ENZYMIC REACTIONS IN HETEROGENEOUS PHASE. PREPARATION OF 17α,20β,21-TRIHYDROXYPREGN-4 [^{14}C] EN-3,11-DIONE.

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SUMMARY

The preparation of 17α, 20β, 21-trihydroxypregn-4 [^{14}C] en-3,11-dione, in quantitative yields, has been achieved using enzymic reduction of cortisone [^{14}C] in water-organic solvent heterogeneous system. Since small volumes and little amount of enzyme are required to carry out the reaction, as compared to the enzymic preparation using an homogeneous aqueous phase, the methodology is useful for preparative purposes.

INTRODUCTION

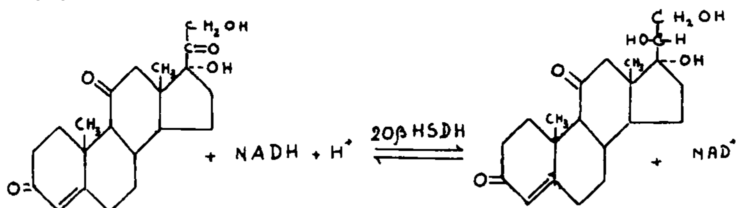
In order to study steroid-protein interactions, labelled 17α, 20β, 21-trihydroxypregn-4 [^{14}C] en-3,11-dione was required.

This adrenal steroid (Reichstein's substance U) is also of particular interest from the point of view of its metabolism (1,2).

As regards its preparation, the chemical synthesis was based on stereospecific reduction of the side chain of cortisone with $NaBH_4$ (3). This reaction leads to incomplete conversion of cortisone to the 20β-hydroxy derivative, and a further purification of the product from the reagent was therefore required.

Enzymic reduction by 3α, 20β hydroxysteroid-dehydroge

nase (4) using NADH as hydrogen donor is a more simple method:



The kinetic data referred to cortisone as substrate suggest that the reduction is 20β specific (with a K_H value of 4.7×10^{-11} M), but the presence of 3α hydroxy steroid: NAD oxidoreductase activity in crystalline preparations of 20β HSDH and, mainly, the low solubility of the steroid in the water phase (10^{-4}-10^{-5} M/l) resulted in a limitation of the enzymic transformation in preparative scale.

In earlier works (5) we have verified the possibility of performing enzymic reactions on steroids in heterogeneous phases obtained by shaking a system in which an aqueous phase contained the enzymes and an organic phase the steroids. When mixing starts a continuous transfer of steroid molecules to the aqueous phase, catalysis and return of the product to the organic phase, is initiated as a function of the partition coefficient.

Since the reaction can proceed for a long period of time at a rate approaching the initial one, it is possible to reach a relatively great quantity of product in small reaction volumes using only small amounts of enzyme.

EXPERIMENTAL PROCEDURE

Material and method

Cortisone carrier, crystallized several times from

ethanol and ethyl acetate, was checked for its purity by TLC in the system methanol/water/ethyl ether/methylene chloride (8:1.2:15:77) on Silica gel GF 254.

Labelled cortisone (NEN 50 mCi/mM, in benzene:ethanol 9:1) was utilized. Butyl acetate-solutions of labelled cortisone with a specific activity of 0.194 mC/mM were employed to performe the reactions.

The purity of β-diphosphopyridinedinucleotide was greater than 90%.

Crystalline preparations of 3α,20β-hydroxysteroid-dehydrogenase (Boehringer) were used.

Reaction methodology was similar to that referred to elsewhere (6). The reaction was followed by measurements of the optical density of the water phase at 340 nm.

Steroid determination was done by TLC and U.V. spectroscopy of the isolated spot.

In experiments employing autoradiography, thin-layer chromatograph plates were kept in close contact with Kodak X-ray film for 48 hours. The position of labelled steroids were localized by development of the film.

RESULTS AND DISCUSSION

First of all it was necessary to determine which, among the different organic solvents, had no influence (by denaturation or inhibition) on the catalytic activity of the enzyme, when it was shaken with the aqueous phase. These reactions were performed under the same working conditions: only the nature of the organic solvent was varied. From the results given in table I it can be seen that the highest conversions were obtained using ethyl or butyl acetate as steroid solvent.

TABLE I - Dependence of cortisone reduction on organic solvents

Organic solvent	20β HSDH activity ΔA_{340}/min a)	Cortisone solubility g x 100 ml	Cortisone conversion b)
n-hexane	41	.002	<5
iso-octane	42	-	<5
methylene chloride	-	-	70
carbon tetrachloride	42	.004	<5
chloro benzene	40	.030	<5
trichloro ethylene	38	-	10
trichloro methane	-	-	80
diethyl ether	16	.017	10
ethyl acetate	12	.270	90
butyl acetate	20	.160	100
---(control)	42		

a) 20β HSDH rates in aqueous solutions saturated with organic solvents. Conditions: 0.6 μmoles of NADH, 0.3 μmoles of cortisone (in 30 μl ethanol), 0.02 units of 20β HSDH in 0.7 ml of 0.2 M phosphate buffer pH 6.5 saturated with organic solvent. Spectrophotometric determinations at 340 nm in 0.2 cm path cell.

b) Conversion of cortisone to 20β hydroxy derivative in heterogeneous phase. Conditions: 7 μmoles NADH and 0.24 units of 20β HSDH in 2.5 ml of 0.1 M phosphate buffer pH 6.5 with 0.3‰ HSA.
4.5 μmoles of cortisone in 1 ml of organic solvent. Shaking rate 40 oscillations per minute. Reaction time 2 hours. Cortisone conversions are expressed as a ratio of the butyl acetate run.

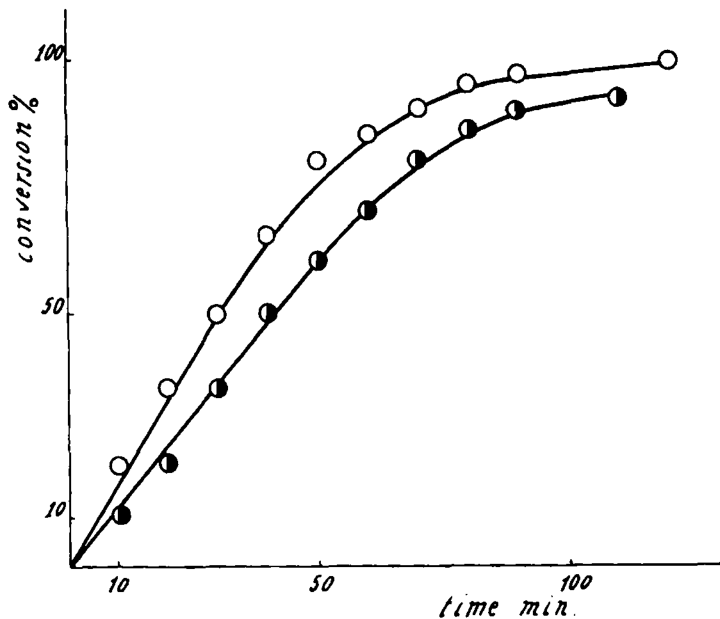
Other solvents such as isoctane or ethyl ether gave poorer yields due to the lower solubility of cortisone.

With some chloride containing solvents the lower stability of the enzyme is responsible for the low reactivity. The efficiency of the reaction in butyl acetate was measured by

the specific radioactivity of the cortisone at the start of the reaction and of the 20β hydroxyderivative at the end.

The time course of the reaction is illustrated in Fig. 1; the curves refer to two similar tests. The first one was carried out in the absence of serumalbumine and the second in the presence of serumalbumine. The effect of added protein is that of enhancing the stability of the enzyme in the presence of the organic solvent.

Fig. 1 - Time dependence of cortisone conversion to 20β-hydroxy derivative.
Conditions: 10 μmoles NADH, 0.24 units of 20β HSDH in 2.5 ml of 0.1 M phosphate buffer pH 6.5., 5.5 μmoles of labelled cortisone in 1.5 ml butyl acetate. Shaking rate 40 oscillations per minute.
(o——o) with 0.3‰ HSA, (●——●) without HSA



From the data obtained it can be shown that the difference between the two curves does not appear too relevant here: in fact we have found that the serumalbumine dependence becomes significant only either at longer reaction time or at lower enzyme concentration. Nevertheless it can be noticed that by carrying out the reaction without serumalbumine the rate is about 30 per cent lower.

The time to reach complete conversion of cortisone to 20β hydroxyderivative (see Fig. 1), is obviously dependent on the amount of the enzyme present in the system.

In order to determine the limiting conditions of work for the system employed we studied the dependence of the conversion as a function of the concentration of the enzyme. The results are shown in Fig. 2.

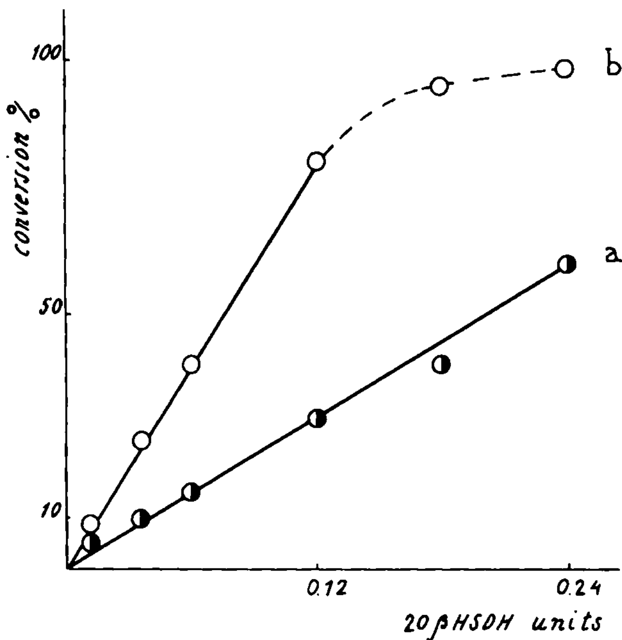
Curve a) is related to the data after 30 min. of reaction and shows an increasing rate proportional to the increasing concentration of the enzyme in the two phase system. These concentrations are lower or equal to that utilized in an enzyme kinetic test.

Curve b) shows the points at which for the higher enzyme concentration the system approaches equilibrium.

The reaction proceeds with rates similar to the initial values and is regulated by the rate of exchange of the steroid between the two phases.

Since we used an enzyme which was not only 20β but also 3α active, it was thus necessary to test the uniqueness of the reaction product. The autoradiography TLC of the organic phase and the U.V. data derived for the reaction product revealed that by enzymic reduction of cortisone, only 17α, 20β, 21-trihydroxypregn-4 en-3,11-dione was obtained.

Fig. 2 - Dependence of cortisone reduction on enzyme concentration.
Conditions: 7 μmoles NADH, 0.012 to 0.24 units of 20β HSDH in 2.5 ml of 0.1 M phosphate buffer pH 6.5 with 0.3‰ HSA.
5.5 μmoles of labelled cortisone in 1.5 ml butyl acetate. Shaking rate 40 oscillations per minute.



This results in an indirect confirmation of the kinetic data (7) that suggested that cortisone is not a substrate for the 3α activity in 3α, 20β-hydroxysteroid-dehydrogenase.

From all the data shown here we can conclude that enzymic reduction in the heterogeneous phase is appropriate for the preparation of labelled steroids. It allows one to obtain

the product easily in almost quantitative yields, with only one step and only one extraction with organic solvent. This extraction is here necessary since the partition coefficient of 17α,20β,21-trihydroxypregn-4 en-3,11-dione is lower than that of cortisone.

As compared with the $NaBH_4$ reduction the enzymic reaction leads to complete conversion of the 20 keto group to the 20β hydroxyderivative. This means that no further purification of the product is therefore required. With respect to the enzymic transformation of steroids in homogeneous aqueous phase, the main advantage, for preparative purposes, of carrying out the reaction in a two phases system is that small volumes are required.

Finally, since the transformation in heterogeneous phase is achieved using low amount of enzyme, it is possible to employ highly purified enzymes with a consequently higher reaction specificity.

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