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# INFLUENCE OF SOME STEROIDS ON THE METABOLISM OF RAT LIVER MITOCHONDRIA AND OF HEPATOMA ASCITES CELLS *IN VITRO*

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**Abstract**—The effects of some steroids of the testosterone and 19-nortestosterone groups on the phosphorylative oxidation of ketoglutarate by isolated rat liver mitochondria and on the $O_2$ uptake and aerobic glycolysis of hepatoma AH 130 ascites cells have been compared.

The ratios between oxidation inhibiting activity and oxidative phosphorylation uncoupling efficiency of some of the steroids tested are widely different.

Both inhibitors of oxidations and uncouplers of oxidative phosphorylations cause an increase of aerobic glycolysis in intact hepatoma ascites cells. Only the oxidation of DPN-dependent substrates is influenced by these compounds. The steroids inhibiting mitochondrial oxidations decrease the rate of external DPNH reoxidation by mitochondria pretreated with hypotonic solutions, some steroids which do not inhibit mitochondrial oxidations have the same effect in a lesser degree. 16α-Hydroxy-17α-methyltestosterone has the highest uncoupling activity that has been so far described for a steroid compound.

THE inhibitory effects of steroids on the respiration of cells and of subcellular preparations has been studied by several authors. The steroids chiefly examined were: progesterone, cortisol, deoxycorticosterone, testosterone, nortestosterone and some of their derivatives, oestrone and oestradiol.

White and his collaborators[1–3] carried out their tests with normal and neoplastic lymphoid tissues, other authors[4–6] used kidney, brain, liver, diaphragm, and others again used either liver homogenates or isolated liver mitochondria.[7–9] Discherl and Schafhausen[10] and Hübener and Cloeren[11] employed Ehrlich carcinoma ascites cells.

The inhibiting action of steroids on respiration has been recently ascribed to an inhibition of the DPNH–cytochrome *c* reductase reaction, localized between the flavoprotein and coenzyme *Q* or cytochrome *b*.[12–16]

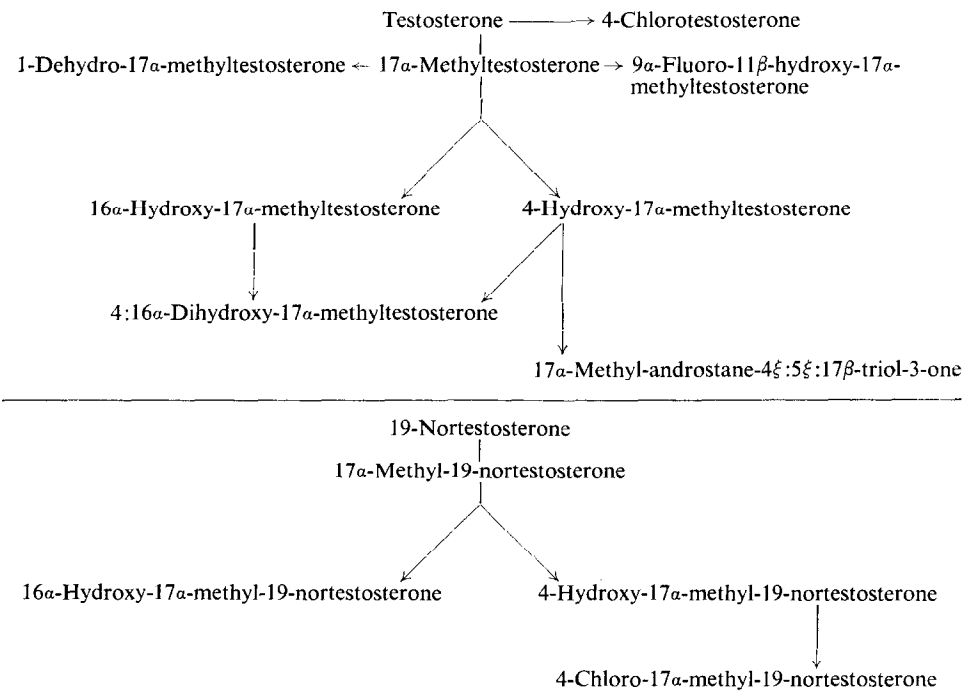
It has been observed, moreover, that some steroids inhibit the oxidative phosphorylation of isolated mitochondria[8, 9] and increase their adenosine-triphosphatase activity.[17–19]

The influence of steroids on aerobic and anaerobic glycolysis of lymphoid tissues and of some tumour tissues has also been studied.[10, 11, 20–24] The tests carried out by several authors showed that the addition of steroids sometimes stimulated and sometimes inhibited aerobic glycolysis; in most cases it decreased anaerobic glycolysis. Blecher and White[23] suggested that this inhibiting effect might be due, at least in some cases, to the stimulating action exerted by some steroids on the ATPase activity of

tissue homogenates, resulting in a decrease of the amount of ATP available for the phosphorylation of carbohydrate.

The present paper deals with the influence that some steroids, which can be considered as derivatives of testosterone and of nortestosterone (Table 1), exert both on the metabolism of hepatoma AH 130 ascites cells and on the oxidative phosphorylation of normal rat liver mitochondria. We have studied particularly the mechanism

TABLE 1




of action of 4-hydroxy-17α-methyl-19-nortestosterone and of 16α-hydroxy-17α-methyltestosterone, which show a striking difference in their effect on mitochondrial metabolism. It was hoped that the comparison of these two steroids, endowed with different biological activity on isolated mitochondria, could throw some light on the mechanism of their overall effect on intact hepatoma ascites cells.

## MATERIALS AND METHODS

*Chemicals*

We used the following products: adenosine triphosphate (sodium salt), cytochrome *c*, α-ketoglutarate and reduced diphosphopyridine nucleotide Boehringer, hexokinase Sigma type II, β-hydroxybutyric acid Fluka, succinic acid Merck. The steroids were supplied by the chemical department of Farmitalia Research Laboratories.

*Hepatoma AH 130 ascites cell suspensions*

The ascitic fluid, taken from rats killed 6–7 days after tumour transplantation, was diluted with the same volume of cold saline and centrifuged for 1 min at 1000 rev/min.

The sediment was washed twice with saline and resuspended in Krebs–Ringer solution, either phosphate or bicarbonate, at pH 7·4,[25] modified for the $CaCl_2$ concentration, which was 1·5 mM.

*Anaerobic glycolysis, oxygen uptake and aerobic glycolysis assays*

We used the conventional Warburg apparatus. Incubations at 37 °C, sixty complete strokes per minute, gas phase either air or 95% $N_2$, 5% $CO_2$ mixture. Each flask contained, in a total volume of 3 ml, 2 ml of cell suspension, containing 12–16 mg dry weight of cells, glucose 30 μmole, the suitable amount of steroids dissolved in 0·3 ml either of 5% ethyl alcohol or 1% propylene glycol. In aerobic measurements 0·2 ml of 20% KOH and a filter paper strip were placed in the centre well. At the end of incubation, 0·5 ml. of 20% trichloroacetic acid were added to each flask, then the content of the flasks was centrifuged, and the lactic acid was determined in the surnatant by the Barker and Summerson's method.[26] Under anaerobic conditions the lactic acid production was measured manometrically.

*Preparation of mitochondria*

Long–Evans rats, kept fasting for 12 hr, were used. The livers were removed from the rats killed by decapitation and the isolation of mitochondria carried out according to the procedure described by Schneider.[27] Ethylenetetra-acetic acid was added to the 0·25 M sucrose solution, to a concentration of 0·002 mole/l.

*Oxidative phosphorylation assays*

The oxidative phosphorylation activity was determined in suspensions of mitochondria (the amount obtained from 0·5 g liver, wet weight) in 3 ml of a solution containing: sucrose 250 μmole, EDTA 2 μmole, substrate 30 μmole, phosphate buffer pH 7·4 40 μmole, adenosine triphosphate 6 μmole, cytochrome *c* 0·03 μmole, glucose 50 μmole, yeast hexokinase 60 KM units, $MgSO_4$ 22·5 μmole, 0·3 ml either of 5% ethyl alcohol or of 1% propylene glycol with or without added steroid. The mixture was incubated in Warburg flasks at 30 °C for 20 min. $O_2$ uptake was measured by the manometric technique. Inorganic phosphate was determined according to Marsh[28] in the supernatant after deproteinization with 0·5 ml of 20% trichloroacetic acid, at zero time and at the end of incubation.

*DPNH oxidation*

The oxidation of external DPNH by mitochondria pretreated under hypotonic conditions for 10 min according to Maley[29] was determined spectrophotometrically. The reaction was run at 25 °C in the Beckman spectrophotometer. The incubation mixtures contained, in a total volume of 2·5 ml, phosphate buffer at pH 7·4 30 μmole, $MgSO_4$ 15 μmole, sucrose 625 μmole, EDTA 5 μmole, DPNH 0·25 μmole, mitochondria corresponding to 2·5 mg of proteins (determined by the biuret reaction), 0·1 ml of 50% propylene glycol with or without the suitable amount of the steroid. Readings were performed at 340 mμ, at 15–30 sec intervals, up to 4 min after the addition of DPNH. The blank consisted in the same mixture, without DPNH.

## RESULTS

I. *Influence of testosterone and nortestosterone derivatives on hepatoma AH* 130 *ascites cells and normal liver mitochondria*

From the data reported in Tables 2 and 3 it is apparent that testosterone and 4-chlorotestosterone, at concentrations of 10–15 μg/ml, show a moderate, if any,

activity both on the aerobic metabolism of hepatoma cells and on the oxidative phosphorylation of ketoglutarate in rat liver mitochondria. The introduction of a methyl group at $C_{17}$ in the testosterone molecule brings about a compound, 17α-methyltestosterone, which influences the metabolism of both cells and mitochondria to a greater extent, namely it inhibits respiration, uncouples phosphorylative oxidation and stimulates aerobic glycolysis.

TABLE 2. INFLUENCE OF STEROIDS ON PHOSPHORYLATIVE OXIDATION OF α-KETOGLUTARATE BY NORMAL RAT LIVER MITOCHONDRIA

| | Per cent changes of $O_2$ uptake | | | Per cent changes of P/O* | | |
|---|---|---|---|---|---|---|
| Steroid concentrations (μg/ml) | 10 | 25 | 50 | 10 | 25 | 50 |
| Testosterone | −11 | −14 | −27 | −3 | −12 | −16 |
| 4-Chlorotestosterone | +3 | +10 | | −17 | −13 | |
| 17α-Methyltestosterone | −27 | −51 | −57 | −21 | −21 | −30 |
| 1-Dehydro-17α-methyltestosterone | −13 | −12 | −13 | −4 | −19 | −38 |
| 4-Hydroxy-17α-methyltestosterone | −13 | −24 | | +16 | −65 | |
| 4:16α-Dihydroxy-17α-methyltestosterone | 0 | 0 | −9 | −9 | −21 | −71 |
| 16α-Hydroxy-17α-methyltestosterone | −11 | −33 | | −93 | −97 | |
| 9α-Fluoro-11β-hydroxy-17α-methyltestosterone | −13 | +19 | +23 | +11 | −8 | −6 |
| 17α-Methyl-androstane-4ζ:5ζ:17β-triol-3-one | −1 | −7 | | −5 | −2 | |
| 19-Nortestosterone | −36 | −23 | −46 | 0 | 0 | 0 |
| 17α-Methyl-19-nortestosterone | −26 | −50 | −80 | +6 | −23 | −27 |
| 4-Hydroxy-17α-methyl-19-nortestosterone | −22 | −64 | | −3 | −85 | |
| 4-Chloro-17α-methyl-19-nortestosterone | −50 | −67 | | +9 | −69 | |
| 16α-Hydroxy-17α-methyl-19-nortestosterone | 0 | 0 | 0 | −17 | −39 | −64 |

* P/O values of controls ranged between 2·5 and 3·5.

TABLE 3. INFLUENCE OF STEROIDS ON RESPIRATION AND AEROBIC GLYCOLYSIS OF ASCITES HEPATOMA AH 130 CELLS

| | Per cent changes of $QO_2$ | | | Per cent changes of Lactic acid production | | |
|---|---|---|---|---|---|---|
| Steroid concentration (μg/ml) | 10 | 25 | 50 | 10 | 25 | 50 |
| Testosterone | −9 | −13 | −34 | +5 | +7 | +12 |
| 4-Chlorotestosterone | 0 | 0 | | 0 | 0 | |
| 17α-Methyltestosterone | −28 | −52 | −62 | +25 | +54 | +66 |
| 1-Dehydro-17α-methyltestosterone | −15 | −36 | −51 | +33 | +55 | +77 |
| 4-Hydroxy-17α-methyltestosterone | −43 | −65 | −71 | +47 | +67 | +78 |
| 4:16α-Dihydroxy-17α-methyltestosterone | +24 | +32 | +47 | 0 | +12 | +83 |
| 16α-Hydroxy-17α-methyltestosterone | −26 | −44 | −46 | +160 | +160 | +162 |
| 9α-Fluoro-11α-hydroxy-17α-methyltestosterone | 0 | 0 | | 0 | 0 | |
| 17α-Methyl-androstane-4ζ:5ζ:17β-triol-3-one | +13 | +8 | 0 | 0 | 0 | 0 |
| 19-Nortestosterone | −17 | −29 | −34 | +13 | +30 | +44 |
| 17α-Methyl-19-nortestosterone | −24 | −49 | −63 | +16 | +66 | +88 |
| 4-Hydroxy-17α-methyl-19-nortestosterone | −60 | −71 | −85 | +39 | +82 | +88 |
| 4-Chloro-17α-methyl-19-nortestosterone | −18 | −56 | −72 | +18 | +58 | +83 |
| 16α-Hydroxy-17α-methyl-19-nortestosterone | +12 | +29 | +20 | +10 | +19 | +95 |

19-Nortestosterone does not seem to possess any uncoupling activity, but it inhibits respiration and enhances glycolysis in hepatoma cell. 17α-Methyl-19-nortestosterone shows the same properties, to a higher degree.

We have examined some derivatives of 17α-methyltestosterone and of 17α-methyl-19-nortestosterone, hydroxylated at different positions. Both 4-hydroxy-17α-methyltestosterone and 4-hydroxy-17α-methyl-19-nortestosterone show a remarkable uncoupling activity on mitochondria and are good inhibitors of $QO_2$ in intact ascites cells, they furthermore stimulate aerobic glycolysis. The properties of 16α-hydroxy-17α-methyltestosterone and of 16α-hydroxy-17α-methyl-19-nortestosterone are very different. The former is a much more effective uncoupler of oxidative phosphorylation, it highly stimulates aerobic glycolysis and inhibits oxygen uptake in both mitochondria and hepatoma cells, while the latter uncouples phosphorylation and stimulates glycolysis to a lesser extent, and does not show any inhibiting effect on respiration.

Among the other compounds tested, 4:16α-dihydroxy-17α-methyltestosterone does not inhibit respiration, but uncouples phosphorylative oxidation and stimulates glycolysis, 9α-fluoro-11β-hydroxy-17α-methyltestosterone and 17α-methylandrostane-4ξ:5ξ:17β-triol-3-one, are scarcely active on both cells and mitochondria.

II. *Influence of 4-hydroxy-17α-methyl-19-nortestosterone and of 16α-hydroxy-17α-methyltestosterone on the phosphorylative oxidation of several substrates by liver mitochondria*

The data reported in Table 4 show that 4-hydroxy-17α-methyl-19-nortestosterone inhibits both ketoglutarate oxidation and the phosphorylation coupled to it, and that the decrease of the oxygen uptake occurs even at steroid concentrations which are ineffective on phosphorylation.

TABLE 4. INFLUENCE OF 4-HYDROXY-17α-METHYL-19-NORTESTOSTERONE (4 HMN) AND OF 16α-HYDROXY-17α-METHYLTESTOSTERONE (16 HMT) ON THE PHOSPHORYLATIVE OXIDATION OF α-KETOGLUTARATE BY RAT LIVER MITOCHONDRIA

| Exper. | Steroid | | Oxygen uptake μatoms | % inhibition | P/O | % inhibition |
|---|---|---|---|---|---|---|
| I | None | | 3·81 | | 2·83 | |
| | 4 HMN | 10 μg/ml | 3·09 | 19 | 3·05 | 0 |
| | 4 HMN | 25 μg/ml | 2·10 | 55 | 0·81 | 71 |
| II | None | | 2·95 | | 3·37 | |
| | 16 HMT | 2·5 μg/ml | 3·20 | 0 | 2·46 | 27 |
| | 16 HMT | 5 μg/ml | 3·22 | 0 | 1·07 | 68 |
| | 16 HMT | 10 μg/ml | 3·01 | 0 | 0·99 | 70 |
| III | None | | 3·41 | | 2·96 | |
| | 4 HMN | 25 μg/ml | 0·40 | 88 | 0 | 100 |
| | 16 HMT | 25 μg/ml | 2·28 | 33 | 0·90 | 97 |

The uncoupling action of 16α-hydroxy-17α-methyltestosterone, on the contrary, is stronger than its inhibiting effect on ketoglutarate oxidation and occurs also at concentrations of 2·5 μg/ml, while the oxygen uptake is irregularly inhibited only at 10–25 μg/ml.

16α-Hydroxy-17α-methyltestosterone uncouples the phosphorylative oxidation of succinate and of β-hydroxybutyrate as well as that of ketoglutarate and does not modify

the oxidation rate of these substrates, contrary to what one could expect from a substance endowed with such a high uncoupling power (Table 5).

III. *Action of 4-hydroxy-17α-methyl-19-nortestosterone and of 16α-hydroxy-17α-methyltestosterone on the aerobic metabolism of hepatoma AH 130 ascites cells* in vitro

The addition of both these steroids, at a concentration of 25 μg/ml, to hepatoma ascites cell suspensions, remarkably decreases the endogenous respiration, both steroids

TABLE 5. EFFECT OF 4-HYDROXY-17α-METHYL-19-NORTESTOSTERONE (4 HMN) AND OF 16α-HYDROXY-17α-METHYLTESTOSTERONE (16 HMT) ON THE PHOSPHORYLATIVE OXIDATION OF β-HYDROXYBUTYRATE AND SUCCINATE BY RAT LIVER MITOCHONDRIA

| Substrate | Steroid | | Oxygen uptake μatoms | % change | P/O | % inhibition |
|---|---|---|---|---|---|---|
| β-Hydroxybutyrate | | | 5·03 | | 2·09 | |
| β-Hydroxybutyrate | 4 HMN | 10 μg/ml | 3·26 | −35 | 1·23 | 41 |
| β-Hydroxybutyrate | 4 HMN | 25 μg/ml | 1·13 | −77 | 0·00 | 100 |
| β-Hydroxybutyrate | | | 4·10 | | 2·35 | |
| β-Hydroxybutyrate | 16 HMT | 25 μg/ml | 3·75 | −10 | 0·00 | 100 |
| Succinate | | | 3·50 | | 1·52 | |
| Succinate | 4 HMN | 10 μg/ml | 4·45 | +30 | 0·90 | 41 |
| Succinate | | | 2·82 | | 1·43 | |
| Succinate | 16 HMT | 10 μg/ml | 2·78 | 0 | 0·00 | 100 |

showing a similar degree of activity. When glucose is present in the medium, 16α-hydroxy-17α-methyltestosterone stimulates glycolysis much more strongly than 4-hydroxy-17α-methyl-19-nortestosterone, on the contrary the latter is a more effective inhibitor of the oxygen uptake (Table 6).

TABLE 6. INFLUENCE OF 4-HYDROXY-17α-METHYL-19-NORTESTOSTERONE (4 HMN) AND OF 16α-HYDROXY-17α-METHYLTESTOSTERONE (16 HMT) ON RESPIRATION AND GLYCOLYSIS OF HEPATOMA AH 130 ASCITES CELL *in vitro*

| Substrate | Steroid | | $QO_2$ | % change | Lactic acid μg/mg dry weight | % change |
|---|---|---|---|---|---|---|
| Endogenous | | | 8·25 | | | |
| Endogenous | 4 HMN | 10 μg/ml | 6·98 | −15 | | |
| Endogenous | 4 HMN | 25 μg/ml | 4·06 | −50 | | |
| Endogenous | 16 HMT | 10 μg/ml | 5·47 | −33 | | |
| Endogenous | 16 HMT | 25 μg/ml | 5·41 | −34 | | |
| Glucose | | | 6·57 | | 76 | |
| Glucose | 4 HMN | 10 μg/ml | 4·91 | −25 | 108 | +41 |
| Glucose | 4 HMN | 25 μg/ml | 2·80 | −57 | 136 | +78 |
| Glucose | 16 HMT | 10 μg/ml | 5·60 | −15 | 215 | +172 |
| Glucose | 16 HMT | 25 μg/ml | 5·57 | −15 | 223 | +190 |

The difference in the action of these steroids is more evident when oxygen uptake and lactate production are determined at time intervals in the course of the experiments (Fig. 1). It is clearly seen that while 4-hydroxy-17α-methyl-19-nortestosterone immediately decreases the oxygen uptake, 16α-hydroxy-17α-methyltestosterone exerts

the same effect only after a certain delay, although it stimulates the glycolytic rate at once.

It might be supposed, in this case, that the inhibition of $QO_2$ is induced by the lowering of pH due to the increased production of lactic acid. Tests carried out with cells suspended in buffers ranging from pH 7·4 to 5·8, showed that the oxygen uptake

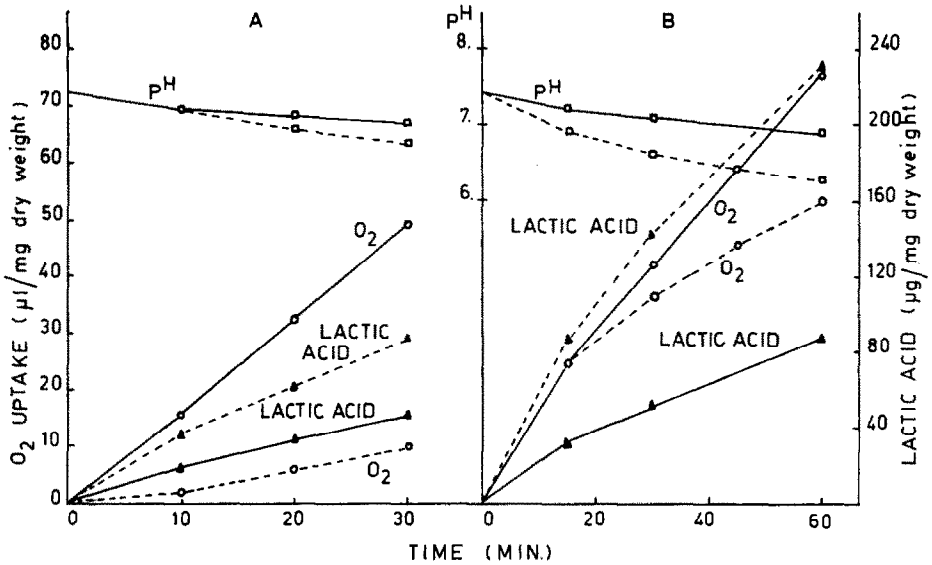



FIG. 1. Influence of 4-hydroxy-17α-methyl-19-nortestosterone (A) and of 16α-hydroxy-17α-methyltestosterone (B) on respiration and aerobic glycolysis of hepatoma AH 130 ascites cells *in vitro*. ——— control; - - - - - with added steroid; □ = pH; ▲ = lactic acid; ○ = $O_2$.

decreases slightly at pH values lower than 6·6; for instance an inhibition of 13 per cent is obtained at pH 6·2. However, in the presence of 16α-hydroxy-17α-methyltestosterone the final pH of the medium is 6·2, and the inhibition of $QO_2$ is as high as30 per cent (Fig. 1). Therefore this cannot be ascribed only to the lowering of pH in the medium.

Under anaerobic conditions, both steroids, at a concentration of 25 µg/ml, cause only a slight, if any, decrease in lactate production.

On the basis of these results, the increase in glycolysis caused by the addition of 4-hydroxy-17α-methyl-19-nortestosterone might be ascribed to an inhibition of both the oxidative and the phosphorylative processes of mitochondria, resulting in a higher supply of some intermediate, such as phosphate, to the glycolytic enzymes. In the case of 16α-hydroxy-17α-methyltestosterone the stimulation of glycolysis could be due, above all, to the strong uncoupling action of this steroid, which also causes a higher availability of phosphate. Therefore we tested the influence of phosphate on the aerobic glycolysis of hepatoma AH 130 cells, and found that an increase in the phosphate concentration in the medium stimulates glycolysis only when this is relatively low, but leaves it unchanged when it exceeds 100 µg of lactate per mg cell dry weight per

hour. However, the addition of the steroids tested further stimulates the lactate production both at low and at high phosphate concentrations, thus showing that phosphate is not the only limiting factor in aerobic glycolysis (Table 6).

IV. *Effect of some steroids on the DPNH dehydrogenase of mitochondria*

We have compared the inhibitory effect of some among the steroids examined on the external DPNH dehydrogenase activity of mitochondria pretreated with hypotonic solutions. It is known that this treatment enables mitochondria to oxidize added DPNH. This reaction can be followed spectrophotometrically (see Methods).

TABLE 7. EFFECT OF 4-HYDROXY-17α-METHYL-19-NORTESTOSTERONE (4 HMN) AND OF 16α-HYDROXY-17α-METHYLTESTOSTERONE (16 HMT) ON AEROBIC GLYCOLYSIS OF HEPATOMA AH 130 ASCITES CELLS, AT DIFFERENT PHOSPHATE CONCENTRATIONS

| | Medium (pH 7·4) | | Lactic acid μg/mg dry weight/hr | | |
|---|---|---|---|---|---|
| Exper. | Phosphate buffer | Tris buffer* | Control | +4 HMN 25 μg/ml | +16 HMT 25 μg/ml |
| I | 0·0016 M | 0·016 M | 143 | 182 | |
| | 0·016 M | — | 142 | 184 | |
| | 0·100 M | — | 140 | 191 | |
| II | 0·0008 M | 0·016 M | 89 | 149 | |
| | 0·0016 M | 0·016 M | 100 | 158 | |
| | 0·016 M | — | 116 | 171 | |
| III | 0·0016 M | 0·016 M | 46 | | 83 |
| | 0·016 M | 0·016 M | 86 | | 196 |
| | 0·16 M | 0·016 M | 102 | | 180 |

* Tris-(hydroxymethyl)-aminometane-HCl buffer.

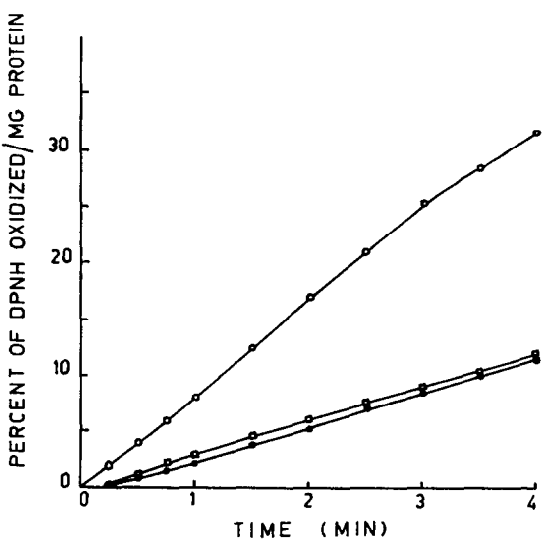



FIG. 2. Influence of 4-hydroxy-17α-methyl-19-nortestosterone (4 HMN) and of 16α-hydroxy-17α-methyltestosterone (16 HMT) on DPNH oxidation by isolated rat liver mitochondria.
——— ○ ——— control, ——— ● ——— 4 HMN 50 μg/ml; ——— □ ——— 16 HMT 50 μg/ml.

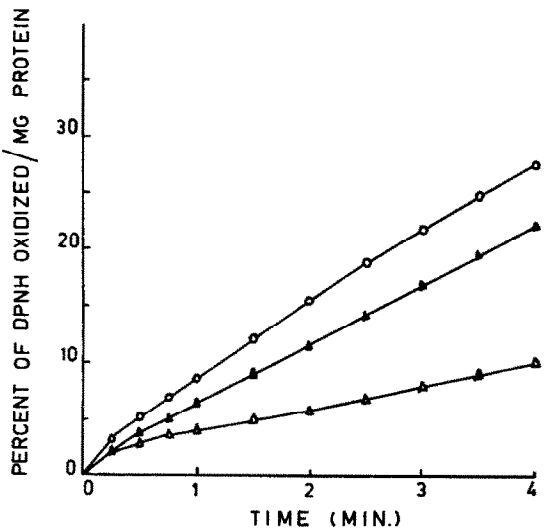



FIG. 3. Influence of cortisone and of 4-hydroxy-17α-methyltestosterone on DPNH oxidation by isolated rat liver mitochondria.
——— ○ ——— control; ——— ▲ ——— cortisone 50 μg/ml; ——— △ ——— 4-hydroxy-17α-methyltestosterone 50 μg/ml.

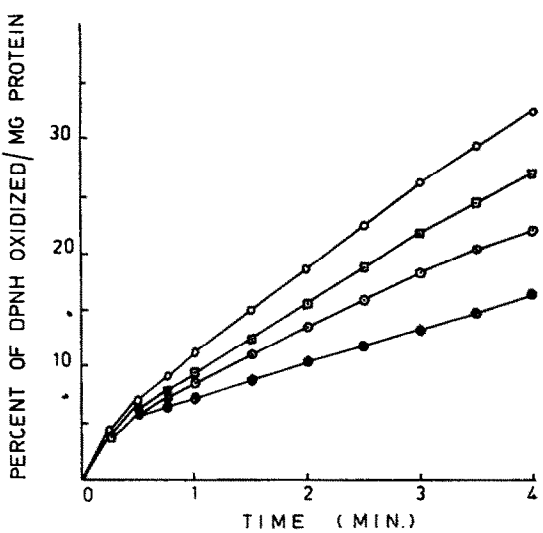



FIG. 4. Influence of 16α-hydroxy-17α-methyl-19-nortestosterone, 4:16α-dihydroxy-17α-methyltestosterone and 17α-methyltestosterone on DPNH oxidation by isolated rat liver mitochondria.
——— ○ ——— control; ——— ⊡ ——— 16α-hydroxy-17α-methyl-19-nortestosterone 50 μg/ml; ——— ○ ——— 4:16α-dihydroxy-17α-methyltestosterone 50 μg/ml ——— ⊕ ——— 17α-methyltestosterone 50 μg/ml.

In Figs. 2, 3 and 4 are plotted (versus time) the percentage of DPNH reoxidized by damaged mitochondria, in the absence of steroids and with addition of them, at the concentration of 50 μg/ml. 17α-Methyltestosterone, 4-hydroxy-17α-methyltestosterone, 4-hydroxy-17α-methyl-19-nortestosterone, and 16α-hydroxy-17α-methyltestosterone

possess the same inhibitory activity on DPNH oxidase, while 16α-hydroxy-17α-methyl-19-nortestosterone is remarkably less active, and 4:16α-dihydroxy-17α-methyltestosterone has an intermediate degree of effectiveness.

## CONCLUSIONS

The data reported confirm that the introduction of an alkyl group at $C_{17}$ increases the ability of testosterone and nortestosterone to inhibit the oxidative phosphorylation of mitochondria.

Among the testosterone and nortestosterone derivatives examined, those having an hydroxyl group at $C_4$ (4-hydroxy-17α-methyltestosterone and 4-hydroxy-17α-methyl-19-nortestosterone) are able to inhibit both oxidations and oxidative phosphorylations: 4-hydroxy-17α-methyl-19-nortestosterone seems to be the most active, even taking into account the experimental variability.

16α-Hydroxy-17α-methyltestosterone is endowed with a remarkable uncoupling activity, which is the highest so far observed in steroids, and with some inhibiting activity on the oxidation. 4:16α-Dihydroxy-17α-methyltestosterone and 16α-hydroxy-17α-methyl-19-nortestosterone differ from the other compounds belonging to this group, since they do not influence the mitochondrial oxidation at all, but have uncoupling activity. By comparing 4-hydroxy-17α-methyltestosterone with 4-hydroxy-17α-methyl-19-nortestosterone on the one hand, and 16α-hydroxy-17α-methyltestosterone with 16α-hydroxy-17α-methyl-19-nortestosterone on the other, it may be observed that the substitution at $C_{10}$ of the methyl group with an hydrogen atom induces a remarkable change in the biological activity, namely the disappearance of the inhibiting effect on the oxidations, only in the steroid bearing an hydroxyl group at $C_{16}$. The disappearance of the oxidation inhibiting activity is also observed when a third hydroxyl group is introduced in 16α-hydroxy-17α-methyltestosterone at $C_4$.

We might tentatively say that the steroids containing a 16α-hydroxyl group are better inhibitors of phosphorylations than of oxidations. The substitution of the hydroxyl group at $C_4$ with a chlorine atom is not very important for the biological activities considered in this work.

Our tests made evident a marked dissociation between the ability of the steroids under examination to inhibit oxidations and the ability to uncouple the oxidative phosphorylation of mitochondria. Some compounds, indeed, possess only uncoupling activity, others, at low concentrations, are only able to inhibit oxidations. Moreover the tests carried out with succinate as substrate, showed that the steroids examined inhibit also the phosphorylations coupled to DPN-independent mitochondrial oxidations, which are not influenced by the same steroids.

The mechanism of action of the steroids tested on the metabolism of hepatoma ascites cells may be explained by the occurrence of a competition between glycolytic enzymes and mitochondrial oxidative system for inorganic phosphate and phosphate acceptors (such as adenosine diphosphate), which has been suggested to explain the Crabtree effect.[30] It is likely that if the glycolytic enzymes can dispose of a higher amount of phosphate and of ADP as a consequence of an inhibition either of the oxidations or of the oxidative phosphorylations, the formation of lactate can be favoured, at least as long as hexokinase activity is not limited by the amount of intracellular ATP. Thus the glycolysis of intact cells can be stimulated either by the steroids

which chiefly inhibit the oxidations such as 17α-methyltestosterone, or by those which do not influence the oxidation but uncouple the oxidative phosphorylations, such as 16α-hydroxy-17α-methyl-19-nortestosterone and 4:16α-dihydroxy-17α-methyl-19-nortestosterone and 4:16α-dihydroxy-17α-methyltestosterone at the highest doses. The stimulation of lactic acid production, induced by the steroids endowed with strong uncoupling activity, might bring about a decrease in pH at some intracellular site, which might cause in turn a $QO_2$ inhibition. However, according to experiments performed by several authors (quoted by Ibsen[30]) and confirmed by ourselves for AH 130 hepatoma ascites cells (unpublished data), this possibility seems to be excluded since dinitrophenol, which causes a very strong increase in lactic acid production contemporarily stimulates cell respiration. The lack of a close correlation between the ability to inhibit the DPNH dehydrogenase activity of mitochondria treated with hypotonic solutions, and the ability to inhibit either the oxidation of DPN-dependent substrates by undamaged mitochondria, or the oxygen uptake by intact cells, indicates that some structural features may prevent a steroid from reaching the sites sensitive to its inhibiting activity. These sites should be "exposed", or easily reached, in the mitochondria which have undergone hypotonic treatment.

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