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The Enzymic Synthesis of Aryl Sulphamates

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(Received 22 April 1959)

Boyland, Manson & Orr (1957) have shown that the administration of certain arylamines to rats or to rabbits is followed by the urinary excretion of the corresponding aryl sulphamates, compounds whose natural occurrence had not previously been recognized. This was followed by the demonstration of the synthesis *in vitro* of 2-naphthyl sulphamate by rat-liver preparations (Roy, 1958) and of phenyl sulphamate by sheep intestinal-mucosal enzymes (Kent & Pasternak, 1958). These results were of interest, as the reactions involved might be expected to be related to those involved in the synthesis of the sulphamate groups of heparin (Foster & Huggard, 1955). In the light of the present ideas on the synthesis of sulphuric acid esters (Lipmann, 1958) it seemed likely that the synthesis of aryl sulphamates would involve sulphate transfer from 'active sulphate', adenosine 3'-phosphate 5'-sulphatophosphate, this reaction being catalysed by a specific sulphokinase, arylamine sulphokinase. The present paper describes an investigation into the enzymic synthesis of aryl sulphamates and, in particular, into the influence of certain steroids on this reaction. A preliminary account of the work has already appeared (Roy, 1959).

EXPERIMENTAL

Preparation of the enzyme system. This was carried out essentially as previously described (Roy, 1956). With 5 g. samples of liver the tissue was treated with 3 vol. of 0.15 M-KCl containing mM-ethylenediaminetetra-acetate (EDTA), pH 7, in a glass homogenizer, for two 60 sec. periods.

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Larger amounts of tissue were treated with 3 vol. of the KCl solution in an Atomix blender (M.S.E. Ltd.) for two 30 sec. periods. The resulting tissue suspension was centrifuged for 2 hr. at 20 000 g and the supernatant precipitated between 1.5 and 2.3 M-(NH₄)₂SO₄, to give the enzymically active fraction. This was dissolved in water so that 1 ml. of the resulting solution corresponded to 500 mg. of liver; the protein concentration was approximately 18 mg./ml.

The sulphate-activating system, a mixture of adenosine triphosphate (ATP) sulphurylase and adenosine 5'-sulphatophosphate phosphokinase (Lipmann, 1958), was prepared by heating the above enzyme solution at 52° (DeMeio, Wizerkaniuk & Schreiber, 1955) for 30 min. and removing the denatured protein by centrifuging.

The sulphate-activating system from baker's yeast was prepared exactly as described by Nose & Lipmann (1958).

The sulphokinase fraction of the enzyme was prepared from the complete enzyme system by dialysing the latter for 48 hr. against repeated changes of 100 vol. of water at 0°, a procedure which destroyed the sulphate-activating system because of the latter's instability in the absence of SO₄²⁻ ions. The resulting suspension was made 0.03 M in glycylglycine and adjusted to pH 8 with N-Na₂CO₃. After the suspension had stood for a few hours any remaining insoluble material was removed by centrifuging. The enzyme suspension obtained after dialysis could be used without further treatment, but a considerable proportion of the sulphokinase was present in the insoluble fraction and was rather unstable in this state, the activity being destroyed on freezing. The soluble sulphokinase preparations obtained after glycylglycine treatment were stable and could be stored frozen for long periods.

Preparation of adenosine 3'-phosphate 5'-sulphatophosphate. This was prepared enzymically by incubating 100 ml. of a mixture containing 50 ml. of the above unfractionated enzyme preparation, 10 ml. of 0.2 M-phosphate buffer (pH 7.2), 10 ml. of 0.04 M-MgSO₄-0.06 M-K₂SO₄ and 400 μmoles of ATP (crystalline sodium salt, Sigma Chemical Co., adjusted to pH 7.2 with N-NaOH) for 1 hr. at 37°.

Proteins were precipitated by adding 3 vol. of ethanol and removed by centrifuging. The ethanol was removed from the supernatant by concentrating it *in vacuo* at a bath temperature of 40° to about 25 ml.; the resulting solution was brought to pH 8 with *N*-NaOH, insoluble material removed by centrifuging and the volume made up to 50 ml. with water. The resulting solution was stored frozen at -10°.

This preparation contained nucleotides other than adenosine 3'-phosphate 5'-sulphatophosphate, but was used for experiments involving assays of the sulphokinase fraction, prepared as above, which cannot utilize ATP and SO_4^{2-} ions for the synthesis of sulphate esters.

Chromatography. Chromatographic separation of the metabolites of 2-naphthylamine, and related arylamines, was carried out by descending chromatography on Whatman no. 1 paper with the solvent system butan-1-ol-propan-1-ol-aq. 0.1*N*- NH_3 (2:1:1, by vol.) as described by Boyland *et al.* (1957). 2-Naphthyl sulphamate and other arylamine metabolites containing a free, or potentially free, amino group were detected as orange-yellow spots after spraying successively with *N*-HCl, 0.1% (w/v) NaNO_2 and 0.5% (w/v) thymol in 2*N*-NaOH. They could also be detected by spraying with Ehrlich's reagent [0.5% (w/v) dimethylaminobenzaldehyde in 0.1*N*-HCl in ethanol] and heating at 37° for 30 min. The R_f values of 2-naphthyl sulphamate and 2-naphthylamine were 0.26 and 0.83 respectively.

Determination of 2-naphthyl sulphamate. This was carried out by hydrolysis to 2-naphthylamine, followed by diazotization and coupling. To 3 ml. of sulphamate solution, free from naphthylamine, was added 0.3 ml. of conc. HCl and the mixture heated for 30 min. in boiling water. After cooling, 1.0 ml. of 0.1% (w/v) NaNO_2 was added, followed after 5 min. by 5 ml. of 0.5% (w/v) thymol in 5*N*-NaOH. The intensity of the resulting orange-yellow colour was read in the Spekker absorptiometer, with Ilford filter 602 (maximum transmission at 470 μ). Calibration curves prepared from 2-naphthyl sulphamate and 2-naphthylamine were identical.

The method could be applied to the determination of other aryl sulphamates, although with some, such as phenyl sulphamate, acid hydrolysis was not required, as the sulphamate decomposed quantitatively during the diazotization.

Assay of 2-naphthyl sulphamate synthesis. The method varied, according to the object of the assay and the enzyme preparation used. With the unfractionated enzyme system the volume of the reaction mixture was 5 ml., containing 0.5 ml. of buffer (see below), 0.5 ml. of 0.04*M*- MgSO_4 -0.06*M*- K_2SO_4 , 1 ml. of 0.02*M*-ATP (crystalline sodium salt, adjusted to the appropriate pH with *N*-NaOH), 1 ml. of 0.01*M*-2-naphthylamine hydrochloride and 2 ml. of enzyme solution. With enzyme preparations from guinea-pig liver the amount used corresponded to approximately 60 mg. of tissue, but with rat preparations some 200 mg. of tissue was required. Incubation was for 2 hr. at 37°. Control assays were simultaneously carried out under identical conditions except that no ATP was added. Values obtained by these controls were identical with those in which the enzyme was incubated separately from the remainder of the reaction mixture (containing ATP).

For the qualitative demonstration of sulphamate synthesis by chromatography the buffer used was 0.2*M*-

potassium phosphate buffer, pH 7.2: this buffer was chosen despite the fact that the pH was not optimum because it was to a large extent eliminated by the subsequent treatment and so did not interfere with the chromatography. The reaction was stopped by the addition of 25 ml. of ethanol, the precipitate removed by centrifuging and the supernatant evaporated to dryness *in vacuo*. The residue was taken up in 0.3 ml. of water, insoluble material centrifuged off and 0.02 ml. samples were used for chromatography as described above.

Quantitative assays were carried out as above but with 0.5*M*-2-amino-2-hydroxymethylpropane-1,3-diol-acetic acid buffer (tris buffer), pH 8.2, and the mixture was deproteinized by the addition of 2 ml. of 25% (w/v) trichloroacetic acid. Proteins were centrifuged off and free amino compounds were removed from the supernatant by shaking it for two 15 min. periods, each with 2 g. of Zeo-Karb 225 H^+ (Permutit Co. Ltd., London). After centrifuging, 3 ml. samples of the resulting solution were taken for the determination of sulphamate as described above.

The technique used in the assay of sulphamate synthesis from adenosine 3'-phosphate 5'-sulphatophosphate by sulphokinase preparations was similar. The volume of the reaction mixture was 5 ml., containing 1 ml. 0.5*M*-tris buffer (pH 8.2), 1 ml. of 0.01*M*-magnesium acetate, 1 ml. of 0.01*M*-2-naphthylamine hydrochloride, 1 ml. of the adenosine 3'-phosphate 5'-sulphatophosphate solution described above and 1 ml. of a sulphokinase preparation corresponding to some 250 mg. of liver. Incubation was at 37°, for 1 hr. with guinea-pig preparations, but for 2 hr. with rat preparations. The reaction was stopped by the addition of 2 ml. of 25% (w/v) trichloroacetic acid and thereafter the procedure was as described above for the assay with unfractionated enzyme preparations. Controls in which adenosine 3'-phosphate 5'-sulphatophosphate was omitted from the reaction mixture were carried out with each set of determinations.

Assay of steroid and aryl sulphate synthesis. This was carried out as previously described by using the methylene-blue technique (Roy, 1956). The ATP concentration used in these assays was 4 mM, not 2 mM as originally described: this higher optimum ATP concentration was apparently due to the use of crystalline ATP preparations in the present series of determinations. The substrate concentrations used were: *p*-nitrophenol, 1 mM; oestrone, 0.1 mM; dehydroepiandrosterone, 0.1 mM; and epitestosterone, 0.1 mM.

RESULTS

The general validity of the methods of analysis was first demonstrated as follows. A qualitative assay was carried out as described above and after removal of a 0.02 ml. sample of the resulting solution for chromatographic analysis the remainder was diluted to about 6 ml. with water and treated with Zeo-Karb 225 H^+ as described above. The solution was neutralized, evaporated to dryness *in vacuo* and the residue taken up in 0.25 ml. of water. A 0.02 ml. sample of this solution was taken for chromatography and to the remainder was added 0.25 ml. of 2*N*-HCl. The mixture was heated on a boiling-water bath for 30 min. and then again

taken to dryness. The residue was dissolved in 0.25 ml. of water and a further 0.02 ml. sample was taken for chromatography. Before the resin treatment, spots corresponding to 2-naphthylamine, 2-naphthyl sulphamate and two unidentified metabolites were obtained; after treatment with Zeo-Karb 225 H⁺ only the spot corresponding to 2-naphthyl sulphamate remained, whereas after the acid hydrolysis only 2-naphthylamine could be detected. The method is therefore satisfactory in that the resin treatment removed all the metabolites of 2-naphthylamine which could be detected by diazotization, or by Ehrlich's reagent, other than 2-naphthyl sulphamate.

The recovery of 2-naphthyl sulphamate added to the reaction mixture without incubation was approximately constant at 90 %. The recovery of the sulphamate after ethanol precipitation of the proteins, as used in the chromatographic experiments, was very poor and could not be increased above about 50 %.

General properties of the enzyme system. These studies were carried out with enzyme preparations from rat or from guinea-pig livers, there being no significant differences between the results obtained with the two species. The optimum ATP concentration was 4 mM; the optimum concentration of SO₄²⁻ was 10 mM and the optimum pH was 8.2–8.3 in 0.05M-tris buffers. The optimum substrate concentration could not be determined as it was apparently considerably greater than 2 mM-2-naphthylamine, the highest concentration with

which it was possible to work because of the limited solubility of the amine in alkaline solutions.

In these conditions there was, with enzyme preparations from guinea-pig liver, a linear relation between the amount of enzyme present and the extent of sulphamate synthesized (Fig. 1) and the reaction was of zero order for at least 2.5 hr. This was not strictly so with rat-liver preparations, with which the reaction velocity did not increase strictly in proportion to the enzyme concentration, as shown in Fig. 1. This behaviour of the rat enzyme was not altered by dialysis of the enzyme preparation against 0.01M-K₂SO₄ in an attempt to remove possible endogenous inhibitors whose presence would account for the shape of the appropriate curve in Fig. 1.

No thorough search for inhibitors has been undertaken, but the synthesis of 2-naphthyl sulphamate is strongly inhibited by EDTA, 0.05M-EDTA inhibiting the reaction by about 99 %. This is to be expected, as it is known that the activation of sulphate depends upon the presence of Mg²⁺ ions (Robbins & Lipmann, 1958). As mentioned below, arylamine sulphokinase also requires Mg²⁺ for its full activity.

The substrate specificity of the enzyme system has not been thoroughly investigated, but, as already briefly reported (Roy, 1958), 1-naphthylamine and aniline can also form sulphamates in this system although aniline reacted much more slowly. Neither benzylamine nor glucosamine inhibited the synthesis of 2-naphthyl sulphamate; this would suggest that these substances are not substrates for this enzyme system.

Role of adenosine 3'-phosphate 5'-sulphatophosphate. From the generally accepted mechanism of sulphate-ester formation (Lipmann, 1958) it seemed likely that adenosine 3'-phosphate 5'-sulphatophosphate would be an intermediary in the formation of sulphamates. Evidence for this was first obtained when it was shown that neither the sulphate-activating system nor the sulphokinase fraction, prepared as described above, could catalyse the formation of 2-naphthyl sulphamate, but that a mixture of the two fractions did catalyse sulphamate formation. The results of a typical experiment are shown in Table 1, which also shows that the sulphate-activating system from yeast could replace that obtained from liver. This seemed good evidence for the hypothesis that adenosine 3'-phosphate 5'-sulphatophosphate was indeed involved in the synthesis of 2-naphthyl sulphamate.

It was then shown chromatographically that, when a preparation of adenosine 3'-phosphate 5'-sulphatophosphate, obtained as described above, was incubated with the sulphokinase fraction in the

Fig. 1. Effect of varying amounts of enzyme on the rate of synthesis of 2-naphthyl sulphamate. Vol. of reaction mixture 5 ml., containing volumes of enzyme solution corresponding to the specified amounts of liver; pH 8.2 in 0.05M-tris buffers; ATP, 4 mM; SO₄²⁻, 10 mM; Mg²⁺, 4 mM; 2-naphthylamine, 2 mM. ○, Guinea pig; ●, rat.

absence of added SO_4^{2-} ions, formation of 2-naphthyl sulphamate occurred. These sulphokinase preparations cannot utilize ATP and SO_4^{2-} ions for sulphamate synthesis (Table 1), so that their presence in the crude preparations of adenosine 3'-phosphate 5'-sulphatophosphate which were available was of no consequence.

From these experiments it therefore seemed justifiable to conclude that sulphate was transferred from adenosine 3'-phosphate 5'-sulphatophosphate to 2-naphthylamine, forming 2-naphthyl sulphamate, by a presumably specific sulphokinase, arylamine sulphokinase. The pH optimum for the reaction was 8.2-8.3 in 0.1M-tris buffers, identical with that for the overall reaction. The transfer reaction was, as expected, independent of the presence of SO_4^{2-} ions, but its velocity was governed by the concentration of adenosine 3'-

phosphate 5'-sulphatophosphate. As shown in Fig. 2, 1 ml. of the standard preparation of adenosine 3'-phosphate 5'-sulphatophosphate described above was sufficient to saturate the enzyme: assuming a quantitative transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate it follows that the concentration of the latter in the solution must be at least 0.25 mM. Arylamine sulphokinase required the presence of Mg^{2+} ions in concentrations of at least 2 mM, before it exhibited its full activity: in the absence of added Mg^{2+} ions the activity was only about 40 % of the maximum.

Arylamine sulphokinase is probably an SH enzyme, as it is inhibited by about 50 % with mM-*p*-chloromercuribenzoate, this inhibition being almost completely reversed by cysteine.

The above results were obtained with both rat and guinea-pig sulphokinase preparations.

Effect of steroids on sulphamate synthesis. If, as indicated above, the synthesis of 2-naphthyl sulphamate involved transfer from adenosine 3'-phosphate 5'-sulphatophosphate then it might be expected that the addition of steroids such as dehydroepiandrosterone to the reaction mixture would cause a drop in the amount of sulphamate synthesized by competing for the adenosine 3'-phosphate 5'-sulphatophosphate, it being known that the enzyme preparations used in this work can form steroid sulphates (Roy, 1956). With enzyme preparations from guinea-pig liver the addition of dehydroepiandrosterone to the reaction mixture did in fact cause an inhibition of 2-naphthyl sulphamate synthesis, 0.05 mM dehydroepiandrosterone causing a 90 % inhibition. It is, however, unlikely that the inhibition is due to a competition for adenosine 3'-phosphate 5'-sulphatophosphate, as dehydroepiandrosterone sulphate similarly inhibited this reaction and also the synthesis of phenyl sulphamate from aniline.

On the other hand, when the influence of similar steroids on the synthesis of 2-naphthyl sulphamate by rat-liver preparations was studied the very surprising result was an increase in the amount of sulphamate produced. These results are summarized in Table 2 and Fig. 3, the latter showing that the effect was most pronounced at concentrations of approximately 10^{-5} M steroid. At concentrations of less than 10^{-5} M the free steroid and its sulphate ester had approximately similar activating effects, but at higher concentrations the effect of the free steroid fell off more rapidly. The magnitude of the effect varied in different enzyme preparations but its occurrence was quite constant; with a concentration of 10^{-5} M-epiandrosterone the activation produced varied from approximately 300 to 500 % with different preparations.

In experiments involving the use of free steroids these were added as the thin film obtained by

Table 1. *Participation of the sulphate-activating system and a sulphokinase in the synthesis of 2-naphthyl sulphamate*

The enzyme fractions were prepared and the assays were carried out as described in the text. The activities are expressed as μm -moles of 2-naphthyl sulphamate formed/ml. of enzyme preparation in 2 hr.

Enzyme preparation	Activity
Unfractionated enzyme	140
Activating system	40
Sulphokinase fraction	0
Sulphokinase fraction + activating system	110
Yeast activating system	0
Yeast activating system + sulphokinase fraction	68

Fig. 2. Effect of varying amounts of the standard adenosine 3'-phosphate 5'-sulphatophosphate preparation (see text) on the rate of synthesis of 2-naphthyl sulphamate by guinea-pig sulphokinase preparations. Vol. of reaction mixture 5 ml., containing enzyme corresponding to 250 mg. of liver; pH 8.2 in 0.1M-tris buffers; Mg^{2+} , 2.5 mM; 2-naphthylamine, 2 mM.

Table 2. Influence of steroids and steroid sulphate on the synthesis of 2-naphthyl sulphamate

The enzyme activity is expressed relative to that in controls with no added steroid (activity 1.0). The steroid concentration was 10^{-5} M and the steroid sulphate concentration 10^{-4} M.

	Steroid	Steroid sulphate
3-Hydroxyoestra-1:3:5-triene	0.9	—
3-Hydroxyoestra-1:3:5-trien-16-one	1.4	—
3-Hydroxyoestra-1:3:5-trien-17-one	1.4	—
3-Methoxyoestra-1:3:5-trien-17-one	1.4	—
3:16 α -Dihydroxyoestra-1:3:5-trien-17-one	0.9	—
3 β -Hydroxy-5 α -androstan-17-one	1.1	—
3 β -Hydroxyandrost-5-ene	1.0	—
3 α -Hydroxy-5 α -androstan-17-one (Androsterone)	2.1	2.4
3 β -Hydroxy-5 α -androstan-17-one (<i>epi</i> Androsterone)	3.3	3.4
3 α -Hydroxy-5 β -androstan-17-one	2.1	2.1
3 β -Hydroxy-5 β -androstan-17-one	2.2	2.6
3 β -Hydroxyandrost-5-en-17-one (Dehydro <i>epi</i> androsterone)	2.4	2.9
3 β -Acetoxyandrost-5-en-17-one	1.8	—
3 β -Methoxyandrost-5-en-17-one	2.5	—
3 β :16 β -Dihydroxyandrost-5-en-17-one	0.8	—
6 β -Hydroxy-3:5- <i>cyclo</i> androstan-17-one	1.3	—
17 α -Hydroxyandrost-4-en-3-one	0.8	1.1
17 β -Hydroxyandrost-4-en-3-one	0.9	0.7
3 β :17 β -Dihydroxyandrost-4-ene	0.9	—
Androst-4-ene-3:17-dione	1.9	—
11 β -Hydroxyandrost-4-ene-3:17-dione	2.5	—
Androst-4-ene-3:11:17-trione	2.1	—
3 α -Hydroxy-5 α -pregnan-20-one	0.8	0.4
3 β -Hydroxy-5 α -pregnan-20-one	0.9	0.7
3 α -Hydroxy-5 β -pregnan-20-one	0.9	0.7
3 β -Hydroxy-5 β -pregnan-20-one	0.9	0.6
3 β -Hydroxypregn-5-en-20-one	0.9	0.5
17 α :21-Dihydroxypregn-4-ene-3:11:20-trione	0.9	—
11 β :17 α :21-Trihydroxypregn-4-ene-3:20-dione	1.0	—

Fig. 3. Influence of steroids (A) and steroid sulphates (B) on the velocity of 2-naphthyl sulphamate synthesis by rat-liver preparations. Conditions as in Fig. 1, but the enzyme concentration was kept constant at that corresponding to 200 mg. of liver. Activity is expressed relative to controls with no added steroid. O, *epi*Androsterone and sulphate; ●, dehydro*epi*androsterone and sulphate; x, androsterone and sulphate.

evaporating to dryness in the reaction tubes a solution of the requisite amount of the steroid in ether. The remainder of the reaction mixture was then added. The low concentrations of steroid required and the relatively prolonged incubation times justified this procedure, which eliminated

any possible complicating effects that might have arisen through using a steroid solvent, such as propylene glycol.

From the results summarized in Table 2 it is obvious that of the large number of steroids studied only those having a keto group in the 17 position, or perhaps in the 16 position, exhibited this activating effect on the synthesis of 2-naphthyl sulphamate by rat-liver preparations. Chromatographic experiments showed that the increase was not due to any general increase in the metabolism of 2-naphthylamine, as only the amount of 2-naphthyl sulphamate was increased in the presence of dehydro*epi*androsterone, the amounts of the other metabolites not being altered.

There appeared to be an antagonism between 17-oxo and 17-hydroxy steroids, especially the 17 α -hydroxy compound, as these depressed the activating effect of the former. In a typical experiment the presence of *epi*androsterone (10^{-5} M) in the reaction mixture increased the amount of sulphamate synthesized to some 300% of the control value: when testosterone or *epitestosterone* of the same concentration was simultaneously present the activity was respectively only 190 or 250% of the control. The presence of 17-deoxy steroids together with *epi*androsterone had little effect on the activation caused by the latter.

For investigation of the possible mechanism of this activation *epiandrosterone* and *dehydroepiandrosterone*, or their sulphate esters, were chosen. The free steroids were used at a concentration of 10^{-5} M and the sulphates at 10^{-4} M. The activating effect of all these compounds depended upon the presence of ATP and SO_4^{2-} ions in the reaction mixture; this would appear to eliminate the possibility which was first considered, namely that the sulphate group was being transferred from the steroid sulphate to the 2-naphthylamine. Such a transfer would not be expected to require the presence of SO_4^{2-} ions provided that a sufficient concentration of the steroid sulphate was present. In a system containing a steroid sulphate the ATP could not be replaced by adenosine 3':5'-diphosphate, which acts as a cofactor in the transfer of sulphate from *p*-nitrophenyl sulphate to phenol (Gregory & Lipmann, 1957). The activation of sulphamate synthesis was apparently a fairly general phenomenon as the rate of synthesis of phenyl sulphamate and of 1-naphthyl sulphamate was also increased by *dehydroepiandrosterone* sulphate.

The influence of steroids and of steroid sulphates on the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to 2-naphthylamine by arylamine sulphokinase was also investigated. The results were similar to those for the overall synthesis of sulphamate from ATP and SO_4^{2-} ions. It therefore follows that the activation of sulphamate synthesis by steroids must be due to an action of the latter on the sulphokinases rather than on the sulphate-activating system. It must again be stressed that the sulphokinase prepara-

DISCUSSION

The above results show that liver preparations can synthesize 2-naphthyl sulphamate, and related aryl sulphamates, from the parent amine in the presence of ATP and SO_4^{2-} ions. This synthesis probably involves the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate, a reaction catalysed by arylamine sulphokinase. Although this enzyme has not yet been separated from the other sulphokinases present in the enzyme preparations used there seems no reason to doubt its separate identity; this view is supported by the high specificity of the known sulphokinases (Nose & Lipmann, 1958) and by the comparative data summarized in Table 3. Arylamine sulphokinase is further distinguished from phenol sulphokinase by the requirement of the former for Mg^{2+} ions, the activity of phenol sulphokinase apparently being independent of the presence of these ions (Gregory & Lipmann, 1957), although the results of Segal (1957) do not confirm this latter claim.

The most interesting observation is the pronounced activation of the synthesis of 2-naphthyl sulphamate by 17-oxo steroids. As yet it has not proved possible to elucidate completely the mechanism of this activation, but a reasonable hypothesis would seem to be that the steroids are functioning in their corresponding enolic forms. Some evidence for this is given in Table 2, which shows that 16-hydroxy 17-oxo steroids, which cannot enolize, do not activate the synthesis of sulphamate. A possible explanation for this activation would seem to be provided by the following reaction sequence:

tions used in these experiments contain sulphokinases other than arylamine sulphokinase and the steroid effect may well be on the former, as is suggested below.

Rates of synthesis of other sulphate esters. The results of these determinations are summarized in Table 3. These figures refer to the overall rate of synthesis of the sulphate ester from ATP and SO_4^{2-} ions. From these data it is obvious that in the rat arylamine sulphokinase must be the limiting enzyme in the synthesis of 2-naphthyl sulphamate, as the enzyme preparations can synthesize *p*-nitrophenyl sulphate at approximately $1 \mu\text{mole/g./hr.}$, and so presumably can synthesize adenosine 3'-phosphate 5'-sulphatophosphate at a similar rate, yet the rate of sulphamate formation is only about 25% of this. In the guinea pig, on the other hand, arylamine sulphokinase is probably not rate-limiting.

The compound primarily responsible for the increase in the amount of sulphamate formed is presumed to be the 17-enol sulphate, formed enzymically from adenosine 3'-phosphate 5'-sulphatophosphate by a reaction catalysed either by a general steroid sulphokinase or, more probably, by a specific 17-hydroxy steroid sulphokinase. Rat-liver preparations, similar to those used in the present experiments, can form steroid 17-sulphates from 17-hydroxy steroids (Roy, 1956; Schneider & Lewbart, 1956) and it may be that the sulphokinase involved could also yield 17-enol sulphates under suitable conditions. Such a 17-enol sulphate would be expected to have a very high sulphate-group potential, comparable with the phosphate-group potential of phosphoenol pyruvate, and it might well be able to transfer its sulphate group to an acceptor such as 2-naphthylamine to form the sulphamate in which the sulphate-group potential

Table 3. *Rates of synthesis of sulphate esters by liver preparations*

The general conditions of assay were as described in the text and the rates of synthesis are expressed as $\mu\text{m-moles synthesized/g. of liver/hr.}$

	Rat		Guinea pig (male and female)
	Male	Female	
<i>p</i> -Nitrophenyl sulphate	1500	1200	370
Oestrone sulphate	78	47	72
Dehydroepiandrosterone sulphate	120	390	260
<i>epi</i> Testosterone sulphate	59	200	150
2-Naphthyl sulphamate	140	320	1200

would certainly be relatively low. No mechanism can yet be suggested for such a transfer; but it cannot involve arylamine sulphokinase, as it is obvious from the data of Table 3 that this enzyme is the rate-limiting one in the synthesis of sulphamate by rat-liver preparations.

The test of this theory would be the synthesis of a 17-enol sulphate and its use as a sulphate donor in the absence of adenosine 3'-phosphate 5'-sulphatophosphate. Unfortunately this has not so far proved possible, as attempts to prepare these compounds chemically have failed and there is no reason to assume that they would accumulate in the enzyme systems used above.

The above sequence of reactions could also account for the failure of 17-oxo steroids to activate the synthesis of 2-naphthyl sulphamate by guinea-pig-liver preparations. As pointed out above, in such preparations the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate is probably the rate-limiting reaction in the overall synthesis of 2-naphthyl sulphamate; if this indeed be the case then activation by the mechanism postulated above could not occur. On the other hand, the synthesis of phenyl sulphamate by guinea-pig preparations is a much slower reaction than the synthesis of 2-naphthyl sulphamate, so that in the former case arylamine sulphokinase must be rate-limiting; activation of phenyl sulphamate synthesis by steroids might therefore be expected. As reported above, dehydroepiandrosterone sulphate inhibits the synthesis of phenyl sulphamate by guinea-pig enzymes; this would suggest that some other explanation must be sought for the failure of steroids to activate sulphamate synthesis in preparations from this species. The reason might lie in the absence of a sulphokinase capable of forming steroid 17-enol sulphates in guinea-pig liver, but no definite evidence on this point is available at present.

It cannot yet be stated whether or not this steroid activation of sulphamate synthesis in the rat has any physiological significance. The concentrations of steroids used are close to the physiological; but the significance of aryl sulphamate synthesis in the normal animal is obscure, as no

such compounds have ever been detected therein. Should steroids have a similar action on the synthesis of the sulphamate group of heparin, however, then the mechanism may well be of considerable physiological importance, as steroids are known to influence mucopolysaccharide metabolism *in vivo*. Aryl sulphamates have been used as sulphating agents *in vitro* (Brodersen & Quaedvlieg, 1933); it is therefore possible that aryl sulphamates might have a similar action *in vivo*, forming sulphate esters by a mechanism not directly involving adenosine 3'-phosphate 5'-sulphatophosphate. There is, of course, no evidence for this at present.

Perhaps the most important point arising from this work is the suggestion that forms of 'active sulphate' other than adenosine 3'-phosphate 5'-sulphatophosphate may exist and that a steroid 17-enol sulphate may be an example of such a compound with a high sulphate-group potential.

SUMMARY

1. A method is described for the assay of 2-naphthyl sulphamate synthesis by liver preparations. The determination of the sulphamate is based on the removal of free amino compounds by treatment with ion-exchange resin, hydrolysis of the sulphamate to the amino compound and its subsequent determination by diazotization and coupling with thymol.

2. The optimum pH of the reaction is 8.2, the optimum concentration of adenosine triphosphate is 4 mM, the optimum sulphate concentration is 10 mM, and the optimum concentration of 2-naphthylamine is greater than 2 mM.

3. 1-Naphthylamine and aniline are also substrates for this enzyme system, but benzylamine and glucosamine are not.

4. The sulphamate is probably formed by the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate, the transfer being catalysed by arylamine sulphokinase, which has an optimum pH of 8.2 and requires the presence of at least 2 mM-Mg²⁺ ions before it shows its activity. The enzyme is inhibited by *p*-chloromercuribenzoate and the inhibition abolished by cysteine.

5. The rate of synthesis of 2-naphthyl sulphamate by rat-liver preparations is increased by about 400 % by 17-oxo steroids in concentrations of approximately 10^{-5} M.

6. The rate of synthesis of sulphamate by guinea-pig preparations is not increased by 17-oxo steroids.

7. It is suggested that the mechanism of this activation involves the formation of steroid 17-enol sulphates and that these enol sulphates would be a form of 'active sulphate', having a high sulphate-group potential.

8. The rates of synthesis of certain other sulphate esters by the enzyme preparations are described.

The author wishes to express his gratitude to Miss Pamela Burton for carrying out many of the preliminary experiments and for making the observations which led to the discovery of the steroid effects. He is also indebted to Miss Isla Sharp for her skilled technical assistance throughout the work.

Thanks are due to Professor G. F. Marrian, F.R.S., and to Dr W. Klyne for generous gifts of steroids, and to Professor J. Baddiley for samples of adenosine 3':5'-diphosphate.

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The Cholesterol Esters Circulating in Human Blood in Health

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(Received 4 May 1959)

The free and esterified cholesterol circulating in human blood has frequently been measured but, because of the experimental difficulties involved, scant attention has been paid to the fatty acids combined with cholesterol. Kelsey & Longnecker (1941), using selective enzymic hydrolysis of the esters (Kelsey, 1939*a, b, c*), identified some of the esters in the serum of lactating cows and, with chromatographic methods, Lough & Garton (1957) have been able to demonstrate the presence of many more. It seemed possible that knowledge of the identity of the fatty acid component of these esters would throw some light on the metabolism of cholesterol in the human.

In the present work, chromatographic methods were employed to isolate the cholesterol esters from the lipids of normal human sera, to establish their identity and estimate the amount of each fatty acid. The results obtained indicate that over 70 % of the acids are unsaturated and more than half of these are polyethenoid.

* Some of this work formed part of a thesis submitted to the University of Edinburgh for the degree of M.D.

EXPERIMENTAL

Materials

Most of the fatty acids used in examining the characteristics of the columns were prepared from commercial specimens by fractional distillation of their methyl esters at pressures of about 0.1 mm. Hg. The commercial samples of stearic acid were, however, found to be heavily contaminated with palmitic acid. A sample of high purity was eventually prepared by hydrogenation of commercial elaidic acid (L. Light and Co. Ltd.). A sample of pure linoleic acid was kindly provided by Unilever Research Laboratories, Port Sunlight, Cheshire. All solvents were of reagent grade except for the ether, which was of micro-analytical reagent grade. The acetone was slightly acid and the resulting small titration blank was allowed for in the determination of the fatty acids.

Blood samples. Two samples of serum and two of plasma were analysed. Sample I consisted of 100 ml. of pooled sera from 25 patients, none of whom was suffering from liver disease. Sample II was 25 ml. of serum from a healthy male aged 39 years. Sample III was 400 ml. of plasma from two separate pints of transfusion-bank blood; this sample was stored at 0° for 6 weeks before it was extracted. Sample IV was 200 ml. of plasma obtained from another