next step is to separate the zinc-sensitive enzyme. This may be an enzyme similar to the mitochondrial lactate dehydrogenase described by Tubbs (1960) or the D-hydroxy acid dehydrogenase of yeast.

SUMMARY

1. Intact cells of *Euglena gracilis* grown in a medium containing sufficient zinc oxidize lactate at high rates (400–800 μ l. of oxygen consumed/hr./mg. of protein nitrogen). Zinc-deficient *Euglena* oxidizes lactate at a rate of about 40 μ l. of oxygen/hr.

2. The failure of lactate oxidation in zinc deficiency is due in part to decreased activities of D-lactate dehydrogenase.

3. A manometric method is described for measuring lactate-dehydrogenase activity in *Euglena* homogenates or frozen and thawed cells.

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The Enzymic Synthesis of Aryl Sulphamates

3. THE SPECIFICITY AND MECHANISM OF THE ACTIVATION OF RAT-LIVER ARYLAMINE SULPHOKINASE BY 17-OXO STEROIDS*

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(Received 17 May 1961)

In the previous papers of this series it was shown that the arylamine sulphokinase of rat liver was activated by 17-oxo steroids (Roy, 1960) and that the kinetics of the activation by 3 β -methoxy-androst-5-en-17-one (Roy, 1961) were consistent with a reaction mechanism formally similar to that found in partial competitive inhibition (Dixon & Webb, 1958). In this type of inhibition the competitive effect is only partial in the sense that

an enzyme-inhibitor-substrate complex can be formed and the velocity constant for the breakdown of this complex into the reaction products is the same as that for the breakdown of the normal enzyme-substrate complex. As in true competitive inhibition the net effect of the addition of the inhibitor is a decrease in the apparent affinity of the enzyme for the substrate. Activation can occur by a mechanism which is formally similar if the net effect of the modifier is to increase the apparent affinity of the enzyme for the substrate.

* Part 2: Roy (1961).

The same steroids were powerful inhibitors of guinea-pig-liver arylamine sulphokinase and again the inhibition seemed to be of the partial competitive type. This paper describes the results of a more intensive study of the action of 17-oxo steroids on rat-liver arylamine sulphokinase which was carried out with a view to obtaining more detailed information on the nature of the interactions of the enzyme, substrate and steroid. During this work it became obvious that the interpretation of the kinetics presented by Roy (1961) was too limited and that the true situation corresponded to a more general type of mechanism. In this investigation, as in the previous one, the endogenous sulphate-activating enzymes of liver were used to provide the adenosine 3'-phosphate 5'-sulphatophosphate required as sulphate donor by the arylamine sulphokinase, which is present in rate-limiting amounts.

EXPERIMENTAL

Steroids. The following workers generously donated the less-common steroids required for this investigation: Dr E. J. Agnello, androst-4,14-diene-3,17-dione (Δ^{14} -androstenedione); Dr R. I. Cox, 3,16 α -dihydroxyoestra-1,3,5-trien-17-one (16 α -hydroxyoestrone) and 3,16 β -diacetoxyoestra-1,3,5-trien-17-one (16 β -hydroxyoestrone diacetate); Dr K. Fotherby, 3 β ,16 α -dihydroxyandrost-5-en-17-one (16 α -hydroxyandrostenolone); Dr M. Huffman, 3-methoxyoestra-1,3,5-trien-16-one (oestrone-16 methyl ether); Professor W. Klyne, androst-4-en-3-one, androst-4-ene-3,11,17-trione (adrenosterone) and 11 β -hydroxyandrost-4-ene-3,17-dione (11 β -hydroxyandrostenedione); Dr G. P. Mueller, 3-methoxy-16 α -chloro-oestra-1,3,5-trien-17-one (16 α -chloro-oestrone methyl ether) and 3-methoxyoestra-1,3,5,15-tetraen-17-one (Δ^{15} -oestrone methyl ether); Dr V. Petrov, 3 β -acetoxy-16 α -chloroandrost-5-en-17-one (16 α -chloroandrostenolone acetate), 3 β -hydroxy-16 β -chloroandrost-5-en-17-one (16 β -chloroandrostenolone) and 3 β -hydroxy-16-methyleneandrost-5-en-17-one (16-methyleneandrostenolone).

3-Hydroxyoestra-1,3,5-triene (17-deoxyoestrone) was prepared by the Clemmensen reduction of oestrone (Marrian & Haslewood, 1932) and converted into the methyl ether by the method of Butenandt & Westphal (1934). This latter method was also used to prepare oestrone methyl ether. 3 β -Hydroxyandrost-5-ene (17-deoxyandrostenolone) was obtained by the Huang-Minlon reduction of androst-5-en-17-one (Shoppee & Kreuger, 1961) and 3 β -hydroxy-D-homoandrost-5-en-17-one (D-homoandrostenolone) by the Tiffeneau ring enlargement (Heusser, Herzig, Furst & Plattner, 1950) of the appropriate amine. These two 3 β -hydroxy Δ^5 -compounds were converted into the corresponding 3 β -methoxy compounds by the methanolysis of their toluene-*p*-sulphonyl esters (Stoll, 1932). 3 β -Hydroxy-16 β -methylandrost-5-en-17-one (16 β -methylandrostenolone) was prepared according to Neumann, Mancera, Rosenkranz & Sondheimer (1955) by the reduction of the corresponding 16-methylene compound. 16 β -Hydroxyoestrone was obtained by the hydrolysis of the diacetate with $N-H_2SO_4$ in methanol (Marrian, Loke,

Watson & Panattoni, 1957), but because of the scarcity of the starting material the free steroid was not purified and the product was therefore almost certainly contaminated with 16-oxo-oestradiol-17 β (Layne & Marrian, 1958).

Assay of 2-naphthyl sulphamate synthesis. The enzyme system was prepared exactly as described by Roy (1960) by the $(NH_4)_2SO_4$ precipitation of an iso-osmotic (0.15M) KCl extract of rat liver. The reaction mixture used in the assay had the following composition: potassium phosphate buffer, 0.02M, pH 7.5; Mg^{2+} ions, 4 mM; SO_4^{2-} ions, 0.01M; adenosine triphosphate (disodium salt; Sigma Chemical Co.), 4 mM; 2-naphthylamine hydrochloride, 2 mM; steroid, 10 μ M unless otherwise stated. The steroid was added as a freshly prepared solution in 1% ethanol so that the final concentration of the latter in the reaction mixture did not exceed 0.2% (Roy, 1961). The amount of enzyme which was used per millilitre of reaction mixture corresponded to that obtained from 50 mg. fresh weight of liver.

In most cases the amount of 2-naphthyl sulphamate formed after incubation of the above reaction mixture for 2 hr. at 37° was determined by the methylene-blue method (Roy, 1961), which is based on the solubility of the methylene-blue salts of sulphate esters in $CHCl_3$. In some cases, which are specified below, it was necessary to use the more specific but less sensitive diazotization method (Roy, 1960), which is dependent on the separation of the sulphamate from the excess of 2-naphthylamine followed by the hydrolysis of the former and the determination of the liberated amine by diazotization and coupling with thymol. The reaction mixtures had identical compositions in both types of assay but the total volume was 1.0 ml. for the methylene-blue method and 5.0 ml. for the diazotization method.

RESULTS AND DISCUSSION

Kinetic studies

The investigations described by Roy (1961) dealt with the kinetics of the activation of rat-liver arylamine sulphokinase by 3 β -methoxyandrost-5-en-17-one and showed that the behaviour of the system was that expected if the mechanism were of the partial competitive type (Dixon & Webb, 1958). This was shown by the increase in the apparent affinity of the enzyme for 2-naphthylamine, K_m (defined as the reciprocal of the intercept on the abscissa in the plot of $1/v$ against $1/s$ at constant steroid concentration), in the presence of the steroid, K_m decreasing with increasing concentrations of activator. On the other hand, in agreement with the general competitive nature of the activation, changes in the concentration of the activator had little, if any, effect on the maximum velocity of the reaction, V' (defined as the reciprocal of the intercept on the ordinate in the above plot). In the present investigation this general behaviour was confirmed for a much wider range of steroids, as is shown by the results in Table 1 which were calculated by the method of Wilkinson (1961), which involves the

calculation of provisional least-squares estimates of K_m and V' by the suitably weighted fit of a linear regression of $1/v$ on $1/s$; these provisional estimates were followed by fine adjustment and calculation of the standard errors by fitting a bilinear regression of v on the corresponding values of the provisionally fitted Michaelis function and its first derivative. From the results in Table 1 it was obvious that V' did in fact vary with changes in both the concentration and the nature of the steroid although in most cases these changes were small. As found previously, K_m was highly dependent upon the concentration of the steroid as well as upon its nature. It is impossible to determine K_m and V in the absence of any added steroid because of the limited solubility of 2-naphthylamine. At the maximum substrate concentration attainable (2 mM) the reaction velocity is still increasing in an almost linear fashion with increasing substrate concentration so that K_m must be very much greater than 2 mM-2-naphthylamine (Roy, 1961).

The observation that V' varied with the nature of the steroid suggested that there must be a non-competitive component in the activation and clearly indicated that the mechanism of the activation of arylamine sulphokinase by 17-oxo steroids could not be of the simple partial competitive type because in such a case V' is independent both of the concentration and the nature of the steroid (Dixon & Webb, 1958; Roy, 1961).

An explanation of the results can be provided in terms of a more general mechanism in which a partial competitive component is combined with a partial non-competitive component. The reactions which occur in such a system are as follows, where E represents the arylamine sulphokinase-adenosine 3'-phosphate 5'-sulphatophosphate complex, S, 2-naphthylamine, and I, the 17-oxo steroid. The various complexes of these compounds and the dissociation constants of the former are represented in the usual manner. Concentrations of the

various reactants are indicated by the appropriate lower-case italic letters.

This differs from the situation in the partial competitive case only in that k is not identical with k' as it is in the latter. The appropriate velocity equation for such a system, which corresponds to one of the mixed types of inhibition briefly mentioned by Dixon & Webb (1958), can be derived in the usual way and shown to be:

$$v = \frac{(kK'_i K_i + k'K_s i) es}{K_s(K'_i K_i + K'_s i) + s(K'_i K_i + K_s i)}$$

This velocity equation can be converted into the usual reciprocal form:

$$\frac{1}{v} = \frac{K_s(K'_i K_i + K'_s i)}{e(kK'_i K_i + k'K_s i)} \frac{1}{s} + \frac{K'_i K_i + K_s i}{e(kK'_i K_i + k'K_s i)}$$

which shows that the plots of $1/v$ against $1/s$ will be linear at constant concentrations of steroid. In such plots the intercepts on the ordinate, $1/V'$, will have the values

$$\frac{1}{V'} = \frac{K'_i K_i + K_s i}{e(kK'_i K_i + k'K_s i)}$$

and the intercepts on the abscissa, $1/K_m$, the values

$$\frac{1}{K_m} = -\frac{K_s(K'_i K_i + K'_s i)}{e(kK'_i K_i + k'K_s i)}$$

All these expressions reduce to the corresponding ones for the partial competitive case (Roy, 1961) when $k = k'$.

For the present purpose the important point is that in the plot of $1/v$ against $1/s$ the intercept on

Table 1. Variations in V' and K_m for the synthesis of 2-naphthyl sulphamate by rat-liver arylamine sulphokinase with the nature and concentration of the activating 17-oxo steroids

The values were calculated by the method of Wilkinson (1961) and are expressed together with the standard error which is based on 15 degrees of freedom for all values except those of androstenedione which have only 3 degrees of freedom.

Steroid concentration (μM)	V' (μM -moles/ml./2 hr.)		K_m (mM-2-naphthylamine)	
	10	2.5	10	2.5
Oestrone methyl ether	98.9 $\pm$ 5.97	126* $\pm$ 12.6	1.10 $\pm$ 0.127	3.34 $\pm$ 0.461
Androstenedione methyl ether	145* $\pm$ 7.85	123* $\pm$ 4.90	0.525 $\pm$ 0.075	0.645 $\pm$ 0.059
D-Homoandrostenedione methyl ether	104 $\pm$ 5.40	107 $\pm$ 5.47	0.973 $\pm$ 0.103	1.760 $\pm$ 0.151
Androstenedione	102 $\pm$ 3.75	98.7 $\pm$ 3.14	0.618 $\pm$ 0.054	1.04 $\pm$ 0.066
11 β -Hydroxyandrostenedione	134* $\pm$ 2.85	97.7 $\pm$ 2.85	0.411 $\pm$ 0.026	0.936 $\pm$ 0.057
Adrenosterone	119* $\pm$ 3.95	103 $\pm$ 4.51	0.671 $\pm$ 0.053	1.48 $\pm$ 0.115

* These values of V' are significantly higher (to at least the 5% level) than the others.

the ordinate, $1/V'$, will vary with the concentration, i , of the steroid and also with the nature of the latter which must influence K'_i and K_i . This is in sharp contrast with the situation in the partial competitive case in which $1/V'$ is independent of the concentration and nature of the steroid although the mixed case will become less and less readily distinguishable from the partial competitive case as k' becomes more nearly equal to k . In the above mixed case, as in the partial competitive case, the intercept on the abscissa, $1/K_m$, will be dependent on both the nature and concentration of the steroid.

The above expressions can represent either an inhibited or an activated reaction of the mixed type: if $k'/k > (K'_i + s)/(K_i + s)$ then the expression refers to an activated reaction; if

$$k'/k < (K'_i + s)/(K_i + s)$$

it refers to an inhibited reaction and if

$$k'/k = (K'_i + s)/(K_i + s)$$

the steroid has no influence on the velocity of the reaction. This point is of importance as certain 17-oxo steroids do inhibit rat-liver arylamine sulphokinase, as shown in Table 2 below.

Table 1 shows that both K_m and V' do vary with i although the changes in V' are on the whole small, being significant only in the cases of androst-4-en-3-one (to the 5% level), 11 β -hydroxyandrost-4-en-3-one (to 0.1%) and adrenosterone (to 1%). This would suggest that the competitive component of the activation is the dominant one and the k and k' must be of similar magnitude: because of this the distinction between the more general mixed case and the partial competitive case could not easily have been made when the behaviour of only one steroid was considered (Roy, 1961).

Table 1 shows that in the activation by oestrone methyl ether V' decreased with increasing concentration of activator. This is contrary to the findings with the other steroids studied, but it is readily explicable on consideration of the expression given above for V' . V' will decrease with increasing concentrations of steroid if $k > k'$. In such a case it is obvious that the steroid is simultaneously behaving as a partial competitive activator through its effect on the affinity of the enzyme for the substrate and as a partial non-competitive inhibitor through its effect on the velocity constant for the breakdown of the enzyme-substrate complex.

It may be concluded therefore that the activation of rat-liver arylamine sulphokinase by 17-oxo steroids probably represents an example of a general type of mixed activation corresponding to one of the cases of mixed inhibition whose occurrence was postulated from theoretical considerations by Dixon & Webb (1958). In the most typical cases both the partial competitive and partial non-competitive components exert activating effects but in at least one case, that of oestrone methyl ether, a partial competitive activation is combined with a partial non-competitive inhibition. As discussed below, inhibition of arylamine sulphokinase by 17-oxo steroids can occur, but the nature of this inhibition has not been investigated because of the lack of suitable compounds.

Specificity studies

The principal results of the study of the specificity of the modifying effects of steroids on the activity of rat-liver arylamine sulphokinase are summarized in Table 2. In this the amount of 2-naphthyl sulphamate formed in the presence of 10 μ M-steroid at a substrate concentration of 2 mM-2-naphthylamine is expressed relative to the amount

Table 2. Activity of arylamine sulphokinase at substrate concentrations of 2 mM-2-naphthylamine in the presence of 10 μ M-steroid expressed relative to control assays (activity 1.0) carried out in the absence of any added steroid

Derivative	Parent steroid		
	3-Methoxyoestra-1,3,5-triene	3 β -Methoxy-androst-5-ene	Androst-4-en-3-one
—	1.0	1.0	1.2
16-Oxo	1.3	—	—
17-Oxo	2.2	4.0	2.2
17 α -Oxo*	—	2.6	—
17-Oxo, 14-ene	—	—	1.2
17-Oxo, 15-ene	1.0	—	—
17-Oxo, 16 α -hydroxy	1.5†	0.75†	—
17-Oxo, 16 β -hydroxy	1.1†	—	—
17-Oxo, 16 α -chloro	1.0	0.77†	—
17-Oxo, 16 β -chloro	—	1.8†	—
17-Oxo, 16-methylene	—	0.96†	—
17-Oxo, 16 β -methyl	—	1.0†	—

* Derivative of the corresponding D-homo steroid.

† Steroid used as the 3-hydroxy compound, or the 3-acetoxy compound (‡), and enzyme activity determined by the diazotization method.

formed under similar conditions but in the absence of any added steroid. In some cases, indicated in the Table, the diazotization method of assay was used. This was necessary as, either for practical reasons or because of the scarcity of material, it was not possible to prepare the 3-methyl ethers of the steroids concerned which could themselves form sulphate esters and so interfere with the determination of the sulphamate by the methylene-blue technique.

It is obvious from these results that, so far as the present series of compounds is concerned, there is an absolute requirement for the presence of either a 17-oxo group or some closely related group in a steroid before this can activate arylamine sulphokinase. Oestrone-16 methyl ether and D-homo-androstenedione methyl ether, with oxo groups in the 16 and 17a positions respectively, were relatively efficient activators as might be expected from their structural relationships to the corresponding 17-oxo steroids. Androst-4-en-3-one also showed a slight, although consistent, activating effect, but this was negligible compared with that of the corresponding 17-oxo compound.

The presence of a double bond in either the 14-15 or 15-16 position eliminated the activating effect of the 17-oxo group. This is hardly surprising when the effect of such double bonds on the shape of the D ring is considered.

The effect of substituents in the 16 position of the 17-oxo steroid was confusing. Although both 16 α -chloro-oestrone and 16 α -chloroandrostenedione were devoid of the activating effects of the parent oxo steroids, the androstenedione derivative in fact having a considerable inhibitory effect, 16 α -hydroxyoestrone was an activator and 16 α -hydroxyandrostenedione an inhibitor of arylamine sulphokinase. Again, although 16 β -chloroandrostenedione was an activator of the enzyme 16 β -methylandrostenedione was without effect on it. It is obviously extremely difficult to explain these results which are presumably complicated by the separate effects of the variations in the steroid structure on the affinity terms (K'_i and K_i) and the velocity constant (k') in the equations given above. It is likely that those 17-oxo steroids which inhibit arylamine sulphokinase still combine with the enzyme but in those cases k'/k must be less than $(K'_i + s)/(K_i + s)$ as previously discussed. Although the results cannot be fully interpreted, the differences in behaviour between the several 16 α - and 16 β -substituted 17-oxo steroids shown in Table 2 indicate that the oxo steroids cannot be acting in their enolic forms as was originally postulated (Roy, 1960) because both these isomers give the same enol. Again, as some members of both the 16 α - and 16 β -series of substituted 17-oxo steroids can behave as activators it seems justifi-

able to conclude that the steroids cannot be combining with the enzyme through the 16-position: if they were so combining it seems likely that one or other of these epimeric forms would be inactive irrespective of the nature of the substituent.

Table 2 suggests that the nature of the A ring has some influence on the activating effects of 17-oxo steroids as androstenedione methyl ether, androstenedione and oestrone methyl ether, which differ primarily in the structure of their A rings, formed a series of compounds with decreasing potencies as activators. When the values of V' are considered (Table 1) the relative activities of the compounds are in the same order although, as discussed above, there is apparently an inhibitory component present in the action of the oestrone derivative which is not present in the effects of the other compounds. Similarly, the nature of the C ring influences the behaviour of the steroid: in particular an oxygen function at position 11 enhanced the activation caused by the 17-oxo group. Expressed as in Table 2 the activating effects of androstenedione, 11 β -hydroxyandrostenedione and adrenosterone were 2.2, 3.0 and 2.5 respectively, and again when V' is considered (Table 1) the same general effect is noted. The mechanism of this oxygen effect is not obvious, but Bush (1956) postulated an action of an 11-oxygen function on the activity of the 16-position in 17-oxo steroids. Roy (1960) has shown that cortisone and cortisol, with an 11-oxo and an 11 β -hydroxy substituent respectively, but both lacking a 17-oxo group, do not activate arylamine sulphokinase.

It is unfortunately not possible to draw from these results any definite conclusions regarding the mode of interaction of the enzyme, steroid and substrate except that an oxo group, either in the 17-position or some closely related position, is involved in the reaction. The results also suggest that although the general shape of the D ring is important the 16 position is not directly involved in any combination of the steroid with the enzyme or the substrate. It would be pointless at this stage to discuss the possible reactions between the steroid and the enzyme or the arylamine: both 17-oxo steroids and arylamines such as 2-naphthylamine are very reactive compounds and their possible modes of combination are numerous indeed. It seems likely that further information on their interactions will become available only after arylamine sulphokinase has been obtained in a pure state, and only then will it be possible to decide whether the steroid acts as a link between the enzyme and the arylamine or whether it simply facilitates their combination by blocking some interfering group on the enzyme.

SUMMARY

1. The kinetics of the activation of rat-liver arylamine sulphokinase by 17-oxo steroids has been investigated, and it has been shown that they are consistent with the mechanism of the activation's being of a mixed type in which partial competitive and partial non-competitive components are combined.

2. The specificity of the activating effect has been studied and it has been shown that the structural requirements for activation are fairly stringent. A double bond in the D ring of the 17-oxo steroid gives an inactive compound. A substituent in the 16 position (α or β) may or may not eliminate the effect of the 17-oxo group.

3. 16-Substituted 17-oxo steroids which are without effect or which inhibit arylamine sulphokinase may still combine with the enzyme but because of the relative magnitudes of the various velocity and dissociation constants activation does not occur.

4. The structures of the A and C rings of the steroid influence the activating effects of 17-oxo steroids.

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Calcium Binding by Rat Submaxillary Glands *in vitro*

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Rat submaxillary glands contain about four times as much calcium as other soft tissues such as liver and kidney (Dreisbach, 1957), and approximately 50% of this calcium is lost after 2 hr. of maximal pilocarpine stimulation of secretion (Dreisbach, 1959a). One possibility is that this labile calcium in rat submaxillary gland is due to the presence of calcium-binding components in the gland which are different, either qualitatively or quantitatively, from the calcium-binding components in plasma. If sufficient binding material is present in the gland, the intracellular unbound calcium concentration could be the same as that present in the extracellular fluid, and no concentration gradient across the cell membrane would occur. The high tissue calcium could thus be maintained without a specific concentrating mechanism.

The present report describes the effects of variations in pH, temperature and calcium concentration on calcium binding *in vitro* by rat submaxillary glands either as slices or as homogenates.

MATERIALS AND METHODS

Submaxillary glands were removed under chloroform anaesthesia from adult Long-Evans rats of either sex. Except for experiments in which glands were used immediately, glands were stored in screw-capped vials at -20° for at least 1 week before use.

Preparation of homogenates. Glands were disrupted in batches of 0.5 g. by grinding for 3 min. in a glass-walled homogenizer of 10 ml. capacity with Teflon pestle. The pestle was driven at 1000 rev./min. and the apparatus was cooled in ice. The medium for disruption consisted of 0.25 M-sucrose or 0.15 M-NaCl, by using 9 vol. of medium for