The Enzymic Synthesis of Aryl Sulphamates

2. THE EFFECT OF 3 β -METHOXYANDROST-5-EN-17-ONE ON ARYLAMINE SULPHOKINASE*

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In the first paper of this series (Roy, 1960) it was shown that an enzyme system in mammalian livers could synthesize 2-naphthyl sulphamate, and related aryl sulphamates, by a series of reactions terminating in the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to the arylamine, this step being catalysed by a specific sulphokinase, arylamine sulphokinase. The rate of synthesis of 2-naphthyl sulphamate by such enzyme preparations from rat liver was greatly increased by the addition to the system of low concentrations of 17-oxo steroids. There was no comparable activation of preparations from guinea-pig liver, which were, on the contrary, strongly inhibited by 17-oxo steroids. It was suggested that the activation depended upon the formation of a steroid 17-enol sulphate, through the action of an unidentified sulphokinase, followed by the transfer of sulphate therefrom to 2-naphthylamine by a reaction which did not involve arylamine sulphokinase and which re-formed the 17-oxo steroid. The truly catalytic action of the steroid was obvious from the quantitative results: in a typical experiment (Roy, 1960) the addition of 50 μ m-moles of epiandrosterone to the reaction mixture gave an increase of 250 μ m-moles in the amount of sulphamate formed.

The present paper deals with the kinetics of the activated reaction. In this work the rather insensitive assay used previously has been replaced by one based on the methylene-blue technique of Roy (1956), which is more sensitive but cannot be used with those steroids which themselves give rise to sulphate esters. The most suitable type of steroid for the investigation would have been an otherwise unsubstituted 17-oxo steroid such as androstan-17-one, but such compounds were not readily accessible in the required amounts and the compound chosen for detailed study was 3 β -methoxyandrost-5-en-17-one, in which the methoxyl group effectively prevented any changes at the 3 position. Under the experimental conditions used it is extremely unlikely that any change could occur elsewhere, except perhaps at position 17.

As the action of the 17-oxo steroid is known to occur at some stage of the reaction subsequent to the formation of adenosine 3'-phosphate 5'-sulphatophosphate (Roy, 1960), it might be argued that it would have been preferable to study only the sulphokinase stage of the reaction with adenosine 3'-phosphate 5'-sulphatophosphate as the sulphate donor. However, as this latter reaction is the rate-limiting step in sulphamate synthesis (Roy, 1960) little would in fact have been gained by this procedure, and in this work the endogenous sulphate-activating enzymes of liver have been used to provide the adenosine 3'-phosphate 5'-sulphatophosphate. It must be stressed, however, that the kinetics of the formation of 2-naphthyl sulphamate which are described below are essentially the kinetics of the sulphokinase reaction.

EXPERIMENTAL

Preparation of the enzyme. The enzyme system was prepared as previously described (Roy, 1960) from the supernatant obtained by centrifuging for 2 hr. at 20 000*g* a 25% homogenate of rat or guinea-pig liver in 0.15*M*-KCl-1 *mm*-ethylenediaminetetra-acetate, pH 7. The active fraction was precipitated between 1.5 and 2.3 *M*-(NH₄)₂SO₄ and was dissolved in water so that 1 ml. of the solution corresponded to 500 mg. of liver. Preparations from rat liver could be stored frozen for considerable periods without loss of activity but those from guinea-pig liver were considerably less stable and often became inactive on storage for 12 hr.

The above preparation (the complete enzyme system) contained the enzymes responsible for the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate from SO₄²⁻ ions and adenosine triphosphate (ATP) as well as several sulphokinases. The sulphokinase fraction was obtained as previously described (Roy, 1960) by inactivation of the adenosine 3'-phosphate 5'-sulphatophosphate-synthesizing enzymes through removal of the protecting SO₄²⁻ ions by dialysis.

Preparation of adenosine 3'-phosphate 5'-sulphatophosphate. A crude preparation of this compound was made exactly as described by Roy (1960).

Assay of 2-naphthyl sulphamate synthesis. For the assay of the synthesis from ATP and SO₄²⁻ ions the reaction mixture had the following composition: 0.1 ml. of 0.2*M*-potassium phosphate buffer, pH 7.5; 0.1 ml. of 0.04*M*-MgSO₄-0.06*M*-K₂SO₄; 0.2 ml. of 0.02*M*-ATP (crystalline

* Part 1: Roy (1960).

sodium salt, Sigma Chemical Co.; adjusted to pH 7.5 with n-NaOH); 0.2 ml. of 0.01 M-2-naphthylamine hydrochloride; 0.2 ml. of aqueous 1% ethanol, or a solution therein of 3 β -methoxyandrost-5-en-17-one (see below), and 0.2 ml. of the complete enzyme system diluted to correspond to 50–75 mg. of rat liver or 30–40 mg. of guinea-pig liver.

For the assay of the synthesis from adenosine 3'-phosphate 5'-sulphatophosphate the reaction mixture differed from the above in that the $\text{MgSO}_4\text{-K}_2\text{SO}_4$ and ATP solutions were replaced by 0.1 ml. of 0.02 M-magnesium acetate and 0.2 ml. of adenosine 3'-phosphate 5'-sulphatophosphate solution respectively. The complete enzyme system was replaced by 0.2 ml. of the sulphokinase fraction.

It should be noted that in these experiments phosphate buffer, pH 7.5, was used in place of the 2-amino-2-hydroxy-methylpropane-1:3-diol (tris)-acetic acid buffer, pH 8.2, used previously (Roy, 1960). This change was made as it was initially planned to link this work to an investigation requiring determination of the 17-oxo steroid; in such determinations phosphate buffers caused less interference than did tris buffers. In phosphate the pH curve of the reaction was rather flat with an optimum at about pH 7.5.

The solution of 3 β -methoxyandrost-5-en-17-one was prepared immediately before use by dilution of 0.05 ml. of a 5 mM solution of the steroid in ethanol to 5 ml. with water: the final concentrations in the reaction mixture of the steroid and of ethanol were therefore 10 μM and 0.2% respectively. At this concentration ethanol was without effect on the reaction. If higher concentrations of steroid were required then the concentration of the stock ethanol solution was adjusted to keep constant the amount of ethanol in the reaction mixture.

After incubation for 2 hr. at 37° the reaction was stopped by the addition of 1 ml. of methylene blue reagent (Roy, 1956), and 5 ml. of CHCl_3 was added. The mixture was shaken vigorously for 1 min. and then centrifuged to break the emulsion. The aqueous layer was sucked off and a sample of the CHCl_3 layer, containing the methylene blue salt of the sulphamate, was dried over Na_2SO_4 . The extinction of the CHCl_3 solution at 650 $\text{m}\mu$ was determined in a Unicam SP. 600 spectrophotometer against a suitable blank. This was normally the CHCl_3 extract prepared from a reaction mixture in which the enzyme had been added to the remaining constituents only immediately before the methylene blue. The amount of 2-naphthyl sulphamate present was calculated from a suitable calibration curve. The recovery of 2-naphthyl sulphamate added to the reaction mixture was 90%.

In the following sections the amount of sulphamate formed in 2 hr. under the above general conditions in the absence of any added steroid is denoted v_0 , and that formed in the presence of 17-oxo steroid is denoted v .

RESULTS

Typical progress curves for the synthesis of 2-naphthyl sulphamate by the complete enzyme system from rat liver are shown in Fig. 1: they are similar to those previously obtained in the syntheses of other types of sulphate ester (Segal, 1955; Roy, 1956), as there is an initial lag phase followed by a linear phase in which the reaction is in the

steady state and the velocity remains constant for at least 2 hr. The general shape of this curve is unaltered by the addition of 17-oxo steroid, although the lag phase is shortened (Fig. 1). This is as expected because of the greater activity of the arylamine sulphokinase in the presence of the steroid: as Segal (1955) pointed out, the length of the lag phase is inversely related to the velocity of the sulphokinase reaction. With enzyme preparations from guinea-pig liver the general shapes of the progress curves were similar to those shown in Fig. 1 except for the strong inhibition of the reaction by the 17-oxo steroid.

The effects of changes in the concentration of the enzyme system from rat liver are shown in Fig. 2. In the complete enzyme system, both in the presence of and in the absence of 17-oxo steroid, there is a critical enzyme concentration below which no activity can be detected. This effect is due to the prolongation of the lag phase at low enzyme concentrations and it is not apparent in the sulphokinase-catalysed reaction, as is also shown in Fig. 2. Presumably at low concentrations the activity of the sulphate-activating enzymes is so small that the amounts of adenosine 3'-phosphate 5'-sulphatophosphate produced are too low to allow the sulphokinase reaction to proceed at a measurable rate. The behaviour of the guinea-pig enzyme was exactly similar to that of the rat enzyme, apart from the inhibition by the 17-oxo steroid.

Fig. 1. Progress curves for the synthesis of 2-naphthyl sulphamate by rat-liver preparations. Volume of reaction mixture, 1 ml.; pH 7.5 in 0.02 M-phosphate buffer; SO_4^{2-} ion, 10 mM; Mg^{2+} ion, 4 mM; ATP, 4 mM; 2-naphthylamine, 2 mM. Control assays, ●; assays in 10 μM -3 β -methoxyandrost-5-en-17-one, ○.

Effect of varying substrate concentrations. Fig. 3 shows the effects of changes in 2-naphthylamine concentration on the rate of sulphamate formation. With the rat enzyme there is a linear relationship between the substrate concentration and the reaction velocity, implying either that the sub-

strate does not combine directly with the enzyme or, more probably, that the highest substrate concentration attainable, 2 mM, is still considerably less than the K_m for 2-naphthylamine. Whichever explanation is correct, values for K_m and maximum velocity V obviously cannot be obtained. In the

Fig. 2. Effect of varying enzyme concentrations on the synthesis of 2-naphthyl sulphamate by the complete enzyme system (A) and the sulphokinase fraction (B) from rat liver. In A the conditions were as in Fig. 1 except that the time of incubation was held at 2 hr.; in B the conditions were similar except that no sulphate was added and the ATP was replaced by 0.2 ml. of a standard preparation of adenosine 3'-phosphate 5'-sulphatophosphate (see text). A relative concentration of 1 corresponds to the amount of enzyme isolated from 50 mg./fresh wt. of liver. Control assays, ●; assays in $10 \mu\text{M}$ - 3β -methoxyandrost-5-en-17-one, ○.

Fig. 3. Effect of varying substrate concentrations on the rate of 2-naphthyl sulphamate synthesis. Conditions were as in Fig. 1 except that the time of incubation was 2 hr. Control assays, ●; assays in the presence of the concentrations of 3β -methoxyandrost-5-ene-17-one indicated in the Figure, ○. The points + were calculated from assays containing double the usual enzyme concentration incubated for 4 hr. in order to obtain accurate values at low concentrations of substrate.

activated reaction, on the other hand, the effect of changes in 2-naphthylamine concentration is that characteristic of an enzyme reaction in which the substrate combines directly with the enzyme.

The corresponding curves for the guinea-pig enzyme are also shown in Fig. 3. With or without the addition of 17-oxo steroid they are typical of a reaction in which the substrate combines directly with the enzyme, but they are peculiar in that there is an apparent critical substrate concentration of between 0.1 and 0.2 mM-2-naphthylamine, below which no enzyme activity could be detected under the usual conditions of the assay. By using high concentrations of enzyme and prolonged times of incubation it was possible to show that the reaction did proceed at low concentrations of substrate but in this region the substrate curve of the reaction was sigmoidal in shape, as shown in Fig. 3.

In Fig. 4 are shown the usual reciprocal plots of $1/v$ against $1/s$ for the rat and of $1/v$ against $1/(s-x)$, where x is the apparent critical substrate concentration for the guinea-pig enzyme. The significance of the latter plot is discussed below. With the enzymes from both species the plots were linear and the effect of the 17-oxo steroid was the

same in both: K_m was altered, as shown by the change in the intercepts on the $1/s$ axis, but V was unaltered, as shown by the constant intercept on the $1/v$ axis. The inhibition of guinea-pig arylamine sulphokinase by β -methoxyandrost-5-en-17-one is therefore of the competitive type, and from the graphs shown in Fig. 3 values of 0.5 mM-2-naphthylamine and $0.2 \mu\text{M}$ - β -methoxyandrost-5-en-17-one can be calculated for K_m and K_i respectively, assuming for the latter that the inhibition is purely competitive. Similarly, the activation of rat arylamine sulphokinase must be of a 'competitive' type as the effect of the steroid is on K_m , but no value for this nor for V of the unactivated reaction can be obtained. As shown in Fig. 4, however, V for the activated reaction apparently does not change significantly with varying concentrations of steroid. In fact, slight alterations in V with changes in steroid concentration do occur but these are small in comparison with the corresponding changes in the apparent K_m . If it be assumed that the intercepts on the ordinate and abscissa of Fig. 4 are measures of V and K_m respectively, then an increase in the steroid concentration from $1 \mu\text{M}$ to $10 \mu\text{M}$ increases V by approximately 10%, whereas the apparent affinity of the enzyme for 2-naphthylamine, as measured by the apparent K_m , is increased by some 200%. It therefore seems justifiable, as a first approximation, to treat the activation as being of a purely competitive nature.

Effect of varying concentrations of 17-oxo steroid.

In Fig. 5 is shown the effect of varying concentrations of β -methoxyandrost-5-en-17-one on the reaction velocity. With rat arylamine sulphokinase the pronounced activation is obvious: this reaches a maximum at $10 \mu\text{M}$ -oxo steroid and decreases slightly at higher concentrations. Also shown in Fig. 5 are the comparable results for guinea-pig arylamine sulphokinase: the strong inhibition at concentrations of $1 \mu\text{M}$ -oxo steroid is obvious. As pointed out above, the inhibition is of the competitive type, but the results shown in Fig. 5 indicate that it is not purely competitive but partially competitive (Dixon & Webb, 1958), as the plot of $1/v$ against i is non-linear. This means that the true value for K_i must be less than the value of $0.2 \mu\text{M}$ - β -methoxyandrost-5-en-17-one given above: as pointed out by Dixon & Webb (1958), the determination of K_i in cases of partial competitive inhibition is difficult and the method they suggest is inapplicable in the present case because of the limited solubility of 2-naphthylamine.

For both rat and guinea-pig arylamine sulphokinases the reciprocal plots of $1/v$ against $1/i$ (where i is the concentration of 17-oxo steroid whether it is an inhibitor or an activator) are non-linear. However, as shown in Fig. 6, the plots of

Fig. 4. Reciprocal plots corresponding to experiments similar to those shown in Fig. 3. With the guinea-pig enzyme, x is the apparent critical substrate concentration (see text).

Fig. 5. Effect of varying concentrations of 3 β -methoxyandrost-5-en-17-one on the rate of synthesis of 2-naphthyl sulphamate under the same general conditions as in Fig. 1 except that the time of incubation was 2 hr. and the substrate concentrations were as indicated on the Figure. ●, Plot of v , the number of μ m-moles of 2-naphthyl sulphamate formed in 2 hr., against the steroid concentration; ○, plot of $1/v$ against the steroid concentration.

Table 1. *Effect of bilateral adrenalectomy and ovariectomy on the ability of rat-liver preparations to synthesize 2-naphthyl sulphamate*

Details of the assay are given in the text and the activities are expressed as μ m-moles of 2-naphthyl sulphamate formed/hr./g. fresh wt. of liver. The values given are means, together with the standard deviations. Both the control and the operated group contained 12 rats.

	Control group	Operated group
Activity without added oxo steroid	169 $\pm$ 47	153 $\pm$ 42
Activity in 10 μ M-oxo steroid	742 $\pm$ 171	699 $\pm$ 141

$1/(v - v_0)$ against $1/i$ are linear in both cases. With the rat enzyme the intercept on the $1/(v - v_0)$ axis varies with the substrate concentration but the intercept on the $1/i$ axis is constant. The significance of this latter intercept is discussed below. With the guinea-pig enzyme the intercepts on both the axes varied with changes in the substrate concentration.

Effect of endogenous steroids. It seemed possible that the activity of rat arylamine sulphokinase in the absence of added steroid could be due to the presence of endogenous 17-oxo steroids in the preparation and that in the absence of these the activity might disappear. To test this possibility rats were bilaterally adrenalectomized and ovariectomized: after 7 days, during which they were maintained with 1% NaCl in their drinking water, they were killed and the complete enzyme system was prepared as usual from samples of their livers. Such treatment must have considerably lowered the level of the endogenous 17-oxo steroids in the

Fig. 6. Reciprocal plot of an experiment corresponding to that shown in Fig. 5. The graph shows the plot of $1/(v - v_0)$, where v_0 and v are the velocities in the absence of and the presence of steroid respectively, against $1/i$, where i is the steroid concentration (μ). Concentrations of 2-naphthylamine: 2 mM, ●; 1 mM, ○.

livers of these animals, but there was no significant difference between the enzyme activity of these preparations and those from a similar group of untreated rats. This is shown by the results in Table 1. It would seem therefore that the activity of rat arylamine sulphokinase cannot be due to the presence of endogenous 17-oxo steroids in the enzyme preparations.

Further evidence for this view is given in Fig. 7, which shows that with enzyme preparations from individual animals the activation caused by the addition of $10\text{ }\mu\text{M}$ - 3β -methoxyandrost-5-en-17-one is independent of the initial activity of the preparations in the absence of added steroid. If the variations in the initial activities were due to variations in the amounts of endogenous 17-oxo steroids in the preparations then it would have been expected that those preparations with a low initial activity would undergo a greater activation than those with a high initial activity. This is not the case, and again it must be concluded that the activity in the absence of added 17-oxo steroid is not due to endogenous compounds of this type.

DISCUSSION

The method adopted to express the velocity of sulphamate formation (the number of μM -moles of sulphamate formed in the 2 hr. period) may be criticized because the reaction is not of zero order

Fig. 7. Comparison of the rates of sulphamate synthesis by enzyme preparations from individual rats in the presence of $10\text{ }\mu\text{M}$ - 3β -methoxyandrost-5-en-17-one and in the absence of added steroid. Each point corresponds to determinations with a single enzyme preparation from untreated female rats (●) and from bilaterally ovariectomized and adrenalectomized rats (○).

throughout this period. However, the departure from zero order is small and the method appears justifiable. In the experiment illustrated in Fig. 1, for instance, the rates of the control and activated reactions are 35 and 107 respectively when measured as described above; when calculated from the slopes of the linear portions of the progress curves the values are 40 and 112 respectively. The differences between these values, particularly for the activated reaction, are too small to justify the much more complex experimental technique which would have been required to determine in each case the slope of the linear portion of the appropriate progress curve.

The atypical shape of the substrate curve for guinea-pig arylamine sulphokinase is interesting. The phenomenon of a critical substrate concentration is of rare occurrence, and apart from its original detection in the hydrolysis of starch by barley amylase (Eadie, 1926) the only other example which appears to have been noted was found in the hydrolysis of phenyl β -glucuronide by mouse-liver β -glucuronidase (Kerr, Graham & Levvy, 1948). With both of these there was apparently a true critical substrate concentration below which no activity whatever could be detected, so that the situation differs from that in guinea-pig arylamine sulphokinase where the substrate curve is sigmoidal at low substrate concentrations. The only immediately obvious explanation of this latter type of effect is that there must be more than one active centre in the enzyme molecule and that combination of the substrate with the first of these centres facilitates the subsequent combination of substrate with the other centres. Whatever be the explanation of the effect it is obvious from the shape of the substrate curve (Fig. 3) that the normal reciprocal plot of the results will be non-linear. However, at concentrations of substrate which are high relative to the apparent critical concentration the experimental results apparently fit an equation of the type

$$v = \frac{V(s-x)}{K_m + (s-x)}, \quad (1)$$

where x is the apparent critical substrate concentration, as the plot of $1/v$ against $1/(s-x)$ is linear at such substrate levels. This is shown in Fig. 4. It follows from equation (1) that in such a reciprocal plot the intercept on the abscissa will be equal to $-1/K_m$.

The reaction sequence which was previously postulated (Roy, 1960) as an explanation of the activation of 2-naphthyl sulphamate synthesis by 17-oxo steroids was:

However, the experimental findings reported above are not in accord with such a scheme. The important discrepancies are as follows. If the formation of the enol sulphate were rate-limiting then the velocity of sulphamate production via this compound, i.e. $(v-v_0)$, should be independent of the 2-naphthylamine concentration. The plots of $1/(v-v_0)$ against $1/i$ should therefore be independent of changes in the naphthylamine concentration: as shown in Fig. 6 this is not the case. Similarly, if the transfer of sulphate from the enol sulphate to the arylamine were rate-limiting then $(v-v_0)$ would be independent of the steroid concentration, provided that this were sufficiently high to ensure that the rate of enol sulphate production were not limiting. This again is not the case. It is obvious therefore that the reaction mechanism described above is not in accord with the experimental findings and some other explanation of the activation of 2-naphthyl sulphamate synthesis by 17-oxo steroids must be sought. The inhibition of the closely related guinea-pig arylamine sulphokinase should clearly be taken into account in any interpretation of the results, as although the effects in the two species could be unrelated this seems unlikely. A satisfactory explanation of the results must therefore account for both the inhibitory and activatory effect of 17-oxo steroids.

As shown by the results in Figs. 4 and 5, the inhibition of guinea-pig arylamine sulphokinase by 3 β -methoxyandrost-5-en-17-one was of the partially competitive type. The reactions which are assumed by Dixon & Webb (1958) to occur in this type of inhibition are:

These symbols have the following significance: E, arylamine sulphokinase-adenosine 3'-phosphate 5'-sulphatophosphate complex (it is necessary to assume that all the enzyme is present in this form in order to simplify the treatment of the kinetics, and this assumption seems justifiable once the reaction has reached the steady state); S, 2-naphthylamine; I, 3 β -methoxyandrost-5-en-17-one; ES, EI and EIS are the appropriate complexes with the dissociation constants K_s , K_i , K'_i and K'_s as indicated. The velocity constant, k , for the breakdown of the binary enzyme-substrate complex is identical with that for the breakdown of

the ternary enzyme-steroid-substrate complex. In the following discussion the concentrations of the various reactants are indicated by the appropriate lower-case letters.

On the basis of these assumptions Dixon & Webb (1958) derived for a reaction of this type a velocity equation of the usual form, which can be rearranged to give

$$v = \frac{Vs}{K_s \left\{ \frac{K_i K'_i + K'_i i}{K_i K'_i + K_s i} \right\} + s} \quad (2)$$

With guinea-pig arylamine sulphokinase the terms in s would be replaced by corresponding terms in $(s-x)$, where x is the apparent critical substrate concentration. This substitution makes no difference to the general form of the equation.

Equation (2) can only be the velocity equation of an inhibited reaction when K'_i is greater than K_s ; that is, when the addition of I decreases the apparent affinity of the enzyme for its substrate. If, on the other hand, the addition of I increases the apparent affinity of the enzyme for the substrate so that K'_i is less than K_s , then equation (2) would in fact be the velocity equation of an activated reaction. This single expression can therefore represent either an inhibited or an activated reaction, depending upon the relative values of the dissociation constants K_s and K'_i . This situation might be that found in the arylamine sulphokinases, as the apparent affinities of the enzymes for 2-naphthylamine vary in the expected way (Fig. 3).

It follows from equation (2) that the plots of $1/v$ against $1/s$, or $1/(s-x)$, should be linear at constant values of i . The intercept on the $1/v$ axis will be $1/V$ and will be independent of i ; the intercept on the $1/s$ axis will be $-1/KK_s$, where K is the term in K_s , K'_i , K_i and i which appears in the velocity equation, and will therefore vary with i . As shown in Fig. 4, this is in fact the situation found in the inhibition of guinea-pig arylamine sulphokinase and in the activation of rat arylamine sulphokinase.

The velocity in the absence of any added steroid, v_0 , is given by the expression

$$v_0 = \frac{Vs}{K_s + s} \quad (3)$$

where the symbols have the same significance as those above and where the terms in s are replaced by $(s-x)$ for the guinea-pig enzyme. From these expressions for v and v_0 the following can be derived:

$$\frac{1}{v-v_0} = \frac{K_i K'_i (K_s + s)^2}{Vs K_s (K_s - K'_i) i} + \frac{(K_s + s)(K'_i + s)}{Vs (K_s - K'_i)} \quad (4)$$

From this it follows that the plots of $1/(v-v_0)$ against $1/i$ at constant values of s will be linear.

The second term on the right is the intercept on the $1/(v-v_0)$ axis and will obviously vary with s , as is in fact the case with both the guinea-pig and rat enzymes (Fig. 6). The intercept on the $1/i$ axis is

$$-\frac{(K'_s + s) K_s}{(K_s + s) K'_s K'_i} \cdot \frac{1}{K'_i}$$

This will also vary with s except when K_s and K'_s are large compared with s , when the expression will reduce to $-1/K'_i$. As is shown in Fig. 6, this simplification is apparently valid with rat arylamine sulphokinase as the intercept on the $1/i$ axis is independent of s and gives a value for K'_i of $1.5 \mu\text{M}$. 3 β -methoxyandrost-5-en-17-one.

The experimental results are therefore in accord with an explanation of the steroid effects based on the assumed formation of a ternary enzyme-steroid-substrate complex with a dissociation constant of K'_s . Rat arylamine sulphokinase is activated by such a mechanism because K'_s is less than K_s , the dissociation constant of the simple enzyme-substrate complex. Guinea-pig arylamine sulphokinase is inhibited by a similar mechanism because in this case K'_s is greater than K_s . It must be stressed, however, that although the kinetics of the reactions are in accord with this mechanism they cannot prove its occurrence, although they do make the explanation a probable one. If this is indeed the explanation of the mechanism of the action of 17-oxo steroids on arylamine sulphokinases then it is the first example of such a situation, which was discussed on theoretical grounds by Dixon & Webb (1958).

If, as suggested by the data of Fig. 3, 2-naphthylamine does not combine directly with the rat-liver enzyme, the first and fourth reactions in the above-mentioned sequence disappear, with the effect of making infinite the value of K_s in the velocity equations. The resulting expressions for the relationships between $1/v$ and $1/s$ and $1/(v-v_0)$ and $1/i$ are of the same general form and will, in general, give linear plots indistinguishable from those described above, except that in the plot of $1/v$ against $1/s$ the intercept on the ordinate will vary with changes in steroid concentration. Considerably more information will be required to allow the distinction to be made between this situation and the purely competitive one described above.

Whether or not the above explanation is correct, it is obvious that the mechanism previously suggested (Roy, 1960) is incorrect and it is not necessary to postulate the formation of a steroid 17-enol sulphate, or at least not its occurrence in the free state. It is conceivable that such a compound could be implicated in the reaction mechanism now suggested provided that it remained bound to

the enzyme. One aspect of the work requires a much more detailed investigation: this is the nature of the linkage between the steroid and the enzyme. Of the steroids so far tested only 17-oxo steroids influenced arylsulphamate synthesis (Roy, 1960). This implies that the D ring of the steroid is involved in the formation of the enzyme-steroid complex, but whether the reaction occurs through the 17-oxo group itself or through the activated 16-methylene group cannot be decided with the information at present available. The observation that 16-hydroxy-17-oxo steroids do not influence sulphamate synthesis (Roy, 1960) perhaps favours the latter view, although steric effects due to the hydroxyl group cannot be excluded. Finally, it should be pointed out that simple cyclic ketones such as cyclopentanone and cyclohexanone do not influence sulphamate synthesis, so that the combination between the steroid and the enzyme cannot simply be a function of the D ring.

The most interesting aspect of the results, whatever their interpretation, is the finding that a 17-oxo steroid can act as a powerful inhibitor of an enzyme in one species and as a powerful activator of the homologous enzyme in another, not very distantly related, species. This is made the more interesting by the fact that the steroid in question is closely related to physiologically active compounds and that similar effects are undoubtedly caused by the active steroids themselves (Roy, 1960). It would seem therefore that these observations are the first to detect species differences in the response to physiologically active steroids at the level of an isolated enzyme. There is as yet no evidence to suggest that the described effects are occurring *in vivo*, but as the concentrations of steroid involved are close to the physiological it would seem that there is no reason why they should not be, provided that the steroid can gain access to the enzymes in the body. Unfortunately the situation *in vivo* cannot be directly investigated as the function of arylamine sulphokinase in the normal animal is unknown.

SUMMARY

1. A method is described for the study of the formation of 2-naphthyl sulphamate by arylamine sulphokinase.
2. The kinetics of the reaction are described, and in particular the kinetics in the presence of 3 β -methoxyandrost-5-en-17-one.
3. This oxo steroid strongly inhibits guinea-pig arylamine sulphokinase and activates rat arylamine sulphokinase. Both effects are pronounced at steroid concentrations of $1 \mu\text{M}$.
4. Rat-liver arylamine sulphokinase has a very high K_m for 2-naphthylamine, certainly much

greater than for 2 mM-2-naphthylamine. Its apparent affinity for 3 β -methoxyandrost-5-en-17-one is high, K_i being 1.5 μ M.

5. The corresponding values for guinea-pig-liver arylamine sulphokinase are: K_m , 0.5 mM-2-naphthylamine; K_i , less than 0.2 μ M-oxo steroid.

6. An explanation of these effects is suggested. It is based on a mechanism formally similar to the partial competitive inhibition of Dixon & Webb (1958), in which a ternary enzyme-steroid-substrate complex (dissociation constant K'_i) can be formed. The rate of breakdown of this complex to give the reaction products is identical with that of the breakdown of the normal enzyme-substrate

complex (dissociation constant K_s). If K'_i is greater than K_s in such a system then the steroid will be an inhibitor of the enzyme; if K'_i is less than K_s the steroid will be an activator.

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Further Experiments on the Inhibition of Aconitase by Enzymically Prepared Fluorocitric Acid

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Peters, Wakelin, Rivett & Thomas (1953) found that fluorocitric acid made by enzymic action inhibits the aconitase in kidney particles more powerfully than does synthetic fluorocitric acid. This finding was in line with the fact that the synthetic fluorocitric acid should be a mixture of four isomers, of which only one or two might be expected to be active. When Morrison & Peters (1954) compared the effects of enzymically and chemically prepared fluorocitrate on a highly purified soluble aconitase, the behaviour of the two was very different. Synthetic fluorocitric acid was a very strong inhibitor, whereas the enzymic fluorocitric acid was much less inhibitory. The inhibition obtained was reversible even after preincubation in the absence of substrate.

These differences were difficult to explain and led to the suggestions either that the synthetic fluorocitric acid did not penetrate properly into the particle, or alternatively that there was some difference in mitochondrial aconitase. The use of another preparation of soluble aconitase has now made it possible to get an inhibition with fluorocitrate which is of the same order as that found in the particle preparation. In addition, a new

method of testing the activity of specimens of fluorocitric acid has been devised. Some of these results have already been published in a preliminary form (Peters, 1959).

There are several problems involved: (1) that of the discrepancy between the effect of enzymically prepared fluorocitrate on mitochondrial and soluble aconitase; (2) that of the best method of testing the relative activity of specimens of fluorocitric acid with soluble aconitase; (3) that of the discrepancy between the activity of enzymically prepared and synthetic fluorocitric acid on soluble aconitase; (4) finally, whether the inhibition is truly reversible with enzymically prepared or synthetic fluorocitric acid.

Of these problems only (1), (2) and (4) are considered here. Problem (3) forms the subject of a separate paper (Ward & Peters, 1961).

METHODS AND PREPARATIONS

Chemicals. These were AnalaR where possible; DL-iso-citric acid (as Na salt at pH 7.4) was prepared from the lactone essentially by the method of Krebs & Eggleston (1944) or Ochoa (1948). Glass-distilled water was used throughout. Phosphate buffer was made by neutralizing KH_2PO_4 solutions with NaOH.

Fluorocitric acid. This was neutralized to pH 7.4, usually

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