The Inhibition of Two Steroid-Activated Nicotinamide-Adenine Dinucleotide (Phosphate) Transhydrogenases by Folic Acid and Folic Acid Antimetabolites

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1. It was found that steroid-mediated nicotinamide-adenine dinucleotide (phosphate) transhydrogenases can be inhibited *in vitro* by folic acid and its antimetabolites. The most potent inhibitor was methotrexate, a drug with a high therapeutic index against experimental cancer. 2. The inhibitions produced by a combination of folic acid and the analogues were additive, as were those between the folic acid compounds and antagonistic steroid hormones.

The mechanism of the hormonal action of steroids has not been definitely established. Various hypotheses have been proposed, but it is probable that these hormones exert their influence in a diversity of ways. The control of phospholipid synthesis (Mueller, Herranen & Jervell, 1958; Gorski & Nicolette, 1963) and the rate of synthesis of template RNA (Hancock, Zelis, Shaw & Williams-Ashman, 1962; Liao & Williams-Ashman, 1962; Wilson, 1963; Edelman, Bogoroch & Porter, 1963) have been suggested as possible mechanisms of steroid influence. In addition, however, various steroid-mediated NAD(P) transhydrogenases have been identified in certain endocrine-dependent tissues and from tumours of these tissues (Hollander, Jonas & Smith, 1958; Baron, Gore & Williams, 1960). Thus it is possible that steroids could elicit their response, at least in part, via these enzymes. Since the nicotinamide nucleotide coenzymes are involved in the biosynthesis of nucleic acids, the inhibition of the NAD(P) transhydrogenase could be an important means of controlling the growth of certain steroid-dependent tissues. From the therapeutic standpoint, such an approach is of importance, for it is known that steroids can depress the growth rate of certain steroid-responsive tumours.

NAD(P) transhydrogenases can be inhibited *in vitro* by antipurines and antipyrimidines (Frearson & Williams, 1964), and the present work describes the inhibition of two such enzyme systems by folic acid and its antimetabolites. In this study the steroid-dependent transhydrogenases have been regarded as single enzymes and not as a system containing two or more linked steroid dehydrogenases (see Hagerman & Vilee, 1959; Jarabak, Adams, Williams-Ashman & Talalay, 1962).

EXPERIMENTAL

Materials

Folic acid and aminopterin were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Methotrexate was given by the American Cyanamid Co., Pearl River, N.Y., U.S.A. These chemicals were purified before use according to the method of Johns, Sperti & Burgen (1961) to remove fluorescent impurities. Androsterone and oestradiol-17 β were purchased from Mann Research Laboratories, New York, N.Y., U.S.A.; testosterone was given by Organon Laboratories Ltd., London. NAD, NADP (sodium salt), glucose 6-phosphate dehydrogenase (specific activity approx. 20 m-moles of substrate oxidized/min./mg. of enzyme protein) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Folic acid and its derivatives were dissolved in 0.1 M-tris-HCl buffer at a pH appropriate for the individual experiments, and the steroids were made up in acetone-water (1:1, v/v). Ammonium sulphate (A.R. grade) was purified by crystallization from water containing EDTA (0.2%). Visking dialysis tubing was soaked in 2% (w/v) NaHCO₃ containing EDTA (0.4%) before use and rinsed in glass-distilled water.

Methods

Preparation of 3 α -hydroxy steroid-activated NAD(P) transhydrogenase from rat liver. The protein fraction containing the 3 α -hydroxy steroid transhydrogenase was isolated according to the procedure of Baron, Gore, Pietruszko & Williams (1963). The fraction precipitating between 45 and 80% saturation of ammonium sulphate was exhaustively dialysed against 5 mM-tris-HCl buffer, pH 7.5, containing EDTA (1 mM). No transhydrogenase activity was found in the absence of added androsterone. The preparations were stored at -15° for many weeks without loss of activity.

Preparation of 17 β -hydroxy steroid-activated NAD(P) transhydrogenase from human placenta. Term placentas were obtained as quickly as possible after delivery and cooled

in ice. Jarabak *et al.* (1962) described an extensive purification of the enzyme, and their procedure was followed to the end of the heat-treatment stage. The supernatant from the heat treatment was collected by centrifugation at 40000g for 1 hr. and dialysed exhaustively against medium C (Jarabak *et al.* 1962). A copious precipitate was removed by centrifugation at 30000g. The supernatant was used as the enzyme. Considerable difficulties were encountered during the storage of the enzyme at 0° for long periods. It was observed that the basal transhydrogenase activity (not steroid-mediated) gradually rose with time until it equalled that found in the presence of oestradiol-17 β . A possible explanation was that conjugated oestrogens present in the preparation (as glucuronides, for example) were being slowly degraded and thus saturating the enzyme with oestradiol-17 β . The increase in basal activity was not prevented by storage in other media at 0° or -15°, and in the presence of the β -glucuronidase inhibitor, glucosaccharo-1 $\rightarrow$ 4-lactone. Storage in medium B (Jarabak *et al.* 1962) was more successful, but the high concentration of glycerol (50%) gave rise to turbid solutions during the enzyme assays. As a consequence, the enzyme was stored in medium C at 0° and the preparation was discarded after 7 days. The basal activity had risen to about 15% of the oestradiol-17 β -mediated activity during this time.

In the subsequent inhibition studies, a small correction was applied to the reaction rates by subtracting the basal rate in the presence of the folic acid derivatives. The effect of these compounds on the basal activity was determined in separate experiments.

Enzyme assay. The assay of transhydrogenase activity was carried out at room temperature in a Hilger Uvispek spectrophotometer, fitted with a Gilford automatic-recording attachment. The constituents of the assay system were as described by Frearson & Williams (1964), with the exception that the amount of NAD(P) present was decreased. Four tubes were used, each containing the following components: 0.10 ml. of 0.1M-tris-HCl buffer, pH 7.5; 0.01 ml. of glucose 6-phosphate dehydrogenase, diluted to 2 mg. of protein/ml. with 3.3M-ammonium sulphate, pH 7.0 (Baron *et al.* 1963); 0.05 ml. of 0.05M-glucose 6-phosphate; 0.025 ml. of 1.0mM-NAD(P). To each of tubes 1 and 2 was added 0.01 ml. of acetone-water (1:1, v/v), and to each of tubes 3 and 4 0.01 ml. of steroid solution (1 μ g./0.01 ml.) (this was androsterone for the rat-liver enzyme and oestradiol-17 β for the human-placenta enzyme). A further 0.1 ml. of tris-HCl buffer, pH 7.5, was added to tube 1, and 0.1 ml. of enzyme (containing 2-3 mg. of protein for the rat-liver preparation and 4-5 mg. for the placental preparation) was added to all the tubes. At zero time, 0.10 ml. of 7.5 mM-NAD was added to tubes 2, 3 and 4. Tube 1 served as a blank and tube 2 as a control for transhydrogenase activity in the absence of added steroid. After thorough mixing, 0.25 ml. samples of the solutions were rapidly transferred to micro-cuvettes of 0.30 ml. capacity (1 cm. light-path) and the extinctions at 340 m μ were measured continuously for 15-25 min. In all cases the reaction rates were calculated from the initial (linear) portions of the graphs.

Enzyme activities are expressed as the change in extinction (ΔE) at 340 m μ . Since the two enzyme preparations were isolated by different procedures and had a different protein composition, the final extracts were diluted appropriately until each gave the same activity under standard conditions. The standard rate for the transhydrogenase

assay was ΔE_{340} 0.015/min. In this way, a direct comparison could be made of the effect of a given amount of folic acid derivative on the two enzyme systems. From the molar extinction coefficient of 6220 at 340 m μ for NADH₂, this corresponds to a specific activity (ΔE_{340} /mg. of protein/min.) of 3.6 μ moles of NADH₂/mg./min. for the 3 α -hydroxy steroid-mediated transhydrogenase preparations and 2.0 μ moles of NADH₂/mg./min. for the 17 β -hydroxy steroid-mediated transhydrogenase preparations.

In the inhibition studies, it was necessary to add the folic acid compounds to the blank, for these compounds adsorb strongly at 340 m μ . Vogel, Snyder & Schulman (1963) reported that pteridine derivatives can inhibit glucose 6-phosphate dehydrogenase, but no inhibition of this enzyme was found with the concentrations of inhibitors that we have used.

Protein concentrations. This was determined by the biuret method, with bovine serum albumin as standard (Gornall, Bardawill & David, 1949).

RESULTS

Inhibitors of 3 α - and 17 β -steroid-activated NAD(P) transhydrogenases. The inhibitions by folic acid, aminopterin and methotrexate are presented in

Table 1. *Inhibition of 3 α -hydroxy steroid-activated NAD(P) transhydrogenase by folic acid and folic acid antimetabolites*

Experimental details are given in the text. The results were reproducible to $\pm 5\%$, and the values have been corrected to the nearest 5%.

Compound	Concn. of compound (μ g./test)	Average inhibition (%)
Folic acid	25	10
	50	25
	75	50
Aminopterin	25	15
	50	40
Methotrexate	25	20
	50	45

Table 2. *Inhibition of 17 β -hydroxy steroid-activated NAD(P) transhydrogenase by folic acid and folic acid antimetabolites*

Experimental details are given in the text. The results were reproducible to $\pm 5\%$, and the values have been corrected to the nearest 5%.

Compounds	Concn. of compound (μ g./test)	Average inhibition (%)
Folic acid	25	25
	50	40
	75	60
Aminopterin	25	20
	50	45
Methotrexate	25	25
	50	55

Tables 1 and 2. The results are the average inhibitions obtained from at least five determinations. The drug with the highest therapeutic value against many experimental rodent cancers, methotrexate, inhibited the enzymes to the greatest extent.

Influence of pH on the action of inhibitors. The inhibitions produced by the pteridine compounds were measured at a series of pH values in 0.1 M-tris-

Fig. 1. Effect of pH (in 0.1M-tris-HCl buffer) of the test solution on the inhibition of 3α-hydroxy steroid-activated NAD(P) transhydrogenase by: ▲, methotrexate (316 μM); ○, folic acid (310 μM). Experimental details are given in the text.

Fig. 2. Effect of pH (in 0.1M-tris-HCl buffer) of the test solution on the inhibition of 17β-hydroxy steroid-activated NAD(P) transhydrogenase by: ▲, methotrexate (316 μM); ○, folic acid (310 μM). Experimental details are given in the text.

hydrochloric acid buffers. Control experiments were performed for each pH in the absence of the drugs, and the results are presented in Figs. 1 and 2. The maximum inhibitions coincide with the optimum pH of both systems, in the range pH 7–8.

Combined effects of folic acid and folic acid analogues. It was important to determine whether the inhibitory effects of folic acid and its analogues were strictly additive, for, if the NAD(P) transhydrogenases do play a role in controlling the rate of growth of certain steroid-dependent tumours, the administration of folic acid together with folic acid antagonists could be of clinical value. In this way, the inhibition of the transhydrogenase systems could be achieved and yet the toxic side effects of methotrexate, for example, could be substantially decreased. Experiments were carried out in the

Table 3. Inhibition of 3α-hydroxy steroid-activated NAD(P) transhydrogenase by antifolic compounds alone and in combination with folic acid

Experimental conditions are given in the text. The results were reproducible to ± 5%, and the values have been corrected to the nearest 5%.

Additions	Concn. of compound (μg./test)	Average inhibition (%)
Folic acid	25	10
Methotrexate	25	20
Methotrexate	50	45
Methotrexate	25	40
plus Folic acid	25	
Aminopterin	50	40
Aminopterin	75	75
Aminopterin	25	40
plus Folic acid	25	
Aminopterin	50	65
plus Folic acid	25	

Table 4. Inhibition of 17β-hydroxy steroid-activated NAD(P) transhydrogenase by antifolic derivatives alone and in the presence of folic acid

Experimental details are given in the text. The results were reproducible to ± 5%, and the values have been corrected to the nearest 5%.

Additions	Concn. of compound (μg./test)	Average inhibition (%)
Methotrexate	25	25
Methotrexate	50	55
Methotrexate	25	55
plus Folic acid	25	
Aminopterin	25	20
Aminopterin	50	45
Aminopterin	25	40
plus Folic acid	25	

presence of both folic acid and its antimetabolites, and the results are presented in Tables 3 and 4. It is evident that a combination of folic acid and the antimetabolites is nearly as effective as an equivalent concentration of methotrexate alone.

Combined inhibition of folic acid derivatives and antagonistic hormones. Certain tumours, such as prostate carcinomas, which require androgens for maximum proliferation, can be successfully ablated by oestrogen therapy. The 3α -hydroxy steroid-mediated NAD(P) transhydrogenase can be inhibited by oestradiol, stilboestrol and other steroids (Williams, Baron & Gore, 1960). Thus a study of the combined effects of antimetabolites and other steroid hormones was undertaken in the hope that further additive inhibitions would be found. Such inhibition was found, in which methotrexate and oestradiol- 17β strongly inhibited the rat-liver

transhydrogenase (Fig. 3), whereas the antifolic acid compound and testosterone together inhibited the placental transhydrogenase system (Fig. 4). In both cases the inhibition was greater than in the presence of methotrexate alone.

DISCUSSION

The finding that the steroid-mediated NAD(P) transhydrogenases can be inhibited by folic acid antimetabolites raises the problem of the mechanism of the inhibition. The inhibition cannot be regarded as specific, since the folic acid antimetabolites are inhibitors of folate reductase. Vogel *et al.* (1963) have attributed the inhibition of a wide field of dehydrogenases by pteridine derivatives to the chelation of essential metal ions. Such a mechanism cannot be in operation with steroid-mediated transhydrogenases, since these enzymes have no requirement for metal ions. However, there is further evidence that these enzymes can be inhibited by a diversity of non-steroid molecules. These include naphthaquinone and flavonoids (Koide, 1962), and dicumarol (McGuire, Pesch & Fanning, 1963). It is therefore difficult to find a common structural feature in these molecules that could be responsible for the inhibition of the transhydrogenase.

There is also some uncertainty as to the importance of the steroid-activated NAD(P) transhydrogenases, since attempts to find such systems in a wide field of steroid-sensitive tissues have been inconclusive, and the reaction rates are much lower than those found with the mitochondrial transhydrogenase (not steroid-mediated) described by Stein & Kaplan (1959). It is suggested that, in the treatment of carcinoma in tissues where these steroid-mediated transhydrogenases have been found, such as mammary gland (Hollander *et al.* 1958) and anterior pituitary (McGuire & Pesch, 1962), the combination of antimetabolite and steroid therapy may prove of value.

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Fig. 3. Inhibition of 3α -hydroxy steroid-activated NAD(P) transhydrogenase by methotrexate and steroid hormones. All samples contained androsterone ($11\mu\text{M}$), together with: A, no further additions; B, oestradiol- 17β ($22\mu\text{M}$); C, methotrexate ($316\mu\text{M}$); D, methotrexate ($316\mu\text{M}$) and testosterone ($22\mu\text{M}$) or progesterone ($20\mu\text{M}$); E, methotrexate ($316\mu\text{M}$) and oestradiol ($31\mu\text{M}$).

Fig. 4. Inhibition of 17β -hydroxy steroid-activated NAD(P) transhydrogenase by methotrexate and steroid hormones. All samples contained oestradiol- 17β ($11\mu\text{M}$), together with: A, no further additions; B, androsterone ($22\mu\text{M}$) or testosterone ($21\mu\text{M}$); C, methotrexate ($316\mu\text{M}$); D, methotrexate ($316\mu\text{M}$) and testosterone ($30\mu\text{M}$).

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