discussed, together with the part played by penicillinase in the production of resistance to ampicillin.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

5. INHIBITION OF CONJUGATE FORMATION BY ETHANOL AND PROPYLENE GLYCOL DURING THE METABOLISM OF [4-¹⁴C]PROGESTERONE BY RAT LIVER *IN VITRO**

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Cooke & Taylor (1963) presented evidence that water-soluble conjugates (mainly glucuronides and sulphates) are formed when [4-¹⁴C]progesterone is incubated with female-rat-liver homogenate at tissue:steroid ratios of 56 000:1. During the investigations it was found that ethanol and propylene glycol inhibit this conjugate formation. These alcohols have been used extensively in studies *in vitro* to dissolve steroids before addition to incubation media because of the low solubility of steroids in water [e.g. Ofner (1955), Wiest (1959) and Dominguez, Acevedo, Huseby & Samuels (1960) used ethanol; Taylor (1954), Wotiz & Lemon

(1954) and Sie & Fishman (1957) used propylene glycol]. Therefore it was decided to investigate the degree and possible mechanisms of this inhibition of conjugate formation by the alcohols. A preliminary report of this work has been published (Cooke & Taylor, 1961).

MATERIALS AND METHODS

Ethanol (The Distillers Co. Ltd.) that had stood over sodium hydroxide pellets overnight was distilled and then redistilled in all-glass apparatus through a 14 in. column packed with Raschig rings. Propylene glycol (propane-1,2-diol) (Laboratory Reagent, British Drug Houses Ltd.) was used without further purification. Assay of radioactivity was carried out as described by Taylor & Scratcherd (1961).

* Part 4: Cooke & Taylor (1963).

† Present address: British Drug Houses Ltd., Graham Street, London, N. 1.

Approx. 4 μ g. of [4-¹⁴C]progesterone was incubated at 37° for 0.5 hr. with 250 mg. wet wt. of liver as homogenate in a medium containing phosphate buffer (pH 7.4), KCl, MgCl₂ and nicotinamide; the total volume was 3 ml. At the end of the incubation period proteins were precipitated by acetone and removed by filtration. The acetone was evaporated in a stream of air at below 50°, and the aqueous residue was extracted with *n*-hexane-chloroform (9:1, v/v) to give the 'free steroid' fraction. The residual aqueous phase was hydrolysed successively by limpet powder, 2N-H₂SO₄-ether (1:1, v/v) and finally by boiling the solution at a pH of less than 1. Extraction after each hydrolytic step gave the limpet-powder, cold-acid and hot-acid fractions. All extracts were washed with alkali and so each fraction contained only neutral metabolites. Full details of these methods have been described by Taylor (1954) and by Cooke & Taylor (1963).

Throughout the present paper the concentrations of added alcohols are expressed as m-moles/3 ml. of incubation medium.

Celite partition chromatography. Celite columns (10 cm. long $\times$ 1.0 cm. diam.) were prepared as described by Butt, Morris, Morris & Williams (1951) and by Taylor (1954), with methanol-water (17:3, v/v; 1 ml./g. of Celite) as stationary phase and hexane as mobile phase. 'Free steroids' were acetylated with 0.2 ml. of pyridine and 0.1 ml. of acetic anhydride at room temperature for 16 hr. after the addition of 250 μ g. of unlabelled progesterone to prevent destruction of the [4-¹⁴C]progesterone. Progesterone was eluted from the columns by 18–34 ml. of the mobile phase. This fraction was collected and its radioactivity assayed.

RESULTS

Table 1 shows the effects of ethanol and propylene glycol on conjugate formation when these alcohols are added to the incubation medium at concentrations close to those normally used in experiments *in vitro*; thus 0.5 m-mole is approximately equivalent to 0.04 ml. of propylene glycol and 0.02 ml. of ethanol. The results confirm those of Cooke & Taylor (1963) in that rather more conjugation occurs in oxygen (73.9–89.8%; Expts. 3, 4, 7, 8 and 9) than in air (64.4–83.3%; Expts. 1, 2, 5 and 6). In those experiments (1 and 2) in which ethanol was added to incubation media with air as the gas phase different degrees of inhibition were found, especially at lower ethanol concentrations: e.g., in Expt. 1, 0.17 m-mole of ethanol decreased conjugation from 64.4 to 62.8%, whereas in Expt. 2 the corresponding values were 71.9 and 59.2%. It is not possible to decide whether this was due to the greater amount of conjugation occurring in the absence of ethanol in Expt. 2 or to some other variations in the metabolic activity of the two livers. Inhibition of conjugation by ethanol was less marked when incubation was carried out under oxygen (cf. Expts. 1 and 2, and 3 and 4). Although the results with propylene glycol were more variable, a general tendency towards decrease in conjugation with increase in concentration of propylene

glycol occurred both in air (Expts. 5 and 6) and in oxygen (Expts. 7–9). As with ethanol, inhibition of conjugation was less in oxygen than in air (cf. Expts. 5 and 6, and 7–9). Further, both in air and in oxygen, propylene glycol had a smaller inhibitory effect on conjugation than had ethanol (cf. Expts 1–4 and 5–9). The amount of [4-¹⁴C]progesterone recoverable after incubation in air was not markedly affected by the presence of relatively high concentrations of ethanol or of propylene glycol in the incubation medium. Thus the amount of 'free steroids' ranged from 16.7 to 35.6% in the absence of alcohols and from 43.4 to 82.6% in their presence, whereas the amount of progesterone remaining unmetabolized increased relatively slightly from about 1 to 6.6%. Table 2 shows the effect of the alcohols on the amounts of radioactivity extractable after successive hydrolytic procedures when incubations were carried out in oxygen. With increasing alcohol concentration the amount of radioactivity extractable before hydrolysis (i.e. 'free steroid') increased: conjugate formation decreased from 89.3 to 29.6% in the presence of ethanol and from 89.3 to 72.8% in the presence of propylene glycol. With ethanol present, as the amount of total conjugation decreased the amount of radioactivity extractable after limpet-powder hydrolysis (probably glucuronides; Cooke & Taylor, 1963) also decreased, but the ratio of the radioactivity of this glucuronide fraction to that of total conjugates remained fairly constant (at 0.17–0.21) after an initial fall from 0.29 to 0.21. Cold-acid-hydrolysable substances (probably sulphates), on the other hand, increased from a ratio of 0.27 to 0.54, then decreased slightly to (0.47 and 0.50) as the alcohol concentration increased. The ratio of hot-acid-hydrolysed substances remained fairly constant (at 0.11–0.19). However, as the alcohol concentration increased there was a definite increase (from 72.5 to 93.3%) in the total amount of radioactivity recovered. The nature of the substances that remain water-soluble after all the hydrolytic procedures has been discussed by Taylor & Scratcherd (1961) and by Cooke & Taylor (1963), and the increase in the total recovery of radioactivity indicates that there was a corresponding decrease in the formation of these water-soluble metabolites. Similar results were obtained on the addition of propylene glycol, except that there was no stimulation of formation of substances hydrolysed by cold acid.

DISCUSSION

Ethanol and propylene glycol inhibit conjugate formation during metabolism of progesterone by female-rat-liver homogenate, and these alcohols themselves form conjugates. Kamil, Smith & Williams (1953*a, b*) isolated ethanol glucosiduronic

Table 1. *Effect of ethanol and propylene glycol on conjugate formation during metabolism of [4-¹⁴C]-progesterone by female-rat-liver homogenate in air and in oxygen*

Approx. 4 μ g. of [4-¹⁴C]progesterone was incubated for 30 min. at 37° with 250 mg. wet wt. of liver as homogenate in a phosphate-buffered medium (final vol. 3 ml.). Experimental details are given by Taylor (1954) and by Cooke & Taylor (1963) (see the Materials and Methods section). Alcohol concentrations are given as m-moles/3 ml.

Alcohol	Expt. no.	Gas phase	Alcohol added to incubation medium (m-moles/3 ml.)	Conjugation (%)	Unmetabolized progesterone (%)
Ethanol	1	Air	0	64.4	1.4
			0.17	62.8	—
			0.86	28.2	—
			1.72	17.4	2.8
	2	Air	0	71.9	0.7
			0.17	59.2	—
			0.86	66.3	—
			1.20	56.3	—
	3	O ₂	1.72	31.4	2.3
			0	73.9	—
			0.86	68.0	—
			1.72	47.3	—
	4	O ₂	0	89.8	—
			0.86	76.4	—
			1.40	69.0	—
			2.06	50.3	—
			2.58	29.6	—
Propylene glycol	5	Air	0	78.7	0.7
			0.68	63.1	—
			1.36	65.5	3.2
			2.05	55.1	—
	6	Air	0	83.3	1.6
			0.55	77.0	—
			0.68	77.0	—
			0.82	72.4	—
	7	O ₂	1.09	52.8	—
			1.23	60.4	—
			1.36	65.6	—
			2.05	56.6	6.6
	8	O ₂	0	73.9	—
			0.82	62.8	—
			1.64	71.0	—
	9	O ₂	0	89.3	—
			0.82	79.8	—
			1.23	79.8	—
			1.64	76.8	—
None (control)	9	O ₂	2.32	72.8	—
			0	88.9	—
			0.68	63.1	—
			1.36	65.5	—
			2.05	55.1	—
			0	0	98.5

acid from rabbit urine after the oral administration of ethanol, although the yield was usually less than 1%. Evidence has been obtained for the formation of primary aliphatic [³⁵S]sulphates, including 'ethanol [³⁵S]sulphate', by rats *in vivo* (Boström & Vestermark, 1960) and *in vitro* (Vestermark & Boström, 1959, 1960). Small amounts of propylene glycol have been reported to conjugate with glucuronic acid in rabbits (Neubauer, 1901; Miura,

1911; Fellows, Luduena & Hanzlik, 1947), but Gessner, Parke & Williams (1960) were not able to confirm this. Fishman & Green (1957) reported that purified liver β -glucuronidase could transfer up to 90% of glucuronic acid from a glucosiduronic acid to various alcohols, including ethanol and propylene glycol. The glucosiduronic acid of the latter was isolated and its acetyl methyl ester prepared. However, Roy (1956) found that propylene glycol

Table 2. *Effect of ethanol and propylene glycol on the amounts of radioactivity, after incubation with [4-¹⁴C]progesterone, in fractions of female-rat-liver homogenate after successive hydrolytic procedures*

The incubation conditions were as indicated in Table 1; the gas phase was oxygen. Experimental details of incubation and of hydrolysis are given by Taylor (1954) and by Cooke & Taylor (1963) (see the Materials and Methods section). The values for total conjugates were obtained by subtracting the percentages of 'free steroids' from 100.

Alcohol	Concn. of alcohol (m-moles/3 ml.)	'Free steroids' (%)	Total conjugates (%)	Radioactivity extractable after hydrolysis (% total counts/min./incubation vessel)			Total recovery of substrate (%)
				After limpet-powder hydrolysis	After cold-acid hydrolysis	After hot-acid hydrolysis	
Ethanol	0	10.7	89.3	26.1 (0.29)	24.2 (0.27)	11.3 (0.13)	72.5
	0.86	23.6	76.4	15.9 (0.21)	33.3 (0.44)	9.1 (0.12)	81.9
	1.38	31.0	69.0	14.1 (0.20)	37.6 (0.54)	7.5 (0.11)	90.2
	2.06	49.7	50.3	9.8 (0.19)	23.7 (0.47)	7.4 (0.14)	90.6
	2.58	70.4	29.6	4.9 (0.17)	15.0 (0.50)	3.0 (0.10)	93.3
Propylene glycol	0.82	20.2	79.8	16.2 (0.20)	31.3 (0.40)	11.7 (0.15)	79.5
	1.23	20.2	79.8	18.9 (0.24)	31.4 (0.40)	10.7 (0.13)	81.2
	1.64	23.2	76.8	13.4 (0.17)	32.8 (0.43)	12.7 (0.17)	82.1
	2.32	27.2	72.8	13.8 (0.19)	32.2 (0.44)	13.7 (0.19)	86.9
None (control)	—	99.4	—	—	—	—	99.4

* The values in parentheses are the ratios of the radioactivity extractable after hydrolysis to that of the total conjugates.

was not conjugated with sulphuric acid to any appreciable extent by rat liver. It is possible, therefore, that ethanol and propylene glycol inhibit conjugation of progesterone metabolites *in vitro* by competing for conjugating enzymes and cofactors.

McGuire & Tomkins (1960) reported that, when ethanol was added to a reaction mixture containing steroid, rat-liver microsomes and a NADPH-generating system, the rate of reduction of 17 α ,21-dihydroxypregn-4-ene-3,20-dione, androst-4-ene-3,17-dione and 21-hydroxypregn-4-ene-3,20-dione was accelerated by ethanol, whereas the reduction of 17 α ,21-dihydroxypregn-4-ene-3,11,20-trione and 11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione was strongly inhibited. The results shown in Table 1 indicate that the amount of [4-¹⁴C]progesterone recoverable after incubation was not markedly affected by the addition of alcohols to the incubation medium. However, the possibility that the alcohols interfered with the formation of some progesterone metabolites cannot be ruled out, particularly when the decrease in formation of water-soluble metabolites (Table 2) is taken into account. The stimulatory effect of ethanol on the formation of cold-acid-hydrolysable metabolites (Table 2) suggests that this alcohol may affect conjugate formation by a mechanism different from that of propylene glycol.

Whatever mechanism is involved in the inhibition of conjugation of progesterone metabolites, it is desirable that the amounts of these substances used to dissolve steroids for experiments *in vitro* be kept as low as possible, or that they should be avoided. The present availability of labelled steroids of

relatively high specific activity makes the use of solubilizing agents unnecessary for most studies *in vitro*.

SUMMARY

1. Ethanol and propylene glycol inhibit conjugate formation during the metabolism of [4-¹⁴C]-progesterone by female-rat-liver homogenate; more inhibition occurs in air than in oxygen.

2. The amount of [4-¹⁴C]progesterone metabolized is not markedly affected by the addition of these alcohols to the incubation media.

3. Possible reasons for these observations are discussed.

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The Biosynthesis of Phenolic Glucosides in Plants

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The first claim that phenolic glucosides were formed when phenols were fed to plants was made by Ciamician & Ravenna (1916). These workers reported that saligenin (*o*-hydroxybenzyl alcohol) was converted into salicin (*o*-hydroxymethyl-phenyl β -D-glucopyranoside) by maize seedlings. Other feeding experiments with a variety of phenols have since been carried out and it is now clear that glucosylation of phenols is a common reaction in plant tissues. These studies, leading to the implication of uridine diphosphate glucose in the reaction, have been reviewed by Pridham (1960*a*). More recent advances have included feeding coumarin and *o*-coumaric acid to white clover with the formation of melilotyl- and *o*-coumaryl-glucosides respectively (Kosuge & Conn, 1959) and allowing the leaves of various plant species to take up cinnamic acid derivatives (Harborne & Corner, 1961). In this latter case the corresponding glucose esters were the main products, but with caffeic acid the 3- and 4- β -glucosides were also formed. Yamaha & Cardini (1960*a*) have now studied in some detail the enzyme from wheat germ which is specific for the formation of glucosides from uridine diphosphate glucose and phenols with 1,4-dihydroxy groupings (cf. Cardini & Leloir, 1957; Pridham & Saltmarsh, 1960) and the enzyme which transfers glucose from uridine diphosphate glucose to arbutin with the formation of a β -gentiobioside (Yamaha & Cardini, 1960*b*; cf. Pridham, 1957, 1960*b*; Anderson, Hough & Pridham, 1960). The wheat-germ enzyme will also transfer glucose from adenosine diphosphate glucose to quinol (Trivelloni, Recondo & Cardini, 1962). Marsh (1960) has transferred glucuronic acid from uridine diphosphate glucuronic acid to quercetin using an enzyme from French beans and Barber (1962) has converted this flavonol into the glycoside rutin with an enzyme preparation from

mung beans in the presence of uridine diphosphate glucose (or thymidine diphosphate glucose) and thymidine diphosphate rhamnose. The formation of phenolic α -glucosides in the absence of nucleotides has been observed with *Aspergillus niger* extracts, maltose being used as a glucose donor (Pridham, 1961). Acidic derivatives of phenolic glucosides are also produced when broad-bean seeds are treated with simple phenols (Pridham & Saltmarsh, 1962).

This study has been undertaken to establish the nature of the glucosides formed by feeding simple phenols to the broad bean (*Vicia faba*) and to compare these products with those obtained from experiments *in vitro*. All possible steps have been taken to determine accurately the structures of the phenolic glucosides produced, but in some cases low yields or instability of the compounds, or both, have prevented a complete chemical and physical identification. We consider that inadequate characterization may have led to error in some previous publications. For example, some doubt exists about the product of glucosylation of saligenin, where isomeric monoglucosides could be formed by reaction with the phenolic or the primary hydroxyl group.

A preliminary account of this work has been published (Pridham & Saltmarsh, 1960).

METHODS

General methods. M.p. values are uncorrected. Ultra-violet-absorption spectra were measured with a Unicam SP.500 spectrophotometer (1 cm. cell). Free phenolic hydroxyl groups were detected by a bathochromic shift in the presence of base (Mansfield, Swain & Nordstrom, 1953) and ene-diol groupings were revealed by a hypsochromic shift on subsequent addition of borate ions to the alkaline