- Money, R. W. & Christian, W. A. (1950). *J. Sci. Fd Agric.* **1**, 8.
- Pithawala, H. R., Savur, G. R. & Sreenivasan, A. (1948). *Arch. Biochem.* **17**, 235.
- Pollard, A. & Kieser, M. E. (1951). *J. Sci. Fd Agric.* **2**, 30.
- Pollard, A., Kieser, M. E. & Sissons, D. J. (1958). *Chem. & Ind.*, p. 952.
- Potter, A. L. & McComb, E. A. (1957). *Amer. Potato J.* **34**, 342.
- Ramaswamy, M. S. & Lamb, J. (1958). *J. Sci. Fd Agric.* **9**, 46.
- Reid, V. W. & Truelove, R. K. (1952). *Analyst*, **77**, 325.
- Saywell, L. G. & Robertson, D. P. (1932). *Plant Physiol.* **7**, 705.
- Seaton, H. L. & Gray, G. F. (1936). *J. agric. Res.* **52**, 217.
- Smith, I. (1960). In *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 1, pp. 323, 324. Ed. by Smith, I. London: Heinemann Medical Books Ltd.
- Stier, E. F., Ball, C. O. & MacIinn, W. A. (1956). *Food Tech., Champaign*, **10**, 39.
- Ulrich, R. (1957). *Annu. Rev. Pl. Physiol.* **9**, 385.
- Williams, K. T. & Bevenue, A. (1954). *J. agric. Fd Chem.* **2**, 472.
- Winsor, G. W. (1959). *Rep. Glasshouse Crops Res. Inst.* 1958, p. 29.
- Winsor, G. W., Davies, J. N. & Massey, D. M. (1962). *J. Sci. Fd Agric.* **13**, 145.
- Winsor, G. W. & Massey, D. M. (1959). *J. Sci. Fd Agric.* **10**, 304.
- Woodmansee, C. W., McClendon, J. H. & Somers, G. F. (1959). *Food Res.* **24**, 503.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

4. CONJUGATE FORMATION DURING THE METABOLISM OF [4-¹⁴C]PROGESTERONE BY FEMALE-RAT-LIVER HOMOGENATE*

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It is well established that steroids that have undergone metabolism *in vivo* are excreted in urine and bile almost entirely in the form of water-soluble conjugates. However, of the many investigations that have been made of steroid metabolism *in vitro*, there have been relatively few reports of any extensive degree of conjugate formation. When conjugate formation has been reported to occur, yields were usually low (e.g. Isselbacher & Axelrod, 1955; Dutton, 1956; Fishman & Sie, 1956; Wotiz, Ziskind, Lemon & Gut, 1956). Previous studies of progesterone metabolism by liver *in vitro* have been carried out at low tissue: steroid ratios (1000:1 or less) (Wiswell & Samuels, 1953; Taylor, 1954) because of the low sensitivity of the analytical methods then available. With the introduction of 4-¹⁴C-labelled steroids of relatively high specific activity, it has now become possible to work at much more nearly physiological levels. The metabolism of [4-¹⁴C]progesterone has therefore been investigated at tissue:steroid ratios of about 56 000:1. In preliminary experiments, not reported in the present paper, it was found that water-soluble metabolites, probably conjugates, were formed when progesterone was incubated with female-rat-liver homogenate. Therefore it was

decided to establish the optimum conditions necessary for conjugate formation during progesterone metabolism *in vitro* and to investigate the nature of the conjugates. Standard hydrolytic methods have been carried out after incubation, and the effect of adding co-factors known to be required for the formation of steroid glucuronides and steroid sulphate to preincubated and non-preincubated homogenates has been investigated. A known method of separating glucuronides and sulphates has also been applied to the conjugates before hydrolysis. Preliminary accounts of this work have been published (Cooke & Taylor, 1960, 1962).

MATERIALS AND METHODS

Nicotinamide and ATP (L. Light and Co. Ltd., Colnbrook, Bucks), NAD⁺, NADH, NADPH (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), uridine diphosphate glucose (UDP-glucose) (98–100% pure, sodium salt, pentahydrate) and uridine diphosphate glucuronic acid (UDP-glucuronic acid) (98–100% pure, ammonium salt) (Sigma Chemical Co., St Louis, Mo., U.S.A.) and potassium hydrogen saccharate (laboratory grade) (British Drug Houses Ltd., Poole, Dorset) were all used without further purification.

The methods used for the purification of solvents were as described by Taylor (1954). Assay of radioactivity was carried out as described by Taylor & Scratcherd (1961). Throughout the present paper quantities of radioactivity are expressed as counts/min. actually determined.

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[4-¹⁴C]Progesterone. [4-¹⁴C]Progesterone (pregn-4-ene-3,20-dione) (8.77 to 20.8 mc/m-mole) in benzene solution (The Radiochemical Centre, Amersham, Bucks.) was diluted with approx. 9 times its weight of twice-recrystallized non-labelled progesterone (m.p. 121–121.5°), recrystallized from hexane and stored at –15°. Stock [4-¹⁴C]progesterone solutions made up in ethanol and stored at –15° were stable for at least 6 months. Aqueous solutions of [4-¹⁴C]progesterone were made up by dissolving in water at 60° the residue obtained after evaporation of a suitable volume of ethanol from stock solution. A clear solution with a final concentration of approximately 4.5 µg. of [4-¹⁴C]progesterone/ml. was obtained. By this method clear aqueous solutions of progesterone of concentrations of up to 25 µg./ml. could be obtained. The concentration of [4-¹⁴C]progesterone in the aqueous solution was determined from extinction and radioactivity measurements. The solutions were prepared immediately before use because solutions that had been stored for a few days at room temperature exhibited a decreased extinction at 240 mµ. Radioautographs of hexane-chloroform (4:1, v/v) extracts of the stored aqueous [4-¹⁴C]progesterone run in 'system A' of Bush (1952) showed that the steroid had been partially converted into numerous compounds more polar than progesterone.

Incubation medium. The final contents of the incubation vessels were (values in parentheses denote µmoles): [4-¹⁴C]progesterone (approx. 0.0143); KH₂PO₄ buffer, pH 7.4 (120); KCl (150); MgCl₂·6H₂O (15); nicotinamide (36); 250 mg. wet wt. of liver as homogenate; the total volume was 3 ml. Incubations were carried out at 37° for 30 min.

The methods used in the preparation of the homogenate and in the extraction procedure were similar to those described by Taylor (1954).

Preparation of homogenate and incubation conditions. Female rats of the Wistar strain, usually about 4 months old, were used. The liver was quickly removed after the animal had been killed, washed in chilled 0.154M-KCl solution and dried between filter papers. It was then passed through a small Latapie mincer and the mince was weighed. After the addition of medium containing nicotinamide, homogenization was carried out in a motor-driven Potter-type homogenizer, consisting of a Teflon pestle and glass mortar. Homogenate (1 ml.) was pipetted into each of the chilled incubation vessels containing medium (1.0 ml.) and [4-¹⁴C]progesterone in aqueous solution (1.0 ml.). A stream of oxygen was passed over the surface of the incubation medium for 30 sec., and the vessels were then tightly stoppered and incubated. [In Expt. 5 some incubation vessels were gassed with N₂ instead of O₂, and in Expts. 1–4 some incubations were carried out in air (see Table 1)]. Immediately after incubation protein was precipitated with 15 ml. of acetone previously cooled to –15°. After the incubation vessels had been heated to approx. 50° they were cooled at –15° for 1 hr.

Extraction procedure. Protein was removed by filtration and acetone was evaporated in a stream of air at less than 50°. To each aqueous residue was added a total of 14 ml. of hexane-chloroform (4:1, v/v), 1 ml. of 0.7M-Na₂HPO₄ and 5 ml. of water. The aqueous solutions (pH 8.0) were then extracted three times with 10 ml. of hexane-chloroform. Centrifuging was necessary each time in order to destroy emulsions. The combined extracts were evaporated

under reduced pressure at less than 50°. The extract so obtained is referred to below as 'free steroids'. Duplicate control incubations were carried out in the absence of steroid. [4-¹⁴C]Progesterone was added after the precipitation of protein by acetone. Radioautographs of hexane-chloroform extracts from these controls gave single spots with the same *R_F* as non-labelled progesterone in 'system A' of Bush (1952).

Hydrolysis with limpet powder. The limpet powder used as a source of β-glucuronidase was that described by Taylor & Scratcherd (1961). It had an activity of 10⁶ units/g. as assayed by the method of Levvy (1952). Hydrolyses were all carried out in the presence of 0.02M-KH₂PO₄ to inhibit the steroid sulphatase present in the limpet powder (Roy, 1956a). The final concentration of saccharo-(1→4)-lactone used in the β-glucuronidase-inhibition experiments was 3.3 mM. It was prepared by boiling potassium hydrogen saccharate in aqueous solution for 30 min. (Levy, 1952) and adjusting the pH to 4.6.

After the extraction of 'free steroids' 0.1 vol. of 2M-acetate buffer, pH 4.6, containing 1 mg. of limpet powder that had been homogenized in the buffer, was added to each aqueous solution. Vessels were then incubated at 46° for 16 hr. After adjustment of the pH to 7–8 with N-NaOH, the solutions were extracted three times with hexane-chloroform.

Hydrolysis with cold acid. The aqueous solutions (approx. 10 ml.) were then subjected to solvolysis with H₂SO₄ (final concn. approx. 2.2N) and ether (Burstein & Lieberman, 1958). The vessels were shaken for 1 min. every 2 hr. throughout a working day for 72 hr. The ether was removed by evaporation in a stream of air at room temperature and the aqueous phase was extracted with hexane-chloroform without further adjustment of pH. Extracts were washed with 3 ml. of phosphate buffer, pH 8.0, and the washings were added to the remaining aqueous solution.

Hydrolysis with hot acid. The residual aqueous solutions, the pH of which was less than 1, were heated in a boiling-water bath for 1 hr. and extracted with hexane-chloroform, and the extracts were washed with phosphate buffer, pH 8.0. The washings were added to the remaining aqueous solution.

Extraction at pH 1. Finally, the aqueous solutions were re-extracted with hexane-chloroform without adjustment of the pH. These extracts were not washed.

Separation of sulphates from glucuronides (Expts. 13 and 14). After the free steroids had been extracted with hexane-chloroform at pH 7–8, the aqueous solutions were 50% saturated with (NH₄)₂SO₄ and extracted with ether-ethanol (Edwards, Kellie & Wade, 1953). After evaporation of the ether-ethanol, aqueous solutions (10 ml.) of the extracts were 50% saturated with (NH₄)₂SO₄, made 1N with respect to ammonia by the addition of aq. ammonia (sp.gr. 0.88) and then extracted twice with 3 vol. of ethyl acetate (Bush & Gale, 1960). The hydrolytic methods were then applied to the two fractions.

RESULTS

Preliminary experiments showed that conjugation consistently occurred when [4-¹⁴C]progesterone was incubated under oxygen with female-rat-liver homogenate at tissue:steroid ratios of

approx. 56 000:1. These conditions have been used throughout the experiments described below unless otherwise stated. Recovery of radioactivity from controls (carried out in duplicate in each experiment) was $98.0 \pm 3.9\%$ (S.D.). The double values given in the tables are from duplicate incubations.

Table 1 shows the effect of carrying out incubations under various gas phases on the amount of conjugates formed. In each experiment less radioactivity was recovered as 'free steroids' in oxygen than in air or nitrogen, indicating that more conjugation takes place in oxygen. Another striking feature is the great variability in intrinsic conjugate formation between different livers, irrespective of the gas phase: approx. 12–40% in oxygen and 16–85% in air. This stimulating effect of oxygen was very marked in Expts. 3–5. The addition of NAD⁺ or UDP-glucose or both to incubation media further increased the amount of conjugation when oxygen was used as gaseous phase (Expts. 4 and 5, Table 3), but no increase in conjugation was found with air as gaseous phase in these experiments. However, differences between the effects of oxygen and air were not so marked in Expts. 1 and 2 (Table 1), indicating that considerable variation occurs in the amount of conjugate formation with air as the gas phase.

Table 2 shows that the addition of a specific β -glucuronidase inhibitor, saccharo-(1 $\rightarrow$ 4)-lactone (Levy, 1952), inhibits the release of radioactivity by limpet powder. No radioactivity was released when no enzyme or boiled enzyme solution was added. The total amount of radioactivity recovered was approximately the same in each experiment after hydrolysis with hot acid. In Expt. 12 incubations were carried out in the presence of NAD⁺ and UDP-glucose, as it was thought that glucuronide formation would be increased (see below). A high concentration of phosphate was present in all hydrolyses with limpet powder to inhibit steroid sulphotase (Roy, 1956*a*). Limpet-powder steroid sulphotase is also highly specific for 3β -hydroxy-5 α - and 3β -hydroxy- Δ^5 -steroid sulphates (Roy, 1956*a*). It is therefore unlikely that hydrolysis of steroid sulphates by limpet powder would occur under the conditions used in these experiments. Thus it can be concluded that conjugates hydrolysed by limpet powder were most probably glucuronides.

UDP-glucuronic acid is the immediate donor of glucuronic acid in the formation of glucuronides (Dutton & Storey, 1954; Storey & Dutton, 1955), and the UDP-glucuronic acid itself is formed by oxidation of UDP-glucose in the presence of NAD⁺. The effect of adding NAD⁺ and UDP-glucose to incubations was therefore studied to see whether or not glucuronide formation could be increased (Table 3). In Expts. 6 and 7 total conjugation was

greatly increased when NAD⁺ or NAD⁺ and UDP-glucose were added. However, glucuronide formation was only increased by an average of 7.4 and 6.7% in Expts. 6 and 7 respectively when NAD⁺ and UDP-glucose were added, and by slightly less when either UDP-glucose or NAD⁺ was added separately. In Expts. 4 and 5 there was no increase in glucuronide formation on the addition of UDP-

Table 1. *Effect of air, oxygen and nitrogen on the formation of conjugates of [4-¹⁴C]progesterone by female-rat-liver homogenate*

Details of the incubation conditions are given in the Materials and Methods section.

Gas phase... Expt. no.	Radioactivity recovered as 'free steroids' (% of total counts/min./incubation)		
	O ₂	Air	N ₂
1	10.0	19.0	—
	12.2	23.7	—
2	12.5	16.3	—
	13.6	16.6	—
3	41.4	82.0	—
	41.1	76.0	—
4	50.0	80.5	—
	37.8	91.0	—
5	11.3	—	72.2
	13.3	—	73.7

Table 2. *Effect of saccharate on the release of radioactivity by limpet powder from aqueous solutions of [4-¹⁴C]progesterone metabolites*

[4-¹⁴C]Progesterone was incubated with female-rat-liver homogenate as described in the Materials and Methods section, with either air (Expt. 11) or O₂ (Expt. 12) as the gas phase. The aqueous solutions of [4-¹⁴C]progesterone metabolites were then investigated by incubation with (Expt. 11): limpet powder (6 mg. in 0.2M-acetate buffer, pH 4.6, when added), saccharate (final concn. 3.3 mM, when added) and KH₂PO₄ (final concn. 0.02M) at 37° for 24 hr.; or with (Expt. 12): limpet powder (200 μ g. in 0.2M-acetate buffer, pH 4.6, when added), saccharate (final concn. 3.3 mM, when added) and KH₂PO₄ (final concn. 0.02M) at 37° for 2 hr.

Additions	Radioactivity recovered (% of total counts/min. of [4- ¹⁴ C]progesterone originally incubated with female-rat-liver homogenate)	
	Expt. 11	Expt. 12
Limpet powder	10.7	10.5
	16.5	13.1
Limpet powder + saccharate	1.2	1.0
	0	0
Boiled limpet-powder solution	—	0.9
	0	1.3
None	0	—
	0	—

glucose and NAD^+ . This suggests that female-rat-liver homogenate has a low and variable concentration of UDP-glucose dehydrogenase or UDP-glucuronate glucuronyltransferase, or that all the steroids capable of forming glucuronides had been formed. The fact that glucuronide formation was not increased by NAD^+ and UDP-glucose in Expts. 4 and 5, in which very small amounts of glucuronide were formed, supports the suggestion that female-rat-liver homogenate has a low concentration of enzymes responsible for glucuronide formation. Results obtained in these and other experiments indicate that the amount of glucuronide formation during the metabolism of $[4\text{-}^{14}\text{C}]$ -progesterone in female-rat-liver homogenate is extremely variable. In each experiment there was

a large increase in radioactivity released by cold acid when NAD^+ and UDP-glucose were added. For example in Expts. 4, 5 and 7 there was an average increase of 17.6, 17.4 and 19.1% respectively when NAD^+ and UDP-glucose were added together, compared with average increases of 8.3, 9.9 and 2.4% with NAD^+ and 5.7, 2.8 and 3.0% with UDP-glucose alone (Table 3). This suggests either that glucuronides not hydrolysable by limpet powder were formed or that the addition of NAD^+ and UDP-glucose caused increased formation of conjugates other than glucuronides.

As the results of these investigations of the effects of NAD^+ and UDP-glucose on glucuronide formation in normal homogenate were inconclusive, experiments were carried out with homogenates

Table 3. *Effect of nicotinamide-adenine dinucleotide and uridine diphosphate glucose on the formation of conjugates of $[4\text{-}^{14}\text{C}]$ progesterone by female-rat-liver homogenate*

Details of the incubation conditions are given in the Materials and Methods section: NAD^+ ($0.74\text{ }\mu\text{mole}$) and UDP-glucose ($0.32\text{ }\mu\text{mole}$) were added as indicated.

Expt. no.	Additions	Radioactivity recovered (% of total counts/min./incubation) after hydrolysis					Total radio-activity recovered
		As 'free steroids'	Limpet-powder hydrolysis	Cold-acid hydrolysis	Hot-acid hydrolysis	Extraction at pH 1	
4	None	50.0	5.0	29.3	5.3	2.9	92.5
		37.8	6.6	18.6	19.2	7.8	89.9
	NAD^+	41.1	8.6	33.0	9.9	8.1	100.6
		31.2	12.4	31.6	5.9	4.6	85.5
	$\text{NAD}^+ + \text{UDP-glucose}$	24.3	5.4	47.1	10.1	3.7	90.5
		26.9	4.5	36.1	16.0	6.3	89.8
	UDP-glucose	33.5	4.8	15.7	28.5	12.2	94.7
		29.6	4.8	20.8	11.4	17.6	84.2
5	None	11.3	23.1	12.2	16.3	6.6	69.7
		13.3	26.6	16.0	11.6	6.5	74.1
	NAD^+	12.9	23.8	24.4	14.1	6.7	82.1
		11.1	20.4	23.7	12.5	9.0	76.7
	$\text{NAD}^+ + \text{UDP-glucose}$	13.1	21.8	37.7	6.3	5.9	83.8
		10.5	19.2	25.5	10.2	8.9	74.3
	UDP-glucose	12.0	25.1	22.2	6.0	8.7	74.0
		14.8	24.3	11.7	11.8	19.5	82.1
6	None	60.9	14.8	—	7.1	4.1	86.9
		55.9	10.4	—	6.1	8.9	81.3
	NAD^+	20.7	15.3	—	14.9	18.4	69.2
		15.5	15.3	—	12.8	21.0	64.7
	$\text{NAD}^+ + \text{UDP-glucose}$	11.3	17.9	—	14.0	16.2	59.4
		12.4	22.2	—	15.7	16.2	66.4
	UDP-glucose	63.7	17.0	—	8.4	7.5	96.7
		42.1	20.3	—	6.5	9.5	78.4
7	None	51.5	18.4	6.8	5.9	5.9	88.5
		44.4	21.8	13.8	5.0	3.1	88.1
	NAD^+	31.0	22.4	12.7	12.0	6.9	84.9
		26.8	24.1	12.7	11.5	9.5	84.5
	$\text{NAD}^+ + \text{UDP-glucose}$	13.7	27.2	28.8	6.6	3.7	80.0
		11.6	26.1	29.8	5.2	4.1	76.8
	UDP-glucose	23.7	25.9	13.4	12.1	5.7	80.8
		17.1	25.5	13.2	10.6	11.0	77.5

Table 4. *Effect of adenosine triphosphate and uridine diphosphate glucuronic acid on the formation of conjugates of [4-¹⁴C]progesterone by preincubated female-rat-liver homogenate*

The homogenate was preincubated for 5 min. at 37° before the addition of [4-¹⁴C]progesterone. Details of the incubation conditions are given in the Materials and Methods section. Expt. 8: NADPH (0.266 μ mole) and UDP-glucuronic acid (0.279 μ mole) (where indicated) were added. Expt. 9: NADPH (0.266 μ mole); UDP-glucuronic acid (0.336 μ mole) (where indicated); ATP (6.0 μ moles) (where indicated) and K₂SO₄ (25 μ moles) were added. Expt. 10: NADPH (0.532 μ mole); UDP-glucuronic acid (0.672 μ mole) (where indicated); ATP (6.0 μ moles) (where indicated) and K₂SO₄ (25 μ moles) were added. In Expt. 10 UDP-glucuronic acid was added 5 min. after the incubation of preincubated homogenate with [4-¹⁴C]progesterone had started.

		Radioactivity recovered (% of total counts/min./incubation) after hydrolysis					Total radio-activity recovered
Expt. no.	Additions	As 'free steroids'	Limpet powder hydrolysis	Cold-acid hydrolysis	Hot-acid hydrolysis	Extraction at pH 1	
8	None	72.7	11.3	1.1	2.6	1.3	89.0
		71.7	12.4	4.4	2.3	0.8	91.6
	UDP-glucuronic acid	59.4	27.0	5.5	2.4	0.5	94.8
		58.5	27.8	5.2	2.1	0.7	94.3
9	None*	39.6	7.0	22.5	0	0	69.1
		41.5	6.4	21.5	0	0	69.4
	None	97.4	0	0	0	0	97.4
		90.6	0	0	0	0	90.6
	UDP-glucuronic acid	100.0	0	0	0	0	100.0
		91.5	0	0	0	0	91.5
	ATP	57.8	7.2	11.4	0	0	76.4
		57.2	7.5	11.7	0	0	76.4
	ATP + UDP-glucuronic acid	65.6	5.0	9.9	0	0	80.5
		63.6	5.8	7.2	0	0	76.6
	None*	14.6	24.4	18.3	9.3	0	66.6
		18.4	30.0	19.0	9.9	0	77.3
10	None	78.5	7.9	4.6	3.0	0	94.0
		83.7	5.1	4.7	3.2	0	96.7
	UDP-glucuronic acid	59.8	23.6	6.4	3.5	0	93.3
		59.0	20.2	6.8	3.1	0	89.1
	ATP	53.8	10.4	13.6	11.0	0	88.8
		54.4	10.0	10.1	11.5	0	86.0
	ATP + UDP-glucuronic acid	43.4	19.0	9.8	8.5	0	80.7
		36.0	20.5	13.4	9.6	0	79.5

* Incubations carried out with non-preincubated homogenate.

preincubated in order to destroy endogenous conjugating systems (Table 4). When UDP-glucuronic acid was added with [4-¹⁴C]progesterone to preincubated homogenate there was an increase in glucuronide formation in Expt. 8 but not in Expt. 9. Increased glucuronide formation with UDP-glucuronic acid could only be consistently demonstrated (three times in three experiments) if homogenates were preincubated with nicotinamide (24 μ moles) for 5 min. at 37°, and if UDP-glucuronic acid was added 5 min. after incubation with [4-¹⁴C]progesterone had started (Expt. 10, Table 4). Increased glucuronide formation did not occur with added UDP-glucuronic acid if homogenates were preincubated without nicotinamide (B. A. Cooke & W. Taylor, unpublished work).

ATP, SO₄²⁻ ions and Mg²⁺ ions, which are necessary for sulphate formation (De Meio &

Tkacz, 1952; De Meio, Wizerkaniuk & Fabiani, 1953), were added to preincubated homogenate. Results from four experiments showed that these substances increased sulphate formation by 12.8, 11.6, 9.4 and 11.9%, compared with 5.1, 0, 0 and 4.7% (average results from duplicate incubations) when ATP was omitted (e.g. Expts. 9 and 10, Table 4). There was also a corresponding increase in substances hydrolysable by hot acid in Expt. 10 (Table 4), suggesting either that hydrolysis with cold acid does not completely hydrolyse the steroid sulphates present or that ATP increased the formation of conjugates other than sulphates.

A known method of separating sulphates and glucuronides (Bush & Gale, 1960) was applied to the conjugates (Scheme 1). The latter were extracted with ether-ethanol (3:1, v/v) from aqueous solutions 50% saturated with ammonium sulphate

Scheme 1. Separation of sulphates from glucuronides in aqueous solutions of [4-¹⁴C]progesterone metabolites. The values given in parentheses, which are percentages of the total amount of radioactivity in the original aqueous solution, are average values from duplicate determinations from two separate experiments (13 and 14 respectively). The conjugates were extracted from aqueous solution (Edwards *et al.* 1953) and then separated into glucuronides and sulphates by the method of Bush & Gale (1960).

after the extraction of 'free steroids' (Edwards *et al.* 1953). Most of the radioactivity was recovered: a total average of 78.8 and 87.3 % of the initial radioactivity incubated in Expts. 13 and 14 respectively. Sulphates were extracted by ethyl acetate from an aqueous solution of the conjugates 50 % saturated with ammonium sulphate. Radioactive compounds extracted by ethyl acetate were hydrolysed mainly by cold acid whereas the other conjugates left in the aqueous phase were hydrolysed mainly by limpet-powder β -glucuronidase, indicating that sulphates had been almost completely separated from glucuronides. However, not all of the conjugates were hydrolysed (Scheme 1), suggesting either that the methods used were inadequate for complete hydrolysis or that other conjugates not hydrolysable by the methods used were present.

DISCUSSION

It has been shown that under the conditions described conjugate formation occurs during the metabolism of [4-¹⁴C]progesterone by female-rat-liver homogenate at tissue:steroid ratios of 56 000:1. Failure to detect conjugation during previous studies of steroid metabolism *in vitro* may be attributed to a number of factors. The addition of ethanol or propylene glycol partially inhibits conjugate formation during [4-¹⁴C]progesterone metabolism (Cooke & Taylor, 1961), so this must be taken into account in studies where these alcohols have been added to incubation media. The

variable effect of air as gaseous phase and the lability of endogenous conjugating co-factors must also be considered. At tissue:steroid ratios of approximately 1000:1 with female-rat-liver homogenate little or no conjugation occurred (B. A. Cooke & W. Taylor, unpublished work), thus explaining the lack of conjugation found in previous studies carried out at this tissue:steroid ratio. Stevens, Berliner & Dougherty (1961) have demonstrated that male-mouse-liver mince is capable of conjugating [4-¹⁴C]cortisol (11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione), corticosterone (11 β ,21-dihydroxypregn-4-ene-3,20-dione) and 3 α -hydroxy-5 α -pregnane-11,20-dione (18.8, 25.4 and 42.3 % conjugated respectively) at tissue:steroid ratios of approx. 60 000:1. Corticosterone and 3 α -hydroxy-5 α -pregnane-11,20-dione conjugates were hydrolysed by a bacterial β -glucuronidase (54.6 and 27.2 % of the conjugates hydrolysed respectively). Corticosterone and 3 α -hydroxy-5 α -pregnane-11,20-dione, but not cortisol, were also conjugated by male-mouse-kidney mince (4.96 and 31.8 % respectively). The β -glucuronidase hydrolysed 29.4 % of the 3 α -hydroxy-5 α -pregnane-11,20-dione conjugate but hydrolysis of the corticosterone conjugates by this enzyme was not detectable.

Evidence obtained in the present study suggests that the main conjugates formed during [4-¹⁴C]progesterone metabolism by female-rat-liver homogenate were most probably glucuronides and sulphates. These are also the main conjugates formed during steroid metabolism *in vivo*. There have been a number of reports of the formation of glucuronides

and sulphates by known steroids *in vitro* by different species and tissues. Fishman & Sie (1956) found that the livers of the hamster, mouse, rabbit, guinea pig, dog (most efficient) and rat were all capable of forming testosterone glucuronide *in vitro*. Depending on the species and amount of liver used, 1–20 % of testosterone was converted into its glucuronide. Twenty-two steroids (mainly derivatives of testosterone) were capable of forming glucuronides in rat liver but few had as high a rate of formation as testosterone. Most of the steroid glucuronides formed still retained ring A intact. Conjugation *in vitro* of the naturally occurring oestrogens (Crépy, 1947) and of stilboestrol in the presence of surviving liver tissue of the rat (Zimmerberg, 1946), guinea pig and rabbit (Crépy, 1947) has been demonstrated. Some evidence was obtained for the sulphate and glucuronide nature of the conjugates. Isselbacher & Axelrod (1955) found that a tetrahydrocortisone (3 α ,17 α ,21-trihydroxy-5 β -pregnane-11,20-dione) conjugate was formed when that steroid (0.2 μ mole) was incubated with guinea-pig-liver microsomes in the presence of UDP-glucuronic acid. In the one experiment reported only 0.042 μ mole of tetrahydrocortisone was recovered after hydrolysis with β -glucuronidase. Newcomer & Heninger (1960) found that microsomal fractions of avian adrenal gland will conjugate cortisol, tetrahydrocortisone and tetrahydrocortisol (3 α ,11 β ,17 α ,21-tetrahydroxy-5 β -pregnan-20-one), but not cortisone, with glucuronic acid in the presence of excess of UDP-glucuronic acid. Wotiz *et al.* (1956) have given evidence for the formation of a water-soluble conjugate of testosterone during the incubation of steroid with surviving human prostatic tissue. The conjugate formed was hydrolysable by β -glucuronidase (Ketodase) but the amounts detected were very small (0.18 and 0.09 % of the testosterone incubated). Dutton (1956) reported the formation of the glucuronides of 5 β -pregnane-3 α ,20 α -diol, 3 α -hydroxy-5 α -androstan-17-one, 5 α -pregnane-3 β ,20 β -diol and 4,4'-dihydroxy- α β -diethylstilbene by mouse liver. Only slight synthesis was found, 0.8–3.5 μ g. of glucuronic acid being conjugated/700 mg. wet wt. of liver.

Enzyme systems responsible for formation of sulphate esters, including steroid sulphates, have been studied in some detail (see review by Roy, 1960). The specificity of the steroid alcohol sulphokinase is apparently low, since microsome-free liver preparations form a wide range of steroid sulphates including 3 α - and 3 β -sulphates of 5 α -, 5 β - and Δ^5 -steroids and of 17 α - and 17 β -hydroxy steroids (Roy, 1956*b*; Schneider & Lewbart, 1956). De Meio, Lewycka, Wizerkaniuk & Salciunas (1958) synthesized the sulphates of dehydroisoandrosterone (3 β -hydroxyandrost-5-en-17-one), testo-

sterone, oestrone [3-hydroxyoestra-1,3,5(10)-trien-17-one] and oestradiol-17 β [oestra-1,3,5(10)-triene-3,17 β -diol], with rat-liver and ox-liver microsome-free preparations.

It is not known if the method of hydrolysis with cold acid (Burstein & Lieberman, 1958) used in these experiments quantitatively hydrolysed the steroid sulphates present. Roy (1956*a*) found that little hydrolysis of 3-hydroxy-5 α - and -5 β -pregnan-20-one sulphates occurred at pH 1 even after heating at 100° for 2 hr. Recovery of more radioactivity after hydrolysis with hot acid and extraction at pH 1 indicates that the steroid sulphates or glucuronides may have been incompletely hydrolysed by the cold acid and β -glucuronidase hydrolyses in the present investigation. Modifications in steroid structure may have taken place as the effects of these hydrolytic methods on small amounts of steroids are not known. The possibility of the presence of other types of conjugates must also be considered. Other steroid conjugates that could occur include phosphates. Oertel & Eik-Nes (1958) found that 70 % of the plasma dehydroisoandrosterone obtained after the administration of 3 β -hydroxypregn-5-en-20-one and 3 β ,17 α -dihydroxypregn-5-en-20-one to dogs was hydrolysed by alkaline phosphatase. Dehydroisoandrosterone combined with phosphate has been obtained from plasma from peripheral human blood (Oertel & Eik-Nes, 1959). Oertel & Kaiser (1962) have provided evidence for the existence in plasma of complexes containing 17-oxo steroid, sulphate, phosphate and lipid. However, such phosphates would have been hydrolysed under the conditions used for hydrolysis with limpet powder in the present investigation, and, as it was shown that hydrolysis with limpet powder was almost completely inhibited by saccharo-(1 $\rightarrow$ 4)-lactone, it is unlikely that phosphates are formed during [4-¹⁴C]-progesterone metabolism.

The demonstration of extensive conjugation in the present investigation suggests that metabolism of [4-¹⁴C]progesterone was taking place under conditions which are more physiological than those used in previous investigations.

SUMMARY

1. Water-soluble conjugates are formed when [4-¹⁴C]progesterone is incubated with female-rat-liver homogenate at tissue:steroid ratios of approx. 56 000:1.

2. Conjugate formation was increased by aerobic conditions but partially inhibited by anaerobic conditions.

3. Evidence is presented that the conjugates formed were probably mainly glucuronides and sulphates.

4. Possible reasons for the absence of conjugation in other studies of steroid metabolism *in vitro* are discussed.

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REFERENCES

- Burstein, S. & Lieberman, S. (1958). *J. biol. Chem.* **233**, 331.
- Bush, I. E. (1952). *Biochem. J.* **50**, 370.
- Bush, I. E. & Gale, M. M. (1960). *Biochem. J.* **76**, 10P.
- Cooke, B. A. & Taylor, W. (1960). *Biochem. J.* **77**, 22P.
- Cooke, B. A. & Taylor, W. (1961). *Biochem. J.* **81**, 12P.
- Cooke, B. A. & Taylor, W. (1962). *Biochem. J.* **82**, 46P.
- Crépy, O. (1947). *Arch. Sci. Physiol.* **1**, 427.
- De Meio, R. H., Lewycka, C., Wizerkaniuk, M. & Salciunas, O. (1958). *Biochem. J.* **68**, 1.
- De Meio, R. H. & Tkacz, L. (1952). *J. biol. Chem.* **195**, 175.
- De Meio, R. H., Wizerkaniuk, M. & Fabiani, E. (1953). *J. biol. Chem.* **203**, 257.
- Dutton, G. J. (1956). *Biochem. J.* **64**, 693.
- Dutton, G. J. & Storey, I. D. E. (1954). *Biochem. J.* **57**, 275.
- Edwards, R. W. H., Kellie, A. E. & Wade, A. P. (1953). *Mem. Soc. Endocrin.* **2**, 53.
- Fishman, W. H. & Sie, H. G. (1956). *J. biol. Chem.* **218**, 335.
- Isselbacher, K. J. & Axelrod, J. (1955). *J. Amer. chem. Soc.* **77**, 1070.
- Levy, G. A. (1952). *Biochem. J.* **52**, 464.
- Newcomer, W. S. & Heninger, R. W. (1960). *Proc. Soc. exp. Biol., N.Y.*, **105**, 32.
- Oertel, G. W. & Eik-Nes, K. B. (1958). *Fed. Proc.* **17**, 400.
- Oertel, G. W. & Eik-Nes, K. B. (1959). *Analyt. Chem.* **31**, 98.
- Oertel, G. W. & Kaiser, E. (1962). *International Congress on Hormonal Steroids, Milan; Excerpta Medica*, no. 51, p. 215.
- Roy, A. B. (1956a). *Biochem. J.* **62**, 41.
- Roy, A. B. (1956b). *Biochem. J.* **63**, 294.
- Roy, A. B. (1960). *Advanc. Enzymol.* **22**, 205.
- Schneider, J. J. & Lewbart, M. L. (1956). *J. biol. Chem.* **222**, 787.
- Stevens, W., Berliner, D. L. & Dougherty, T. F. (1961). *Endocrinology*, **68**, 875.
- Storey, I. D. E. & Dutton, G. J. (1955). *Biochem. J.* **59**, 279.
- Taylor, W. (1954). *Biochem. J.* **56**, 463.
- Taylor, W. (1956). *Biochem. J.* **62**, 332.
- Taylor, W. & Scratcherd, T. (1961). *Biochem. J.* **81**, 398.
- Wiswell, J. G. & Samuels, L. T. (1953). *J. biol. Chem.* **201**, 155.
- Wotiz, W. H., Ziskind, B. S., Lemon, H. M. & Gut, M. (1956). *Biochim. biophys. Acta*, **22**, 266.
- Zimmerberg, H. (1946). *J. biol. Chem.* **166**, 97.

Biochem. J. (1963) **86**, 372

The Effects of Anoxia on Metabolism in the Adult Housefly, *Musca domestica*

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In contrast with most mammals many insects are able to remain alive under completely anoxic conditions for several hours and in some cases even for days (Brand, 1946). Changes in the concentrations of the intermediates of basal metabolism under conditions of anoxia are therefore of considerable interest, for in the insect these changes are, presumably, reversible and of such a nature that they do not have any serious deleterious effect on the insect's metabolism. The effect of anoxia on the concentrations of glycolytic end products in insects has been investigated by various authors. Gilmour (1941) showed that the amount of lactate accumulating in larvae of *Tenebrio molitor* under anoxic conditions was not sufficient to account for the glycogen and sugar utilized. Barron & Tahmisian (1948) and Humphrey (1949) found that

there was only a small accumulation of lactate, and that pyruvate did not accumulate at all in their preparations of cockroach-leg muscle unless glycogen was added. Chefurka (1958) observed that the sum of pyruvate, lactate and α -glycerophosphate accumulating in cockroach-leg muscle under anoxic conditions was less than the amount of hexose diphosphate utilized. These observations suggested that some end product, in addition to those mentioned above, was accumulating in these insect preparations. It has been suggested (Price, 1961b) that this end product might be α -alanine, and in the present work evidence for this suggestion is examined and the effects of anoxia on various amino acids, on citric acid-cycle intermediates and on the incorporation of amino acids into protein are investigated.