planchet); counts of urine and bile were corrected to infinite thinness by reference to appropriate self-absorption correction curves. All samples were counted for at least 1000 counts/min., so that counting errors did not exceed 5%. Day-to-day instrumentation errors were corrected by the use of poly(^{14}C)methyl methacrylate reference standards (The Radiochemical Centre). Under the above conditions counting efficiency was estimated to be 45–48%. Throughout this paper quantities of radioactivity are expressed as counts/min. actually determined.

RESULTS

Table 1 shows the excretion of radioactivity in the bile of cats after a single rapid injection (Table 1) or after 1 or 3 hr. infusion of [^{14}C]progesterone. The volumes of bile collected and the concentrations of total solids are also included to show that biliary function was maintained throughout the collection period. There was wide variation in the volume of bile produced by different animals, ranging from 0.1 (cat no. 5) to 3.9 ml./hr. (cat no. 2). In general, however, there was a definite decrease in bile volume with time, presumably due, at least in part, to the absence of the choleric activity of bile salts that, under normal conditions, would have been returned to the liver via the entero-hepatic circulation. Since all samples collected were golden-brown in colour it can be concluded that essentially liver bile was collected, and this is borne out by the concentrations of solids in the samples collected. A sample of gall-bladder bile obtained from eight cats used for other purposes was very dark-green and contained 11.2 g. of solids/100 ml. Only the bile of cat no. 6 approached this value and it should be noted that this animal excreted only a small volume of bile.

Table 1 shows that, after a single dose of progesterone administered in 10 sec., excretion of metabolites was rapid, over 60% of the dose being recovered after 4 hr. Excretion of radioactivity reached a maximum during the first or second hour after injection of the steroid and declined thereafter. A similar trend in excretion of radioactivity can be seen from the results of the experiments in which the steroid was infused over 1 or 3 hr., but in these experiments the trends are less definite because of the longer period over which the steroid was given. The amount of radioactivity recovered after infusion was considerably less (26.2–55.3%) than after a single injection (65.6–67.1%). This was not due to the length of the collection period after the end of the administration of the steroid, since bile was collected from cat no. 5 for 5 hr. after the end of the 1 hr. perfusion and a total of 35.2% was recovered, whereas, on the other hand, after a single dose of progesterone cats nos. 1 and 2 had excreted approximately 60% of the dose in less than 5 hr. These findings suggest that a single rapid dose of steroid is handled differently in the body from a similar dose administered over a longer period. Table 1 also shows the distribution of excreted neutral radioactivity after hydrolysis, the amount of radioactivity remaining water-soluble and the percentage recovery of radioactivity after application of the successive hydrolytic methods.

Table 2 gives the corresponding results for the urines obtained from the same six cats. As with

Table 1. *Excretion of radioactivity in bile of male cats after intravenous injection of [4-¹⁴C]progesterone*
A single dose was given to cats 1 and 2; cats 3 and 4, and 5 and 6, were dosed by continuous infusion for 3 and 1 hr. respectively.

Cat no.	Wt. (kg.)	Dose of steroid		Collec- tion period (hr.)	Bile				Neutral, ether-soluble radioactivity					Total recovery after all hydro- lyses (%)	
		(mg.)	(10 ⁻⁶ × counts/ min.)		Bile vol. (ml.)	Total solids		(10 ⁻⁶ × total counts/ min.)	(% of dose)	Non- conju- gated	Gluc- uronide	Cold- acid hydro- lysed	Hot- acid hydro- lysed		Water- soluble
						(mg.)	(g./ 100 ml.)								
1	3.7	0.922	10.00	1-0 2-0 3-0 4-0 5-0 5-5	2.9 2.6 2.5 2.1 1.7 1.4	73 62 57 45 38 32	2.5 2.4 2.3 2.1 2.2 2.3	2.551 1.634 1.077 0.637 0.544 0.272	0.5 0.5 0.3	25.6 10.3 5.9	12.2 22.0 32.2	11.1 10.7 6.9	46.3 47.1 50.2	95.7 90.6 95.5	
Total															
2	3.7	1.390	9.56	1-0 2-0 3-0 4-0	1.4 1.3 0.7 1.0	55 39 27 39	3.9 3.0 2.9 3.9	1.813 2.307 1.098 1.096	0.5 0.6 0.3 0.5	40.1 20.9 13.5 10.8	9.4 13.2 14.9 17.9	13.3 20.0 18.4 20.5	24.7 34.3 42.6 47.9	88.0 89.0 89.7 97.6	
Total															
3	3.0	2.580	30.30	4-5	8.6	260	3.0	16.77	0.5	24.5	26.4	4.5	38.2	94.1	
4	4.1	2.592	31.34	1-0 2-0 3-0 4-0 5-0 6-0	3.5 3.6 3.7 3.8 3.3 2.7	98 72 71 67 63 54	2.8 2.0 1.9 1.8 1.9 1.9	0.667 1.820 1.942 2.194 1.470 1.090	1.3 0.7 1.0 0.9 0.7 0.7	15.7 10.6 8.8 8.6 6.3 3.7	31.8 25.5 23.8 24.2 21.8 18.6	25.6 25.5 28.6 27.0 26.1 27.0	24.0 25.8 27.4 33.7 35.2 43.7	98.4 88.4 89.7 94.4 90.1 93.7	
Total															
5	2.8	1.251	10.125	1-0 2-0 3-0 4-0 5-0 6-0	0.6 0.6 0.6 0.5 0.1 0.3	30 27 22 18 6 16	5.0 4.5 3.6 3.6 6.0 5.3	0.058 0.782 1.026 0.828 0.229 0.618	0 0.5 0.4 0.4 0.1 0.2	43.1 36.6 18.4 10.5 9.6 9.2	19.0 24.1 30.3 28.9 34.1 38.3	37.9 24.7 34.0 33.0 35.8 26.9	3.4 7.4 5.8 17.8 13.0 22.0	103.7 93.3 88.9 90.6 92.6 96.6	
Total															
6	4.0	2.055	8.756	1-0 2-0 3-0 4-0	0.3 0.5 0.7 0.3	32.5 39.9 43.6 19.0	10.0 7.5 5.8 5.5	0.005 0.460 1.287 0.543	0 0.4 0.3 0.4	28.0 22.2 15.8 10.3	40.0 18.3 22.5 10.8	29.5 10.2 10.4 9.2	0 42.5 50.0 58.0	97.5 93.6 99.0 88.7	
Total															

Table 2. *Excretion of radioactivity in urine of the cats of Table 1*

Cat no.	Collection period (hr.)	Urine vol. (ml.)	Urine			Distribution of radioactivity (% of total counts in sample) after successive hydrolyses					Total recovery after all hydrolyses (%)	
			Total solids		(10 ⁻⁴ × total counts/ min.)	Neutral, ether-soluble radioactivity						
			(g.)	(g./100 ml.)		Non- conjugated	Gluc- uronide	Cold-acid hydrolysed	Hot-acid hydrolysed			
										Water- soluble		
1	5.5	15.6	2.43	15.6	4.70	0.5	1.3	5.0	6.3	11.7	70.2	94.5
2	4.0	4.8	1.28	26.9	4.60	0.5	1.0	5.8	8.2	10.2	69.6	94.8
3	4.5	20.0	1.47	7.3	24.00	0.8	3.7	7.4	11.7	1.2	66.7	90.7
4	6.0	11.0	2.80	25.4	8.55	0.28	5.4	2.7	3.2	4.1	77.2	92.6
5	2.0	21.8	1.11	5.1	2.20	0.22	1.4	5.5	6.2	7.5	74.3	94.8
	4.0	16.9	0.86	5.1	2.40	0.24	1.5	3.3	4.5	8.4	73.4	91.1
	6.0	7.4	0.56	7.6	1.87	0.19	1.3	3.1	4.4	6.6	77.6	93.0
					Total	0.65						
6	2.0	4.0	0.56	13.9	0.764	0.09	1.3	5.5	6.0	4.3	80.0	97.1
	4.0	4.9	0.82	16.7	2.730	0.31	1.0	1.5	4.8	6.3	70.0	83.6
						Total	0.40					

bile there was wide variation among animals in the volume of urine excreted, ranging from 4.8 ml. in 4 hr. (cat no. 2) to 47.1 ml. in 6 hr. (cat no. 5), and also in the total urinary solids, 5.1 % (cat no. 5) to 26.9 % (cat no. 2). This variation probably reflects the degree of hydration of the animals at the time of the experiments. However, there appears to be no correlation between the volumes of bile and urine produced by an animal: e.g. cat no. 5 produced a large volume of relatively dilute urine (Table 2) but only a small volume of concentrated bile (Table 1), whereas cat no. 2 excreted the smallest volume of the most highly concentrated urine (Table 2) and a bile of intermediate volume and concentration (Table 1).

The amount of radioactivity excreted in the urine ranged from 0.28 to 0.8 % of the dose and appeared to be independent of the method of administration of the steroid. Table 2 also shows the amounts of neutral radioactivity recovered after hydrolysis, the amount of radioactivity remaining water-soluble after hydrolysis and the percentage of radioactivity recovered from the sample analysed after the successive hydrolyses.

Table 3 shows the results of experiments carried out to investigate the effect of boiled saccharate solution and phosphate on the release of ether-extractable, neutral radioactivity by incubation of bile and urine with limpet powder. It can be seen that 0.03M-saccharate almost completely inhibited the release and 0.02M-phosphate had no effect.

Table 3. *Effect of boiled saccharate and potassium dihydrogen phosphate on liberation of radioactivity from bile and urine by limpet powder*

Each vessel contained 0.2 ml. of bile or 1.0 ml. of urine, 400 µg. of limpet powder and 0.05M-acetate buffer, pH 4.5, in a final vol. of 3.5 ml. Final concentrations of inhibitors, when added, were 0.03M-saccharate and 0.02M-KH₂PO₄. Incubations, which were carried out in duplicate, were for 2 hr. at 37°.

Additions to incubation medium (Boiled enzyme)	Neutral counts/min. extracted	
	Bile	Urine
Nil	106	47
	94	30
Saccharate	26 100	1040
	26 000	1010
Saccharate + KH ₂ PO ₄	182	52
	162	45
KH ₂ PO ₄	141	43
	128	37
KH ₂ PO ₄	27 000	1050
	26 000	1070

DISCUSSION

Under the experimental conditions employed the administered progesterone is rapidly metabolized and excreted by male cats. A most striking feature of the results is that whereas up to 67.1% of the dose appeared as biliary metabolites, in no animal did urinary radioactivity exceed 1%. Rats and mice excrete an oral, intramuscular or intraperitoneal dose of [21-¹⁴C]progesterone mainly in the bile, but 22–34% appears in the urine (Grady *et al.* 1952; Shen *et al.* 1954). This difference between cats and rodents may be related to the fact that the former were anaesthetized during the whole period of the experiment whereas the latter were not. Also the steroid was administered intravenously to the cats but by other routes to the rodents. However, Sandberg & Slaunwhite (1958) and Wiest *et al.* (1958) administered [4-¹⁴C]progesterone intravenously to human subjects and found a considerable, and usually larger, proportion of radioactive metabolites in the urine. Therefore it would seem that excretion of progesterone metabolites by the cat differs from that by rodents and human subjects. But other factors may be involved in this apparent species difference. The cats were anaesthetized with chloralose and urethane, both of which have been reported to act as cholagogues, although their use is recommended for studies on bile production (Sobotka, 1937). Also, it is well established that enterohepatic circulation of steroid-hormone metabolites occurs under normal conditions, but in the present investigation such circulation could not occur since no bile reached the gastrointestinal tract. Grady *et al.* (1952) and Shen *et al.* (1954), however, found that in rodents urinary excretion of metabolites of [21-¹⁴C]progesterone appeared to be independent of the enterohepatic circulation, and the same conclusion for human subjects was drawn by Sandberg & Slaunwhite (1958). It might also be supposed that the surgical procedures and anaesthetics used on the cats might have had some adverse effect on renal blood flow, thus preventing adequate urine formation and excretion of metabolites. But a good degree of renal function seems to have been maintained in the cats (Table 2), since urine volumes and total urinary solids compare favourably with those reported for normal, non-anaesthetized cats (Prentiss, Wolf & Eddy, 1959; Wolf, Prentiss, Douglas & Swett, 1959).

Nature of the conjugates

The conjugates detected in the present study are those which it is generally accepted are formed during the metabolism of steroid hormones. Non-conjugated radioactivity was extractable before hydrolysis from both bile and urine, but a greater

proportion of such excreted radioactivity was present in urine than in bile. Sandberg & Slaunwhite (1958) found that this also occurred in human subjects. It cannot be decided whether this radioactivity consists of unconjugated metabolites or of conjugates partially extractable from aqueous solution by ether.

Tables 1 and 2 show that appreciable amounts of neutral radioactivity became extractable by ether after incubation of bile and urine with limpet powder at pH 4.5. The limpet powder almost certainly contained sulphatases of various kinds (see Materials and Methods for reference), but the relative specificity of limpet steroid sulphatase and the fact that incubations were carried out in the presence of phosphate reduced the possibility of hydrolysis of steroid sulphate metabolites at this stage. Further evidence that glucuronides were present in bile and urine is provided by the results of the experiments shown in Table 3, which reveal almost complete inhibition by saccharate of release of radioactivity by limpet powder. Robinson & Williams (1958) found that cats did not excrete glucuronides in the urine after administration of a variety of non-steroidal substances known to be converted readily into such conjugates by other species of animals. Borrell (1958) investigated the urinary excretion of corticosteroids in cats, but observed no significant increase in Porter–Silber chromogens after incubation of the urine with β -glucuronidase. These findings could be reconciled with those of the present investigation by assuming that the cat excretes glucuronides almost exclusively in the bile. However, Dutton & Greig (1957) could not detect glucuronide formation in cat-liver slices under conditions in which the liver of other animals readily formed the conjugates of *o*-aminophenol and (–)-menthol. Therefore any firm conclusions about the identity of the glucuronides produced during the present investigation must await a fuller identification of the material hydrolysed by limpet powder.

It is generally recognized that sulphate esters of hydroxy steroids are readily hydrolysed by cold acid (Burstein & Lieberman, 1958), and it is probable that part at least of the radioactivity released by 2N-sulphuric acid was originally present as ester sulphates.

The nature of the metabolites released by hot-acid hydrolysis remains obscure. Any glucuronides and sulphates not hydrolysed in the previous steps would probably appear in this fraction, but the existence of conjugates other than these cannot be ruled out. Of particular interest is the presence in both bile and urine of appreciable amounts of radioactivity not extractable after all forms of hydrolysis. The urine contained a much higher proportion of such metabolites, in contrast with

human subjects in whom the bile contained a greater proportion of these metabolites (Sandberg & Slaunwhite, 1958). Those workers used chloroform as the extracting solvent and did not wash the extracts. In the present investigation it was noticed that at every stage of hydrolysis and extraction significant amounts of radioactivity were present in the alkali and aqueous washings of the ether extracts, hence these washings were always returned to the original aqueous solution before the next hydrolysis stage. This indicates that part at least of the non-extractable metabolites consists of phenolic or acidic substances, which may be non-hydrolysed conjugates or as yet unknown breakdown products of progesterone.

Variation in excretion of conjugates with time

Table 1 shows that when bile samples were collected for 1 or 2 hr. periods the proportions of the different conjugates in the samples varied with time. The most consistent variation was in glucuronide excretion, which showed progressive decrease in successive samples. A similar trend can be seen to occur in urine (Table 2), although insufficient samples were investigated to allow definite conclusions to be drawn. In some animals (nos. 1, 2 and 5) the fall in glucuronides was accompanied by an increase in cold-acid-hydrolysed metabolites, whereas in others (nos. 4 and 6) the proportion of these metabolites decreased in successive samples. No consistent change with time occurred in the proportion of hot-acid-hydrolysed radioactivity, but in four out of the five animals (nos. 2, 4, 5 and 6) there was a definite increase in the proportion of radioactivity present as non-extractable metabolites.

Many complex factors are involved in the secretion of substances into the bile. These include the concentration of the substances in the blood, the rate of uptake by the liver cells, the length of time they are bound by intracellular protein and the mechanism of secretion into the bile, whether by simple diffusion or by some active secretory mechanism. Sperber (1959) has discussed these factors at length, particularly with respect to the excretion of organic anions in the bile. It is not possible to say how the different proportions of conjugates detected in successive bile samples in the present investigation are related to the above factors. A knowledge of the rate of clearance of the various conjugates from the blood would have been of value, but it was felt that withdrawal of serial blood samples might have aggravated the effects of the experimental procedures by decreasing hepatic and renal blood flow. The splanchnic nerves of the animals had in fact been cut in order to minimize vasomotor effects of the operation and anaesthetic on splanchnic blood flow.

Therefore, for instance, it cannot be decided whether the higher proportion of glucuronides appearing in early bile samples and the higher proportion of water-soluble radioactivity in the later samples was the result of differences in rate of uptake of progesterone or metabolites, or both, from the blood, the degree of binding with intracellular protein and the rate of metabolism, or in the rate of secretion by the liver cells. It is well established that competition for secretion into the bile occurs, particularly with organic anions (Sperber, 1959), and this competition may play some part in controlling the secretion of the progesterone metabolites.

Hsia, Dowben & Shaw (1960) showed that certain progesterone metabolites, as well as synthetic and natural progestational agents, can inhibit the conjugation with glucuronic acid of *o*-aminophenol, phenolphthalein and bilirubin *in vitro* by rat and guinea-pig liver. Thus the fall in proportion of glucuronide excreted may to some extent be due to an inhibitory effect of the administered progesterone and its metabolites on the conjugating mechanism in the liver of the experimental animals.

It is apparent therefore that, under the experimental conditions employed, the bile is the major route of excretion for progesterone metabolites in the cat. It would be of interest to determine whether other steroid-hormone metabolites are excreted in the bile to the same extent by this animal. In human subjects approximately 50% of an intravenous dose of progesterone is excreted as urinary metabolites, whereas, under similar conditions, 80–90% of a dose of a variety of other steroids, including corticosteroids, androgens and oestrogens, is excreted in the urine (see Sandberg & Slaunwhite, 1958, for reference). The experiments described confirm that, in man and other animals, progesterone is metabolized and excreted by a number of pathways.

SUMMARY

1. [4-¹⁴C]Progesterone has been administered intravenously to anaesthetized male cats as a single injection or as a 1 or 3 hr. infusion. Bile and urine were collected for periods of up to 6 hr. after the start of the injection, and the excretion of radioactive metabolites was determined.

2. The major part of the dose of progesterone (up to 67.1%) was excreted in the bile, whereas in no cat did urinary excretion of metabolites exceed 1%.

3. Bile and urine samples were hydrolysed by standard methods so that the following ether-soluble neutral fractions were obtained: non-conjugated (extractable before hydrolysis); glucuronide, extractable after incubation with limpet

powder; cold-acid hydrolysed; hot-acid hydrolysed. In addition there was a significant quantity of metabolites remaining water-soluble after all forms of hydrolysis.

4. When 1 or 2 hr. fractions of bile were analysed the proportion of radioactivity excreted as glucuronide always decreased in successive samples, whereas water-soluble metabolites generally increased. In some animals the proportion of cold-acid-hydrolysed metabolites increased and in others decreased. No consistent change was detectable in hot-acid-hydrolysed radioactivity. A similar trend was seen in successive urine samples.

5. The relationship of these results to similar studies of progesterone in man and other animals is discussed.

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REFERENCES

- Borrell, S. (1958). *Biochem. J.* **70**, 727.
 Burstein, S. & Lieberman, S. (1958). *J. biol. Chem.* **233**, 331.
 Davis, M. E. & Plotz, E. J. (1957). *Recent Progr. Hormone Res.* **13**, 347.
 Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.

- Dutton, G. J. & Greig, C. G. (1957). *Biochem. J.* **66**, 52P.
 Grady, H. J., Elliot, W. H., Doisy, E. A., jun., Bocklage, B. C. & Doisy, E. A. (1952). *J. biol. Chem.* **195**, 755.
 Heard, R. D. H., Thompson, L. M. & Yates, C. H. (1952). *Rev. Canad. Biol.* **11**, 66.
 Hsia, D. Y.-Y., Dowben, R. M. & Shaw, R. (1960). *Nature, Lond.*, **187**, 693.
 Levvy, G. A. (1952). *Biochem. J.* **52**, 464.
 Marrian, G. F. (1949). *Recent Progr. Hormone Res.* **4**, 3.
 Marrian, G. F. (1954). In *La Fonction Lutéale* (Publication des Colloques sur la Fonction Lutéale: April, 1954). Paris: Masson et Cie.
 Pearlman, W. H. (1957). *Biochem. J.* **67**, 1.
 Prentiss, P. G., Wolf, A. V. & Eddy, H. A. (1959). *Amer. J. Physiol.* **196**, 626.
 Riegel, B., Hartop, W. L., jun. & Kittinger, G. W. (1950). *Endocrinology*, **47**, 311.
 Robinson, D. & Williams, R. T. (1958). *Biochem. J.* **68**, 23P.
 Roy, A. B. (1956). *Biochem. J.* **62**, 41.
 Sandberg, A. A. & Slaunwhite, W. R. (1958). *J. clin. Endocrin.* **18**, 253.
 Shen, N.-H. C., Elliott, W. H., Doisy, E. A., jun. & Doisy, E. A. (1954). *J. biol. Chem.* **208**, 133.
 Sobotka, H. (1937). *The Physiological Chemistry of the Bile*. London: Baillière, Tindall and Cox.
 Sperber, I. (1959). *Pharmacol. Rev.* **11**, 109.
 Taylor, W. & Scratcherd, T. (1960). *Biochem. J.* **77**, 22P.
 Wiest, W. G., Fujimoto, G. I. & Sandberg, A. A. (1958). *J. clin. Endocrin.* **18**, 972.
 Wolf, A. V., Prentiss, P. G., Douglas, L. G. & Swett, R. J. (1959). *Amer. J. Physiol.* **196**, 633.

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Purification and Properties of Muscle Phosphoglycerate Kinase

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The enzyme phosphoglycerate kinase catalyses the reversible transphosphorylation:

This reaction is an integral part of the Embden-Meyerhof glycolytic scheme. The existence of the reaction was originally mentioned by Warburg & Christian (1939), and the first detailed investigations of the enzyme involved were made by Bücher (1947). The kinase has been partially purified from pea seeds by Bandurski & Axelrod (1953) and has been detected in blood by Blancher, Williams & Weiss (1955) and in liver by Ichihara & Greenberg (1957). Some statements about the behaviour of this enzyme upon fractionation of an

aqueous extract of muscle have been made by Beisenherz *et al.* (1953), but, despite some erroneous statements in the literature, he has not described the crystallization of phosphoglycerate kinase. The present paper deals with the purification and properties of the enzyme from rabbit skeletal muscle. Some differences in properties of the enzymes from yeast and from muscle are also described. A preliminary report was presented earlier by Rao & Oesper (1959).

MATERIALS AND METHODS

Abbreviation. P_i, Inorganic phosphate.

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co., St Louis, Mo., or Schwarz Bio-Research Inc., Mount Vernon, New York. All optical