The proportion of the dose excreted in the bile of the rabbit is small, as shown by the slight effect of ligating the bile duct on the contents of the gastro-intestinal tract and faeces (Table 4), and it appears that a comparable amount reaches the gut by other routes. In the rat a much larger percentage reaches the gut, the great majority being carried in the bile. These conclusions were confirmed by the experiments with cannulated animals (Table 5), which show that in rabbits active material still reached the gut from extra-biliary sources in amounts generally of the same order as that excreted in the bile. In the rat 11% of the dose reached the gut in 1·75 hr. but over three times as much was found in the bile.

Naphthylamine or its metabolites appear very rapidly in the bile (Fig. 2), the rate of excretion reaching a maximum within 15 min. in rabbit 17. Apart from a somewhat sharper maximum the curve closely resembles those showing variation of concentration in the blood (Figs. 1 and 3). Within the accuracy of our measurements the concentration in the bile does not lag behind that in the blood. The transfer of the material from the blood to the bile is not merely a matter of diffusion through a membrane, for the concentration in the bile is much higher than in the blood, strikingly so in the case of rabbit 20, despite the fact that the actual volume of bile excreted by this animal was very high.

SUMMARY

1. Rabbits injected intraperitoneally with 2-[8-^{14}C]naphthylamine (approx. 1 mg.) retain radioactivity in the blood for several weeks. This activity is shared between the plasma and cells. In rats, activity is detectable in the blood for about the same length of time, but after 11 days it is entirely in the cells.

2. In one day after intraperitoneal injection of approximately 1 mg. of 2-[8-^{14}C]naphthylamine in rabbits about 90% of the dose is excreted in the urine and faeces. The initial excretion in the urine is very rapid (over 50% of the dose in 2 hr., compared with 22% for rats). Total urinary excretion in the rabbit is about 80%.

3. Excretion of radioactive material into the gut of rabbits, both by the biliary route and otherwise, is demonstrated. The total amount reaching the gut is much less than in rats, mainly because excretion in the bile is much lower. Reabsorption of active material from the gut is shown to occur.

4. The concentration of radioactivity in the bile and the blood of rabbits varies in a closely similar manner. The actual concentration in the bile at a given time is considerably higher than in the blood and may exceed it from 3 to over 30 times in anaesthetized rabbits with cannulated common bile ducts.

We are indebted to Miss P. M. Andrews, Mrs R. Coombes and Miss E. Teichmann for technical assistance.

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The Enzymic Synthesis of Steroid Sulphates

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(*Received* 2 *November* 1955)

Soluble enzyme preparations from rat liver have been known for some time to be capable of synthesizing aryl sulphates from the appropriate phenol and sulphate ions in the presence of adenosine triphosphate (Bernstein & McGilvery, 1952*a*; DeMeio, 1952). There had been no investigation of the synthesis of steroid sulphates by such enzymes until very recently, when DeMeio & Lewycka (1955) were able to demonstrate the probable synthesis thereby of dehydro*epi*androsterone sulphate (DHAS), although the product was not characterized. The synthesis of such steroid sulphates is of particular interest as these compounds must play a part in the metabolism of steroids, and their synthesis is probably more representative of the true function of the enzyme system involved than is the commonly studied formation of the sulphates of toxic phenols. Furthermore, the molluscan enzyme hydrolysing these steroid sulphates, steroid sulphatase, showed

a high degree of specificity (Roy, 1955), and it was of interest to investigate the corresponding specificity of the more widely distributed synthetic system. The method of assay used in the present investigation allowed the ready investigation of a large number of different substrates and so was of more general application than the previously available methods for the assay of sulphate ester synthesis.

EXPERIMENTAL

Preparation of the enzyme. This was prepared by the method of Bernstein & McGilvery (1952*a*). Portions of rat liver were homogenized in a Potter & Elvehjem (1936) homogenizer with 3–4 vol. of 0·15 M-KCl containing 0·001 M ethylenediaminetetraacetic acid, pH 7. The temperature at this and all subsequent stages was kept at 0°. The homogenate was centrifuged for 2 hr. at 20 000 *g* and the supernatant, containing the enzyme, separated. The enzyme was then purified by fractional precipitation with ammonium sulphate, and the fraction precipitated between 1·7 M- and 2·3 M-$(NH_4)_2SO_4$ contained the bulk of the enzyme. This fraction was dissolved in water (0·7 ml./g. of liver) and the solution stored at −20°, at which temperature it was stable for at least 4 weeks. For assay by the method described below, preparations from male and female animals were diluted with 1 and 3 vol. of water respectively.

Assay of steroid sulphate synthesis. The following method was used in the assay of DHAS synthesis, but it was directly applicable to the assay of the synthesis of any steroid sulphate.

Buffered adenosine triphosphate (ATP) was prepared immediately before use by mixing equal vol. of 0·3 M-KH_2PO_4 (pH 6·8), 0·03 M-K_2SO_4, 0·005 M-$MgCl_2$, and 0·33 vol. of 0·04 M ATP, pH 6·8. The ATP was the disodium salt (L. Light and Co., Colnbrook, Bucks). To 0·5 ml. of buffered ATP was added 0·1 ml. of 0·4 mM dehydro*epi*-androsterone (DHA) in propylene glycol followed by 0·4 ml. of enzyme solution. The final concentrations of ATP and DHA were therefore 2·0 and 0·04 mM respectively. The mixture was incubated for 2 hr. at 37° and the reaction then stopped by the addition of 5 ml. of ethanol. After standing for 15 min. the tubes were capped, and the precipitated protein was removed by centrifuging. A sample of the clear supernatant (5 ml.) was taken for the assay of DHAS as described below.

Blank determinations were simultaneously carried out, in which the enzyme was incubated separately from the remainder of the reaction mixture and added after the ethanol. Assays and blanks were always performed in duplicate.

Determination of DHAS. DHAS was assayed by a micro-modification of the method already described by Roy (1956), which makes use of the solubility in chloroform of the complexes formed between methylene blue and sulphuric acid esters. This method can be applied to the determination of any steroid sulphate ester (Roy, 1956). The methylene-blue reagent used in the present method was prepared from that previously used (Roy, 1956) by diluting with an equal volume of water.

The 5 ml. sample of the protein-free solution obtained above was concentrated in a boiling-water bath for 20 min. This did not give a completely dry residue but left about 0·1 ml. of a viscous liquid, presumably propylene glycol. After cooling, the residue was taken up in 2 ml. of methylene-blue reagent, 5 ml. of chloroform was added and the mixture shaken vigorously for 20 sec. The mixture was centrifuged, the aqueous layer removed and a sample of the chloroform layer taken into a test tube and dried with Na_2SO_4. The intensity of the resulting blue solution was read in the Spekker absorptiometer with micro cells (1 cm. light path) and Ilford filter no. 608 (maximum transmission 700 mμ.) against the appropriate blank.

A calibration curve for the above method was prepared by dissolving known amounts of DHAS in a medium of composition identical with that of the assays, except that water was added in place of enzyme, followed by ethanol precipitation, etc., as described above. It is essential that the blank and calibration tubes have a composition identical with that of the assays as variations in their composition may cause variations in the shape of the calibration curve, which is not strictly linear. Ethanol precipitation minimizes these effects by the removal of the bulk of the inorganic ions which may interfere.

The recovery of known amounts of DHAS added to the incubation mixture was satisfactory, the mean recovery in the range 5–25 μg. of DHAS being 95% from solutions containing 8 mg. of protein/ml., the usual concentration in routine assays. With amounts of DHAS less than 5 μg. the recoveries were less and not so reproducible.

Isolation of DHAS. To characterize the product formed during the enzymic reaction the isolation of DHAS was attempted. To 10 ml. of buffered ATP and 1 ml. of 2 mM DHA was added 8 ml. of undiluted enzyme solution, and the mixture was incubated for 2 hr. at 37°, after which a further 1 ml. of DHA solution was added. The incubation was continued for a total of 5 hr. and the proteins were then precipitated by the addition of 100 ml. of ethanol. The mixture was filtered, and the filtrate concentrated *in vacuo* to about 2 ml. of a viscous liquid; this was taken up in 10 ml. of methylene-blue reagent and the methylene-blue complex extracted by shaking with three 50 ml. portions of chloroform. The extract was dried with Na_2SO_4 and taken to dryness *in vacuo*, and it gave a small blue residue which was dissolved in 0·5 ml. of ethanol and diluted to 5 ml. with water. To convert the methylene-blue complex into the sodium salt this solution was gently shaken for 15 min. with 200 mg. of Zeo-Karb 225 Na^+ (Permutit Co. Ltd., London), and then filtered. Traces of methylene blue were removed from the filtrate by washing twice with 2 ml. portions of chloroform; traces of chloroform were then removed from the aqueous phase by warming *in vacuo*, and the volume was made up to 10 ml. with water. The resulting solution was shown, as described below, to contain DHAS.

RESULTS

Properties of the enzyme system. Preliminary experiments indicated that the enzyme system forming DHAS had the same general properties as that forming aryl sulphates (Bernstein & McGilvery, 1952*a*, *b*; DeMeio, Wizerkaniuk & Fabiani, 1953). The synthesis of DHAS was ATP-dependent and showed its optimum activity at a concentration of 2 mM ATP (Fig. 1). Higher concentrations of ATP caused a significant inhibition of the reaction, so

that at 8 mM ATP the activity of the enzyme was only some 80% of the maximum. The optimum substrate concentration was approximately 0·04 mM DHA (Fig. 2) and substrate inhibition occurred to a considerable extent. With *epi*androsterone and androsterone the optimum substrate concentration was again 0·04 mM, as shown in Fig. 2. The enzyme system was relatively insensitive to the concentration of SO_4^{2-} ions, as the maximal activity was reached by a concentration of 0·01 M-K_2SO_4, and no decrease in activity was noted at concentrations as high as 0·1 M-K_2SO_4 (Fig. 1). In these experiments,

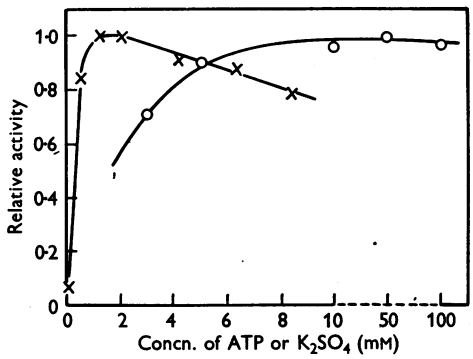

Fig. 1. Influence of different concentrations of ATP and K_2SO_4 on the synthesis of DHAS. Reaction mixture (vol. 1·0 ml.) contained 0·4 ml. of enzyme solution, 0·1 ml. of 0·4 mM DHA in propylene glycol, and 0·5 ml. of buffered ATP (see text) containing varying amounts of ATP or K_2SO_4. ×, Synthesis of DHAS with different concentrations of ATP, and ○, synthesis of DHAS with different concentrations of K_2SO_4.

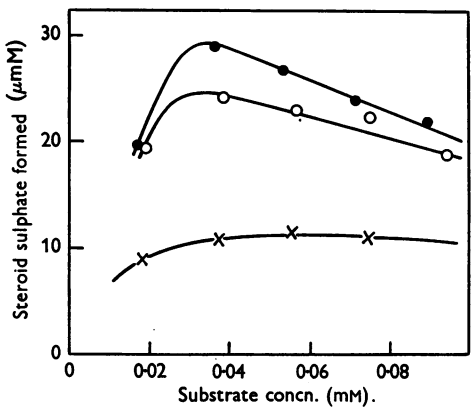

Fig. 2. Effect of substrate concentration on steroid sulphate synthesis. Reaction mixture (vol. 1·0 ml.) contained 0·5 ml. of buffered ATP (see text), 0·4 ml. of enzyme solution and 0·1 ml. of solutions of varying concentration of the steroid in propylene glycol. Incubated for 2 hr. at 37°. ●, DHA; ○, *epi*androsterone; ×, androsterone.

the enzyme preparations used were dialysed against 0·2 M phosphate buffer, pH 6·9, at 0° to reduce the concentration of SO_4^{2-} ions in the enzyme to negligible amounts. Dialysis under these conditions caused about 30% inactivation of the enzyme; dialysis against distilled water caused complete inactivation. The enzyme was thus relatively insensitive to changes in the concentration of SO_4^{2-} ions, and the dialysed enzyme was very unstable. Dialysis was therefore not normally carried out.

As corticosteroids and salicylates have been reported to inhibit the synthesis of ester sulphates (Kodicek & Loewi, 1955) the effect of these substances on the synthesis of DHAS was investigated. Neither salicylic acid nor acetylsalicylic acid in mM concentrations exerted any effect on the synthesis of DHAS. Cortisone, cortisone acetate, and hydrocortisone all inhibited the formation of DHAS to approximately 20% at mM concentrations, as shown in Table 1.

During the course of the present work it was noted that enzyme preparations from the livers of female rats were consistently more active in the synthesis of DHAS than were those from the livers of male rats. That this is a true difference is readily seen from the following figures. The synthetic activity of preparations from the livers of four male rats ranged from 80 to 165 μmM of DHAS/g. of liver/2 hr., with a mean value of 130 μmM/g., while corresponding preparations from the livers of four female rats had activities ranging from 235 to 325 μmM/g., with a mean of 305 μmM/g.

Specificity of the enzyme system. This was investigated under the general conditions described above, and the results of some of the experiments are shown in Figs. 2 and 3. These figures show the synthesis of the sulphates of a number of androstan-3-ol-17-one isomers. It is obvious that although the enzyme system is relatively non-specific, in that all the isomers are conjugated with

Table 1. *Influence of inhibitors on the synthesis of DHAS*

Reaction mixture (vol. 1·0 ml.) contained 0·4 ml. of enzyme, 0·1 ml. of 0·04 mM DHA, and 0·5 ml. of buffered ATP. The water-soluble inhibitors were dissolved in the buffered ATP and the water-insoluble compounds in the substrate solution. The activity is expressed relative to the controls containing no inhibitor, activity 1·0.

Inhibitor (mM)	Activity
Sodium salicylate (1·0)	0·99
Acetylsalicylic acid (1·0)	0·98
Cortisone (1·0)	0·86
Cortisone acetate (1·0)	0·79
Hydrocortisone (1·0)	0·80
Sodium sulphite (1·0)	1·0
m-Aminophenol (1·0)	0·65
m-Aminophenol (0·5)	0·73

sulphate, there is a considerable variation in the rate of the reaction. The results presented in Fig. 3 show that when the reactions have reached their appropriate steady states the velocities differ in each case. This point is discussed below.

A number of other steroids have been investigated as possible substrates of the reaction, but the results are not quoted in detail as they were obtained under arbitrary conditions, not necessarily optimum for the substrate in question. The substrate concentration was 0·1 mM and the time of incubation 2 hr. Taking the rate of conjugation of DHA under comparable conditions to be 1·00, the rate with pregn-5-en-3β-ol-20-one or 5α-pregnan-3β-ol-20-one as substrate was 1·0, with 5β-pregnan-3β-ol-20-one, 0·6; with oestrone and *epi*testosterone, 0·4; with testosterone, 0·25; with cortisone, 0·1; and with hydrocortisone about 0·05. These results confirm that the sulphate-conjugating system shows a relatively low degree of substrate specificity.

Several compounds other than steroids were also tested as possible substrates under the same conditions, except that the substrate was dissolved in water rather than in propylene glycol. Neither *cyclo*hexanol nor β-phenylethanol nor propylene glycol was conjugated with sulphate at a detectable rate. Salicylic acid reacted very slowly, but phenol was conjugated at a rate comparable to DHA and *p*-nitrophenol at about three times the rate of DHA. When the two latter phenols were dissolved in propylene glycol rather than in water, there was a 40% diminution in the rate of the conjugation of phenol, but with *p*-nitrophenol the rate was unaltered.

Although the properties of the enzyme system forming DHAS suggest that it is identical with the system forming aryl sulphates, direct proof of this assumption has not been possible. Good evidence for the identity of the two systems is given, however, by the fact that *m*-aminophenol, which is known to be rapidly conjugated with sulphate by such preparations (Bernstein & McGilvery, 1952*a*), is a powerful inhibitor of the synthesis of DHAS, a concentration of 0·001 M *m*-aminophenol causing a 40% inhibition of DHAS synthesis (Table 1).

Characterization of DHAS. The product of the enzymic reaction was isolated as described above and was identified as DHAS. Characterization of the compound by classical means was not attempted and the substance was identified by its behaviour on hydrolysis.

Enzymic hydrolysis of the reaction product was carried out with a preparation of the steroid sulphatase of *Patella vulgata* under the conditions already described (Roy, 1954). The reaction product behaved exactly similarly to a standard solution of DHAS when submitted to this enzymic hydrolysis. A further sample was hydrolysed in 0·1 N-H_2SO_4 under the conditions described by Roy (1956), and again the behaviour was identical with that of an authentic sample of DHAS. The results of these experiments are shown in Fig. 4.

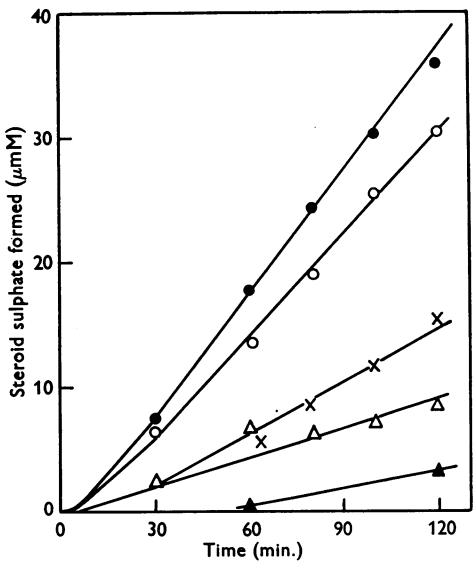

Fig. 3. Course of formation of steroid sulphates. General conditions as in Fig. 2, except that the concentration of the substrate solution was 0·4 mM, giving a final substrate concentration of 0·04 mM, and the time of incubation was varied. △, 5β-Androstan-3β-ol-17-one; ▲, 5β-androstan-3α-ol-17-one. Other symbols as in Fig. 2.

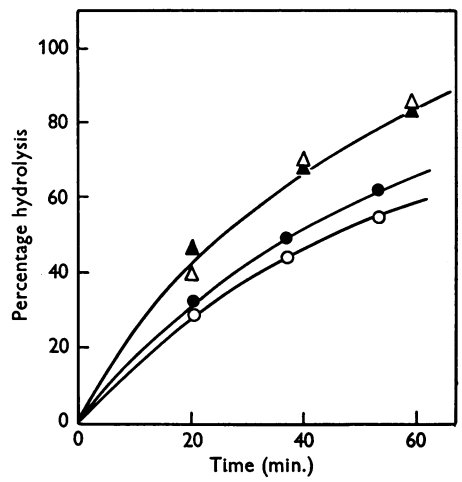

Fig. 4. Hydrolysis of authentic DHAS and DHAS prepared enzymically as described in the text. ▲, Authentic, and △, synthetic DHAS, hydrolysed by 0·1 N-H_2SO_4 at 100°. ●, Authentic, and ○, synthetic DHAS, hydrolysed by steroid sulphatase at pH 4·5 and 37°.

Further proof of the identity of the reaction product with DHAS was obtained by characterization of the steroids produced on acid hydrolysis of the reaction product. A 1 ml. sample of the solution of the product was hydrolysed for 1·5 hr. with 1 ml. of 2N-HCl at 100° and, after cooling, the steroid was extracted with two 5 ml. portions of chloroform. The extract was dried with Na_2SO_4 and taken to dryness *in vacuo*. The residue was dissolved in methanol and submitted to partition chromatography on paper, with the Bush (1952) solvent system *A*.

The steroids produced on the acid hydrolysis of the reaction product were identical with those similarly produced from authentic DHAS, namely, large amounts of chloroandrostenone and small amounts of DHA.

The above experiments were repeated on the reaction product obtained when androsterone was used as substrate. The product behaved then exactly as did authentic androsterone sulphate on hydrolysis by acid, the hydrolysis products being similar in the two cases. The reaction product was not hydrolysed by steroid sulphatase, as was expected from the specificity of that enzyme (Roy, 1956).

There is therefore no doubt that the method described above does indeed measure the rate of formation of steroid sulphates.

DISCUSSION

Although the reaction product has in no case been isolated, these experiments show that when DHA is used as substrate the reaction product is DHAS. The product is an acid-labile conjugate of DHA, which is hydrolysed both by acid and by steroid sulphatase at rates identical with those observed for the corresponding hydrolysis of authentic DHAS (Fig. 4). Further proof that the reaction product is DHAS is afforded by the fact that the reaction is dependent upon the presence of SO_4^{2-} ions in the reaction mixture. The only other product which could conceivably be formed is dehydro*epi*androsterone phosphate, but phosphate esters do not appear to be estimated by the methylene-blue technique (unpublished observations).

The general properties of the enzyme system synthesizing DHAS are similar to those of the system synthesizing *m*-aminophenyl sulphate. Both enzymes are precipitated between 1·7 and 2·3M-$(NH_4)_2SO_4$ and require the presence of 2 mM ATP and 10 mM-SO_4^{2-} ions to exhibit their maximum activity. Again, both enzyme systems are inactivated by dialysis against distilled water and both show typical lag-phase curves for the course of the reaction. It seems reasonably certain that the same enzyme system is involved in the synthesis of both types of compound, but direct evidence for this has not been obtained, except that the synthesis of DHAS is strongly inhibited by *m*-aminophenol. The enzyme system apparently shows a relatively low degree of specificity for steroid substrates, as all sterols so far tested are conjugated with sulphate. It should be pointed out, however, that one enzyme system has not yet been demonstrated to be involved in each case. The only noteworthy result is shown in Fig. 3, which demonstrates that 5β-androstan-3β-ol-17-one is conjugated more rapidly than the corresponding 3α-hydroxy isomer. This is surprising, as the 3β-hydroxyl group is in the axial, therefore hindered, position. In the 5α-androstane series the equatorial hydroxyl group of the 3β isomer reacts considerably more rapidly than the axial group of 3α isomer (Fig. 3), as would be expected.

As shown in Fig. 3, the kinetics of conjugation of the androstanolone isomers were similar to those described by Bernstein & McGilvery (1952*b*) for the conjugation of *m*-aminophenol in that in both there was a lag phase, during which the reaction velocity steadily increased to a maximum value which was maintained for a prolonged period, the linear phase. Bernstein & McGilvery (1952*b*) interpreted these results as indicating the formation of an 'active sulphate' followed by a coupling reaction between this 'active sulphate' and the hydroxyl-containing compound, this second reaction being the rate-limiting one. Segal (1955) developed this theory and provided a mathematical formulation of the results as follows. The two reactions were:

$$A + SO_4^{2-} \xrightarrow{k_1} I,$$

$$I + R.OH \xrightarrow{k_2} R.SO_4^-,$$

the second reaction being rate-limiting. From these assumptions Segal (1955) showed that such a reaction would exhibit a lag phase, the length of which would be governed by k_2, and a linear phase when the system had reached the steady state and when the velocity would be governed solely by k_1. The equations expressing these findings were as follows (Segal, 1955). The velocity at the steady state is

$$\frac{dP}{dt} = k_1, \qquad (1)$$

and the total amount of reaction product (P) formed at any time (t) is

$$P = k_1 t - \frac{k_1}{k_2}(1 - e^{-k_2 t}). \qquad (2)$$

Equation (1) above indicates that the velocity at the steady state is independent of the nature of the substrate, R.OH, as this velocity depends only on k_1. Fig. 3 shows, on the other hand, that the

velocity at the steady state does vary with the substrate. It is therefore obvious that the theory of Segal (1955) is incorrect. The experimental results presented in Fig. 3 can, however, be explained by a very slight modification of the above theory. The modification required is the not improbable one that the first reaction, the formation of 'active sulphate', is reversible. Retaining the nomenclature of Segal (1955) and calling the velocity constant for this reverse reaction k_3, it can be shown that the velocity at the steady state is not independent of the nature of the substrate. As the velocity of the second reaction is rate-limiting, the overall reaction velocity is

$$\frac{dP}{dt} = k_2 I.$$

At the steady state

$$\frac{dI}{dt} = k_1 - k_2 I - k_3 I = 0,$$

$$I = \frac{k_1}{k_2 + k_3},$$

$$\frac{dP}{dt} = \frac{k_1 k_2}{k_2 + k_3}. \quad (3)$$

This equation (3) shows that the velocity at the steady state depends not only upon k_1 but also upon k_2 and k_3. As k_2 must vary with the substrate used, so also must the velocity at the steady state. This does not alter the rest of the argument of Segal (1955) but only makes the equation describing the total amount of reaction product formed at time t rather more complex, as follows:

$$P = k_1 t - \frac{k_1}{k_2 + k_3}(1 - e^{-(k_2+k_3)t}). \quad (4)$$

The form of equation (4) is identical with that of equation (2), but it does not allow the evaluation of k_1 and k_2, as does equation (2), because of the occurrence of a third variable, k_3. None of the constants k_1, k_2 and k_3 is a true velocity constant as they must contain various concentration terms which are not varied under the experimental conditions used. This theory would therefore seem to explain the experimental results more correctly than that of Segal (1955).

There have been many reports in the literature of the inhibition of sulphate-ester synthesis by corticosteroids and by salicylates, but most of these apply to the synthesis of mucopolysaccharides by tissue slices and so are difficult to interpret, as has been stressed by Kodicek & Loewi (1955). The results described above confirm the inhibition of sulphate-ester synthesis by corticosteroids, but show that salicylates have no effect on this process, at least in DHAS synthesis. It must therefore be presumed that previous reports (DeMeio & Tkacz, 1952) of the inhibition by salicylates of aryl sulphate synthesis in liver slices were based on the inhibition of some energy-yielding process involved in the much more complex systems studied by these authors.

One of the most interesting results of the present investigation is the demonstration in rat liver of an enzyme system capable of forming the sulphate esters of a number of steroid alcohols. This shows to be incorrect the statement of Anderton, Smith & Williams (1948) that before a hydroxyl group can be conjugated with sulphate it must have a pK in the range 7–10 and must occur in an enolizing system such as –C:C(OH).C. Of the steroids tested above only oestrone satisfies these conditions, all the other steroids being typical alcohols. It might be argued that the claims of Anderton *et al.* (1948) were based on *in vivo* studies, but there is no reason to suppose that the *in vitro* reactions do not also occur *in vivo*, especially as many of the steroid sulphates have been isolated from urine. It is of interest, however, that none of the simpler alcohols studied showed the ability to conjugate with sulphate, although simple phenols did. It would therefore seem that the claim of Anderton *et al.* (1948) was an oversimplification of the situation, and that the ability of any particular hydroxyl group to conjugate with sulphate is not governed only by its pK value but also by the structure of the molecule as a whole.

SUMMARY

1. A method is described for the assay of steroid sulphate synthesis by soluble enzyme preparations from rat liver. The substrate used is dehydro*epi*androsterone, but the method may be used with any suitable steroid as substrate.

2. The properties of the enzyme system synthesizing dehydro*epi*androsterone sulphate are identical with those of the system which synthesizes aryl sulphates, and it is probable that the same enzyme system is involved in the synthesis of both types of compound.

3. The synthesis of dehydro*epi*androsterone sulphate is inhibited by *m*-aminophenol, cortisone, cortisone acetate and hydrocortisone, but is uninfluenced by salicylates.

4. The substrate specificity of the enzyme system has been investigated and has been shown to be relatively low.

5. An interpretation of the kinetic data is presented.

The author is indebted to Professor G. F. Marrian, F.R.S., and to Dr W. Klyne for gifts of steroids. He is also indebted to Mr K. Fotherby for carrying out the chromatographic analyses and to Miss Isla Sharp for her skilled technical assistance.

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The Inhibition of Carbohydrate Metabolism in Ascites-Tumour Cells by Ethyleneimines

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(*Received* 11 *November* 1955)

The influence of cytotoxic agents upon carbohydrate metabolism has been the subject of several investigations. For example, mustard gas was shown to inhibit glycolysis in Ehrlich rat sarcoma (Jany & Sellei, 1935) and in Jensen sarcoma (Berenblum, Kendal & Orr, 1936). Barron, Bartlett, Miller, Meyer & Seegmiller (1948) found that the respiration of a number of rat tissues was inhibited by methyldi-(β-chloroethyl)amine hydrochloride. Suspensions of Sarcoma 37 obtained from mice after administration of α-peltatin had a decreased ability to utilize glucose anaerobically (Waravdekar, Paradis & Leiter, 1955).

The potent growth-inhibitory properties of the ethyleneimines demonstrated by Hendry, Homer, Rose & Walpole (1951) suggested that a study of the effect of these compounds on carbohydrate metabolism in normal and malignant mammalian cells would be of interest. Cardinali (1954) has already shown that aerobic glycolysis in Rous sarcoma and benzopyrene sarcoma is inhibited by ethyleneimine picrate. In the present work, attention was concentrated mainly on the effect of 2:4:6-triethyleneimino-1:3:5-triazine (TEM) on anaerobic glycolysis in ascites-tumour cells, since this material is convenient for following changes in the concentration of cellular constituents in response to the action of metabolic inhibitors.

A preliminary account of this work has been reported (Roitt, 1955).

EXPERIMENTAL

Ascites-tumour cells. In the majority of experiments, the Krebs II carcinoma in ascites form was used and was maintained in heterozygous albino mice. The animals were killed 7–9 days after inoculation with ascites tumour and the cells were washed with isotonic saline into tubes containing heparin. The ascitic fluid was removed after centrifuging for 5 min. at 2000 *g* and the cells were resuspended in saline, spun at 100 *g* for 3 min. and the bulk of the red cells, still in suspension, removed. The resuspension and low-speed spinning were repeated until contamination with red cells was negligible. The cells were finally suspended in Krebs–Ringer phosphate solution containing 0·1% glucose for aerobic studies, and in Krebs–Ringer bicarbonate containing 0·4% glucose for experiments under anaerobic conditions. The packed-cell volume was determined by centrifuging in a Wintrobe tube for 30 min. at 800 *g*. These preparations contained approximately 5% by number of inflammatory cells, representing less than 1% of the total cell mass.

Respiration and anaerobic glycolysis. The conventional Warburg techniques were used. The media used for studying the metabolism of ascites-tumour cells were also employed for parallel studies on the other mammalian tissues. Substrates were dissolved in isotonic NaCl and the pH was adjusted to 7·4. In a few cases anaerobic lactic acid production was measured (Barker & Summerson, 1941) and shown to be paralleled by the CO_2 evolved. The buffering capacity of TEM was negligible at pH 7·4, so that no correction for retained CO_2 was necessary.

Enzyme preparations

Hexokinase. The activity of a deoxycholate-treated particulate fraction of bullock brain was followed by measuring glucose disappearance (Crane & Sols, 1953; preparation *CD*). Glucose was estimated by the anthrone method (Fairbairn, 1953) after precipitation of phosphates and protein with $Ba(OH)_2$ and $ZnSO_4$.

Triose phosphate dehydrogenase. A dialysed extract of rabbit-muscle acetone powder was used with fructose diphosphate as substrate (Green, Needham & Dewan, 1937). The evolution of CO_2 from a bicarbonate medium