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METABOLIC CLEARANCE RATES OF PREGNENOLONE, 17-ACETOXYPREGNENOLONE AND THEIR SULPHATE ESTERS IN MAN AND IN RABBIT

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SUMMARY

The metabolic clearance rates, distribution volumes and half-lives of pregnenolone, 17-acetoxypregnenolone and their sulphate esters were determined in man and in the rabbit.

Pregnenolone was cleared about three times more quickly than pregnenolone sulphate in both species.

The metabolic clearance rate of 17-acetoxypregnenolone sulphate is greater in the rabbit than in man, whether expressed in absolute terms or in relation to the metabolic clearance rate of the free steroid.

The slow clearance rate of 17-acetoxypregnenolone sulphate in man is similar to that of testosterone sulphate, dehydroepiandrosterone sulphate and cholesterol sulphate; but is unlike that of pregnenolone sulphate, which is cleared approximately ten times as quickly.

INTRODUCTION

Androgen sulphates have no androgenic activity in the rat, rabbit, chick and mouse (Howard, 1962; P. C. Williams, personal communication). However, before a conclusion could be reached concerning the activity of these compounds in man, the clearance of steroid sulphates in man and laboratory animals was compared. It was found that the sulphate esters of dehydroepiandrosterone and testosterone are cleared much more slowly from the peripheral circulation in man than in the rabbit or rat (Wang, Bulbrook, Sneddon & Hamilton, 1967).

Although androgen sulphates are inactive, the free compounds are androgenic. Deghenghi & Revesz (1965) have reported that 17-acetoxypregnenolone and its sulphate have progestational properties when administered orally to rabbits. The sulphate was also active in this respect when administered intravenously. Pregnenolone and its sulphate were inactive. The possibility that 17-acetoxypregnenolone sulphate is active because it may be cleared differently from the C-19 steroid sulphates, or pregnenolone sulphate, was investigated. At the same time the opportunity was taken to compare the clearance of these compounds in man and rabbit.

METHODS

Analar-grade reagents were used and all solvents were distilled before use. [7α-^{3}H]Pregnenolone (3·8 mc/mg.) was obtained from the Radiochemical Centre, Amersham. Pregnenolone was purified by paper chromatography using the A-type solvent system (toluene:2,2,4-trimethylpentane:methanol:water, 5:45:40:10, by vol.) described by Bush (1952).

Preparation of [7α-^{3}H]17-acetoxypregnenolone

This compound was prepared starting from the commercially available [7α-^{3}H]pregnenolone (10 mc, 2·12 mg.) diluted with 100 mg. of inactive steroid. Introduction of the 17α-oxygen function was achieved according to the method of Bailey, Barton, Elks & Templeton (1962) by autoxidation to the 17α-hydroperoxide and subsequent reduction to the 17α-ol. Acetylation of both the 3β and 17α-hydroxyl groups, followed by partial hydrolysis (Turner, 1953), gave [7α-^{3}H]17α-acetoxypregnenolone (15 mg.). To 7·5 μg. of the product (estimated by serial dilution) was added 100 mg. of carrier 17-acetoxypregnenolone (m.p. 232°). The diluted material was re-crystallized from acetone:hexane and counted as follows:

	mg.	c.p.m./mg.
Diluted sample	100	1255
1st recrystallization	57·3	1177
2nd recrystallization	31·6	1184

Accordingly, the radiochemical purity of the undiluted sample was 94·4% with a specific activity of 29·7 mc/mM. This product was further purified by paper chromatography using Whatman 3 MM. paper and the solvent system dekalin:toluene:methanol:water:nitromethane (18:2:7:3:10).

Preparation of steroid sulphates

The method used for the preparation of the pregnenolone and 17-acetoxypregnenolone sulphates was that described by Levitz (1963) for the preparation of oestrone sulphate. The steroid sulphates were purified as their sodium salts by recrystallization from ethanol. Tritiated steroid sulphates were purified by partition chromatography on Celite (*tert.*-butanol:2,2,4-trimethylpentane:N-NH_4OH, 5:2:5, by vol.) and paper chromatography using the solvent system *n*-butyl ether:*tert.*-butanol:NH_4OH:water (10:10:2:8, by vol.) (Schneider & Lewbart, 1959).

Animals

Two Sandy Lop strain rabbits weighing 4·1 and 4·3 kg. were used. At least 10 days were allowed to elapse between serial injections of radioisotope into any one rabbit.

Clinical tests

The steroids were also administered to male volunteers, in hospital for minor surgical treatment.

Administration and extraction of radioactive steroids

The isotopically labelled steroids were intravenously administered to man (7 μc) or rabbit (1 μc), blood was obtained and radioactivity extracted from the blood by procedures described previously (Wang *et al.* 1967).

Isolation of pregnenolone and 17-acetoxypregnenolone

Extracts containing free steroids were chromatographed on silica gel thin-layer plates (250 μ thick, activated for 30 min. at 115°) in the system acetone:benzene (40:60, v/v) for pregnenolone or benzene:acetone:methanol (85:9:6, by vol.) for 17-acetoxypregnenolone. The sulphates of both compounds remain at the origin in these systems. The free compounds were detected by spraying with Rhodamine G and inspection under u.v. light. The relevant areas were removed and twice eluted with 5 ml. ethanol or methanol (containing 5% (v/v) water). The pooled alcoholic extracts were dried and the residues chromatographed on paper (Whatman 3 MM.). The solvent systems were toluene:2,2,4-trimethylpentane:methanol:water (5:45:40:10, by vol.) for 6 hr. and dekalin:toluene:methanol:water:nitromethane (18:2:7:3:10, by vol.) overnight, for pregnenolone and 17-acetoxypregnenolone, respectively.

Isolation of pregnenolone sulphate and 17-acetoxypregnenolone sulphate

Fractions containing the steroid sulphates were chromatographed on thin-layer plates in the system *tert.*-butanol:ethyl acetate:N-NH_4OH (1:1:1, by vol.). The steroids were located using a Rhodamine G spray and inspection under u.v. light. The free steroids run in the solvent front in this system and away from the steroid sulphates. The area containing the steroid sulphate was removed and 5 ml. 0·1 N-H_2SO_4 saturated with NaCl was added. This solution was partitioned with ethyl acetate (2 × 15 ml.) and solvolysed (Burstein & Lieberman, 1958). The unconjugated steroids released by this hydrolytic step were then treated in exactly the same manner as described for the free steroids, pregnenolone and 17-acetoxypregnenolone.

Recovery of steroids or steroid sulphates

Recovery of steroids or steroid sulphates (added to blood before processing) was determined either by gas–liquid chromatography or by estimating the recovery of added ^{14}C-labelled steroid. The columns used for the gas-liquid chromatography were 1% XE 60 for pregnenolone with progesterone as an internal standard and 2% JXR for the trimethyl silyl ether derivative of 17-acetoxypregnenolone with cholestane as internal standard.

Determination of radioactivity and calculation of results

Both were carried out as previously described (Wang *et al.* 1967). A and B refer to percentages of administered radioactivity as shown in Fig. 1, and A' and B' refer to fractions of administered radioactivity as shown in Table 1.

RESULTS

Metabolic clearance rates, distribution volumes and slopes for 17-acetoxypregnenolone, pregnenolone and their sulphate esters

Man

All the steroids studied gave a two-pool pattern of clearance (see Figs. 1 and 2). The values for the metabolic clearance rates, distribution volumes and slopes are shown in Table 1.

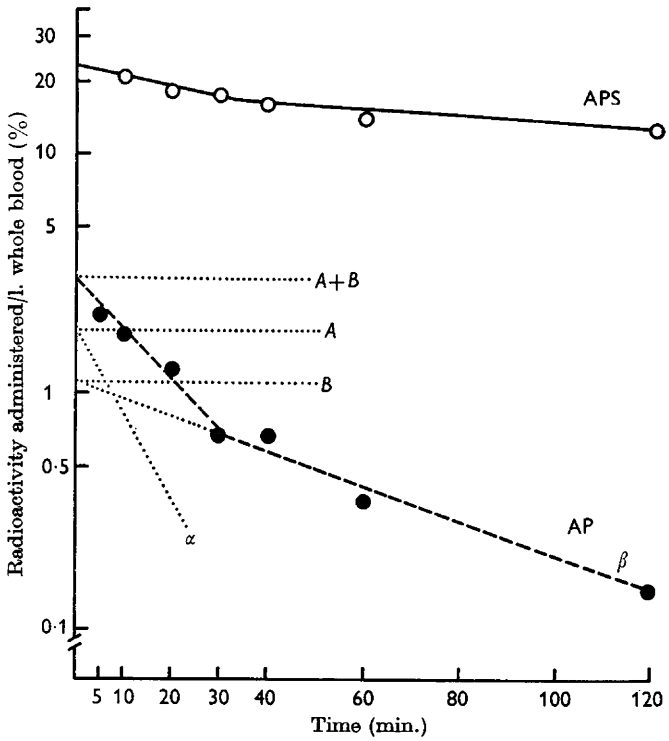

Fig. 1. Disappearance from the peripheral blood of 17-acetoxypregnenolone (AP) and 17-acetoxypregnenolone sulphate (APS) in man. $A+B$ and B are percentages at zero time obtained by extrapolation. A is obtained by subtraction of these two lines. α and β are the slopes of the lines.

The steroid sulphates had lower rates of metabolic clearance than the corresponding free steroids. These differences were due to smaller volumes of distribution compared with the free compounds and, to a lesser degree, to longer half-lives. However, although these sulphates were cleared more slowly than the free compounds, pregnenolone sulphate was cleared approximately seven times more rapidly than 17-acetoxypregnenolone sulphate.

Rabbit

As in man, all the steroids studied showed a two-pool pattern of clearance (see Figs. 3 and 4). The values obtained for the metabolic clearance rates, distribution volumes and slopes are shown in Table 1.

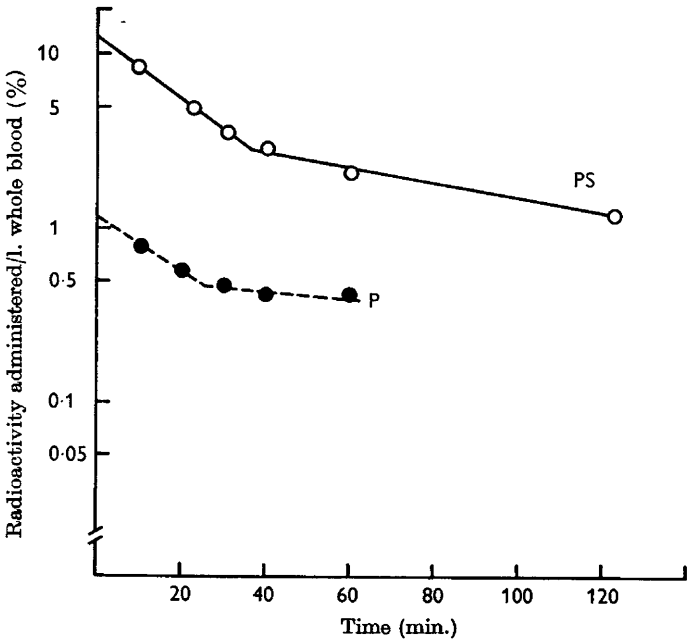

Fig. 2. Disappearance from the peripheral blood of pregnenolone (P) and pregnenolone sulphate (PS) in man.

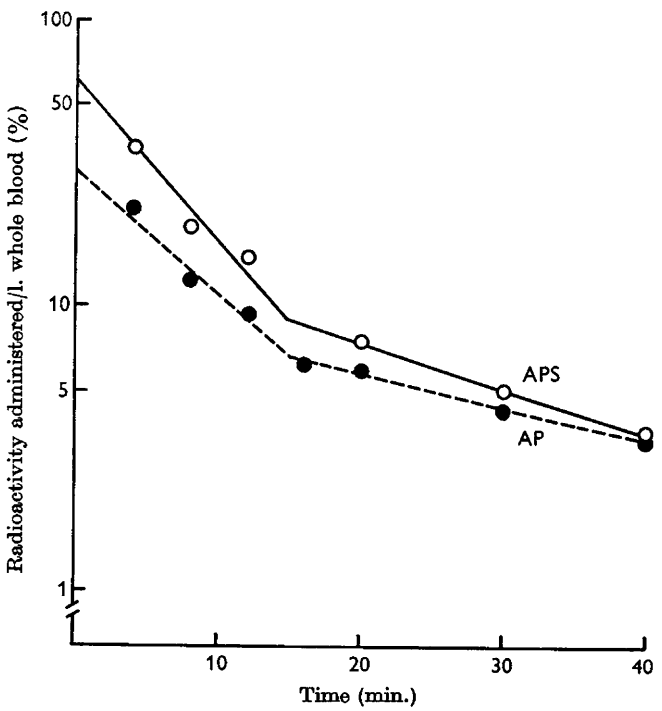

Fig. 3. Disappearance from the peripheral blood of 17-acetoxypregnenolone (AP) and 17-acetoxypregnenolone sulphate (APS) in the rabbit.

The metabolic clearance rates of the steroid sulphates were smaller than those of the corresponding free compounds, due mainly to smaller distribution volumes of the sulphates, rather than gross differences in half-lives. The metabolic clearance rates of the two sulphates studied are similar and are about a third of that of the corresponding free steroids. The ratio of the metabolic clearance rates of pregnenolone and its sulphate was of the same order as that found in man; this did not apply to 17-acetoxypregnenolone and its sulphate.

Table 1. *Rates of disappearance, distribution volumes and metabolic clearance rates of pregnenolone, 17-acetoxypregnenolone and their sulphate esters in man and rabbit*

	Body weight (kg.)	A' (Fraction of dose administered/l. blood)	B'	α (min.$^{-1}$)	β (min.$^{-1}$)	V_1 (l./kg.)	V_2 (l./kg.)	MCR (l./day)	MCR (blood volume/ day)
				Pregnenolone					
Man	70	0·0054	0·0055	0·1066	0·0050	1·314	1·065	1256	251
	74	0·0022	0·0045	0·0770	0·0100	2·041	0·640	3045	576
Rabbit	*	0·7483	0·2041	0·5332	0·0666	0·250	0·330	322	1479
	—	0·4855	0·0892	0·4077	0·0541	0·414	0·572	507	1857
				Pregnenolone sulphate					
Man	70	0·0887	0·0412	0·0630	0·0101	0·110	0·092	263	52·6
	70	0·0471	0·0317	0·0478	0·0083	0·181	0·117	299	59·8
Rabbit	—	1·1975	0·4695	0·1925	0·0433	0·143	0·088	84·3	309
	—	1·4663	0·5747	0·1925	0·1066	0·117	0·010	111	407
				17-Acetoxypregnenolone					
Man	63·5	0·0178	0·0118	0·0990	0·0173	0·534	0·340	1680	371
Rabbit	—	0·1950	0·1035	0·1733	0·0289	0·798	0·604	306	1121
	—	0·1331	0·1126	0·2390	0·0322	0·969	0·638	355	1300
				17-Acetoxypregnenolone sulphate					
Man	76	0·0496	0·1587	0·0128	0·0043	0·063	0·007	37·5	6·9
	76	0·0365	0·1961	0·0444	0·0053	0·064	0·009	37·9	7·9
Rabbit	—	0·4623	0·1550	0·1733	0·0355	0·386	0·280	104	381
	—	1·0884	0·1316	0·3150	0·0204	0·195	0·599	145	531

A' and B' are the fractions of radioactivity per litre of whole blood extrapolated to zero time as shown in Fig. 1; α and β are the slopes of the lines corresponding to A' and B' and are calculated as $\log_e 2$/half-life in min.; V_1 and V_2 are distribution volumes and are defined as $V_1 = 1/(A'+B')$ and $V_1+V_2 = (A'\beta^2+B'\alpha^2)/(A'\beta+B'\alpha)^2$. The metabolic clearance rate (MCR) $= [\alpha\beta/(A'\beta+B'\alpha)] \times 1440$l./day.

* The mean weight of the rabbits was 4·2 kg. Calculations are based on 71·4 ml./kg. and 65 ml./kg. of blood for man and rabbit, respectively.

Table 2. *Metabolic clearance rates of steroid sulphates in man*

Compound	Metabolic clearance rate (l./24 hr.)	Reference
Dehydroepiandrosterone sulphate	7 (plasma)	Sandberg, Gurpide & Lieberman (1964).
Dehydroepiandrosterone sulphate	16 and 32 (whole blood)	Wang *et al.* (1967).
Testosterone sulphate	22 and 25 (whole blood)	Wang *et al.* (1967).
Cholesterol sulphate	11 (plasma)	Gurpide, Roberts, Welch, Bandi & Lieberman (1966).

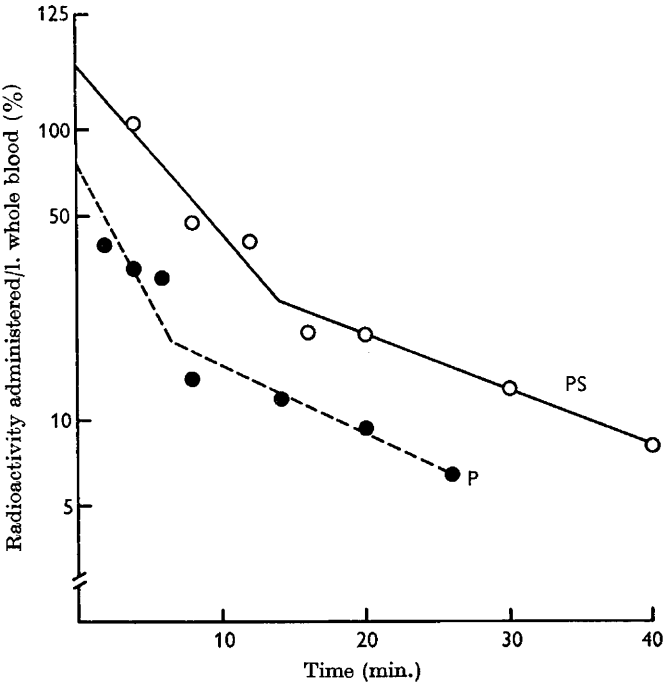

Fig. 4. Disappearance from the peripheral blood of pregnenolone (P) and pregnenolone sulphate (PS) in the rabbit.

DISCUSSION

Only approximate determinations of metabolic clearance rates can be obtained by single injection techniques of compounds which do not occur naturally (Nelson, 1961). There is good evidence that pregnenolone and its sulphate are secreted by the human adrenal (Wieland, DeCourcy, Levy, Zala & Hirschmann, 1965; Fukushima, Bradlow, Hellman & Gallagher, 1963; Arcos, Gurpide, VandeWiele & Lieberman, 1964; Conrad, Pion & Kitchin, 1967). However, there is no evidence that 17-acetoxypregnenolone and its sulphate are natural products. Nevertheless, large differences were found between the metabolic clearance rates of the free and sulphated steroid which are of the same order as those found for dehydroepiandrosterone, testosterone and their sulphates (Wang *et al.* 1967).

Table 2 shows values for the metabolic clearance rates of some steroid sulphates in man. Pregnenolone sulphate with a clearance of about 280 l. whole blood/day is cleared rapidly compared with the steroid sulphates mentioned above but the rate is similar to that for corticosterone sulphate (Kielmann, Stachenko & Giroud, 1966). It is unlikely that the rapid clearance of pregnenolone sulphate is due to a high sulphatase activity (Calvin & Lieberman, 1966).

An implication of this relatively rapid clearance of pregnenolone sulphate is that the blood production rate (Tait, 1963) is appreciable. Thus assuming the haematocrit volume is 50% (see Spector, 1956) and that the level of pregnenolone sulphate in male plasma is 10 μg./100 ml. (Conrad, Pion & Kitchin, 1967), the blood production rate would be approximately 14 mg./day.

In man the progestational compound 17-acetoxypregnenolone sulphate (Deghengi & Revesz, 1965) is cleared much more slowly than the free compound; its metabolic clearance rate is similar to those quoted in Table 2. However, in the rabbit the clearance of the free and conjugated compounds is much the same. Since 17-acetoxypregnenolone is about twice as active as the sulphate as a progestational agent in the rabbit, it would be of interest to know whether 17-acetoxypregnenolone sulphate, because of its slow clearance in man, is a potent progestational compound. Comparison of the clearances of pregnenolone, 17-acetoxypregnenolone and their sulphate esters in the rabbit shows that the sulphates are cleared more slowly than the free compounds. Except for pregnenolone and its sulphate, this difference is not as marked as that found in man for 17-acetoxypregnenolone, testosterone and dehydroepiandrosterone and their sulphates (Wang *et al.* 1967).

The behaviour of steroids and their sulphates in the rabbit resembles that in man in only a few cases. In this and another study (Wang *et al.* 1967) only the clearances of pregnenolone and its sulphate were similar in rabbit and man. Results on the biological activity of these steroids and their sulphates obtained in laboratory animals may therefore not be applicable to man.

Lindner (1964) has reported that in domestic ruminants only 10% of circulating cortisol was bound to blood proteins and Harrison & Paterson (1965) have suggested that this is the reason for the rapid clearance of cortisol in sheep. The possibility that disparities in the clearance of steroid sulphates between man and the rabbit are due to differences in protein binding has been investigated and these results are reported in the following paper.

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REFERENCES

Arcos, M., Gurpide, E., VandeWiele, R. L. & Lieberman, S. (1964). Precursors of urinary pregnanediol and their influence on the determination of the secretory rate of progesterone. *J. clin. Endocr. Metab.* **24**, 237–245.

Bailey, E. J., Barton, D. H. R., Elks, J. & Templeton, J. F. (1962). Compounds related to steroid hormones. IX. Oxygenation of steroid ketones in strongly basic medium: a new method of preparation of 17α-hydroxypregnenolone-20-ones. *J. chem. Soc.* pp. 1578–1591.

Burstein, S. & Lieberman, S. (1958). Hydrolysis of ketosteroid hydrogen sulfates by solvolysis procedures. *J. biol. Chem.* **233**, 331–335.

Bush, I. E. (1952). Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. *Biochem. J.* **50**, 370–378.

Calvin, H. I. & Lieberman, S. (1966). Studies on the metabolism of pregnenolone sulfate. *J. clin. Endocr. Metab.* **26**, 402–424.

Conrad, S. H., Pion, R. J. & Kitchin, III, J. D. (1967). Pregnenolone sulfate in human pregnancy plasma. *J. clin. Endocr. Metab.* **27**, 114–119.

Deghengi, R. & Revesz, C. (1965). The oral activity of 17α-substituted pregnenolones and their sulphates. *J. Endocr.* **31**, 301–302.

Fukushima, D. K., Bradlow, H. L., Hellman, L. & Gallagher, T. F. (1963). Further studies on the origin of pregnanetriol in adrenal carcinoma. *J. clin. Endocr. Metab.* **23**, 266–273.

Gurpide, E., Roberts, K. D., Welch, M. T., Bandi, L. & Lieberman, S. (1966). Studies on the metabolism of blood-borne cholesterol sulfate. *Biochemistry, N.Y.* **5**, 3352–3362.

Harrison, F. A. & Paterson, J. Y. F. (1965). The specific activity of plasma cortisol in sheep after rapid intravenous injection of [1,2-$^3H_2$]cortisol, and its relation to the rate of cortisol secretion. *J. Endocr.* **33**, 477–490.

Howard, E. (1962). Steroids and bone maturation in infant mice: relative activities of dehydroepiandrosterone and testosterone. *Endocrinology* **70**, 131–141.

Kielmann, N., Stachenko, J. & Giroud, C. J. P. (1966). Production rate of corticosterone sulfate in normal human adults. *Steroids* **8**, 993–1025.

Levitz, M. (1963). Synthesis of estrone-6,7-^{3}H sulfate-^{35}S. *Steroids* **1**, 117–120.

Lindner, H. R. (1964). Comparative aspects of cortisol transport: lack of firm binding to plasma proteins in domestic ruminants. *J. Endocr.* **28**, 301–320.

Nelson, E. (1961). Kinetics of drug absorption, distribution, metabolism and excretion. *J. pharm. Sci.* **50**, 181–192.

Sandberg, E., Gurpide, E. & Lieberman, S. (1964). Quantitative studies on the metabolism of dehydroisoandrosterone sulfate. *Biochemistry, N.Y.* **3**, 1256–1267.

Schneider, J. J. & Lewbart, M. L. (1959). Fractionation and isolation of steroid conjugates. *Recent Prog. Horm. Res.* **15**, 201–225.

Spector, W. S. (1956). *Handbook of biological data*. Philadelphia and London: W. B. Saunders Co.

Tait, J. F. (1963). Review: The use of isotopic steroids for the measurement of production rates *in vivo*. *J. clin. Endocr. Metab.* **23**, 1614–1621.

Turner, R. B. (1953). Acylation of 17-hydroxy-20-ketosteroids. *J. Am. chem. Soc.* **75**, 3489–3492.

Wang, D. Y., Bulbrook, R. D., Sneddon, A. & Hamilton, T. (1967). The metabolic clearance rates of dehydroepiandrosterone, testosterone and their sulphate esters in man, rat and rabbit. *J. Endocr.* **38**, 307–318.

Wieland, R. G., DeCourcy, C., Levy, R. P., Zala A. P. & Hirschmann, H. (1965). $C_{19}O_2$ steroids and some of their precursors in blood from normal human adrenals. *J. clin. Invest.* **44**, 159–168.