The Inhibition of Nicotinamide-Adenine Dinucleotide-Linked 3 α -Hydroxy Steroid Dehydrogenase by Certain Antimetabolic Drugs

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(Received 12 October 1964)

1. The inhibition of NAD-linked 3 α -hydroxy steroid dehydrogenase *in vitro* by some of the more therapeutically effective antimetabolic drugs has been investigated. 2. Antipurine and antifolic acid drugs inhibit this system, as does folic acid. 3. Combinations of antifolic acid drugs and folic acid itself produce an additive but not synergic inhibition. 4. Antimetabolic drugs give inhibitions that are additive to those produced by antagonistic steroid hormones.

Since the introduction of the folic acid antagonists in 1947, much work has been carried out to determine the mechanism of their chemotherapeutic action in neoplastic disease. The efficacy of these substances in acute leukaemia and in choriocarcinoma has been well documented. Treatment with antimetabolites is, however, always accompanied by the handicap of drug resistance. However, combinations of antimetabolites and hormones may offer a means of partially overcoming this difficulty, and numerous examples of combined therapy have been reported (e.g. Goldin & Mantel, 1957).

Steroid hormones alone have proved valuable in cancer chemotherapy, particularly in the clinical treatment of certain hormone-dependent tumours. Although the precise mechanisms involved in endocrine therapy are unknown it has been suggested (Talalay & Williams-Ashman, 1958; Williams-Ashman, 1961) that some of the physiological effects of steroids can be interpreted on the basis of control of hydrogen transfer between nicotinamide nucleotides by certain steroid dehydrogenases. The response of such systems to hormonal and antimetabolic treatment is therefore of particular interest.

The hydroxy steroid dehydrogenases are widely distributed enzyme systems that catalyse the inter-conversion of specific hydroxyl and ketone groups in the steroid molecule, with coenzymes functioning as hydrogen acceptors. An enzyme capable of transferring hydrogen from the hydroxyl group of a 3 α -hydroxy steroid (e.g. androsterone) to NAD has been studied by a number of workers, and such a system from rat liver was purified by Tomkins (1956). This enzyme has a dual specificity for NAD and NADP. Similar systems are present in micro-organisms, a typical example being the enzyme isolated from *Escherichia freundii*, which is speci-

fically NAD-linked (Hayaishi, Sato, Jakoby & Stohlman, 1955):

3 α -Hydroxy steroid dehydrogenase has also been detected in extracts of human breast tissues, both normal and malignant (Baron, Gore & Williams, 1960). A similar system has been found in tumours of liver, prostate and uterine tissues of the rat (D. C. Williams, unpublished work). Since these workers observed no difference in hormone response between the steroid dehydrogenase systems isolated from malignant and non-malignant tissues, normal rat liver has been used as the enzyme source in all the experiments recorded below.

The experiments of Frearson & Williams (1964) and Mainwaring & Williams (1965) have indicated that the NAD(P)-transhydrogenating system obtained from rat liver can be inhibited by a number of antimetabolites, including 6-mercaptopurine and its riboside, methotrexate and aminopterin. Moreover, the most effective inhibitors of the transhydrogenase are those having the most marked effect on the dehydrogenase.

In the present work we have studied the inhibitory effects of the antifolates methotrexate and aminopterin, of several other antimetabolic substances and of some steroid hormones on the dehydrogenase system. Many of the experiments detailed below have been performed with both the NAD- and the NADP-dependent 3 α -hydroxy steroid dehydrogenases. Similar results were obtained with both systems, and experiments with NAD are described as being typical of both systems.

MATERIALS

Methotrexate (4-amino-*N*¹⁰-methylpteroylglutamic acid) was a gift from the Lederle Laboratories Division of the

American Cyanamid Co., Pearl River, N.Y., U.S.A. Folic acid, aminopterin, 6-mercaptopurine, 6-mercaptopurine riboside, 8-azauracil, 2,6,8-trichloropurine, testosterone, oestradiol-17 β and diethylstilboestrol were obtained commercially.

Folic acid was freed from a number of fluorescent impurities essentially as described by Johns, Sperti & Bergen (1961) by chromatography on a column of DEAE-cellulose. The folate was eluted in 0.5M-tris-HCl buffer, pH 7.5, and precipitated by acidification to pH 3.0. The purified material was washed with dilute acetic acid, redissolved by adjusting the pH to 7.0 and freeze-dried. Paper chromatography of methotrexate and aminopterin on Whatman no. 3 paper, with butanol-acetic acid-water (4:1:1, by vol.) as mobile phase, followed by elution from the dried chromatogram with 0.1M-tris-HCl buffer, pH 7.5, did not alter the dehydrogenase-inhibiting properties of these antimetabolites. In all subsequent experiments the unpurified commercial material was used.

METHODS

Preparation of enzyme. Two male rats were anaesthetized with ether and their livers perfused *in vivo* with 0.9% NaCl solution containing heparin (5 units/ml.) by inserting the needle of an intravenous-infusion apparatus into the hepatic artery. The hepatic vein was cut and the perfusion continued until most of the blood had been removed from the liver. The liver was removed, cooled in ice, cut into small pieces and suspended in an ice-cold solution of nicotinamide (10 μ M), cysteine (1 μ M) and EDTA (1 μ M) in glass-distilled water containing 20% (v/v) of glycerol. The liver suspension was treated in an Ultra-Turrax microhomogenizer for 1 min. with external ice cooling and ultracentrifuged at 105000g for 1 hr. The supernatant was poured off and maintained at 0°. Ice-cold saturated ammonium sulphate solution was added dropwise, with stirring, and the fraction that precipitated between 0 and 50% saturation was centrifuged at 25000g for 30 min. The supernatant was adjusted to 80% saturation by the dropwise addition of saturated ammonium sulphate and was centrifuged at 2500g for 30 min. The residue was dissolved in the minimum of glass-distilled water and dialysed overnight at 3° against 2l. of 5mM-tris-HCl buffer,

pH 7.4, containing 10mg. of EDTA. The enzyme solution was stored at -20°.

Estimation of NAD-dependent 3 α -hydroxy steroid dehydrogenase. This activity was estimated spectrophotometrically by measuring the initial linear increase in extinction at 340m μ in a Hilger Uvispek spectrophotometer with a Gilford recording attachment. Three cuvettes were prepared containing the ingredients shown in Table 1. The reaction was initiated by adding 0.2 ml. of enzyme solution to the cuvettes. A fourth cuvette containing the same components as cuvette 3 was used as a duplicate in all experiments. Cuvettes 1 and 2 served as blank and control respectively. One unit of enzyme activity is that which produces a change in extinction of 0.001/min. Under the test conditions shown in Table 1 the activity of the preparations varied between 70 and 135 units/ml. In the inhibition experiments the antimetabolites were dissolved in 0.1M-tris-HCl buffer, pH 8.4, and added to each cuvette before the enzyme. Steroids and diethylstilboestrol were dissolved in acetone-water (3:1, v/v) before addition to the test system.

RESULTS

Effect of pH on the inhibitory action of methotrexate and aminopterin on NAD-linked 3 α -hydroxy steroid dehydrogenase. This is illustrated in Fig. 1. The upper curve shows the inhibition produced by 100 μ g. of methotrexate per test at a series of pH values in 0.1M-tris-hydrochloric acid buffers. The lower curve illustrates a similar series of experiments with 50 μ g. of aminopterin per test. In general the maximum inhibition produced by antimetabolites was at pH values between 7 and 8. In all subsequent experiments, however, tris-hydrochloric acid buffer, pH 8.4, was used as this is the pH optimum for dehydrogenase activity. Both the NAD- and NADP-linked dehydrogenase systems were inhibited to about the same extent by the antimetabolites at any particular pH.

Table 1. Contents of cuvettes for the estimation of 3 α -hydroxy steroid-dehydrogenase activity

The final concentration of androsterone in the cuvettes was 94 μ M.

Cuvette no. ...	Vol. added (ml.)		
	1	2	3
0.1M-Tris buffer, pH 8.4	2.0	1.9	1.9
NAD, 500 μ g. in 0.1 ml. of tris buffer	—	0.1	0.1
Acetone-water (3:1, v/v)	0.02	0.02	—
Androsterone solution (60 μ g. in 0.02 ml. of acetone-water)	—	—	0.02
Enzyme solution	0.2	0.2	0.2
Total volume	2.22	2.22	2.22

Fig. 1. Effect of pH (in 0.1M-tris-HCl buffer) of the test solution on the inhibition of NAD-dependent 3 α -hydroxy steroid dehydrogenase: ●, by 100 μ g. of methotrexate; ▲, by 50 μ g. of aminopterin. Inhibition is expressed as a percentage of the dehydrogenase activity in a test solution at the same pH containing no inhibitor.

Inhibition by other antimetabolites and by steroids. Antimetabolites were screened for their inhibitory activity against NAD-linked 3 α -hydroxy steroid dehydrogenase and the inhibition produced by the more effective substances is shown in Table 2.

Table 2. *Inhibition of NAD-dependent 3 α -hydroxy steroid dehydrogenase by antimetabolites and other antagonists*

Experimental details are given in the text. The results are corrected to the nearest 5% and the method is reproducible within these limits. The experiments with oestradiol-17 β and stilboestrol were limited by the low solubility of these compounds and in the experiments marked with an asterisk (*) part of the inhibitor was in suspension. One unit of enzyme activity produces an extinction change of 0.001/min. The enzyme activity of uninhibited control experiments varied from 14 to 27 units per test.

Compound	Concn. (μ M)	Inhibition (%)
Methotrexate	50	10
	100	25
	200	40
Aminopterin	50	5
	100	20
	200	45
Folic acid	50	20
	100	30
	200	40
6-Mercaptopurine	130	30
	270	40
	500	80
6-Mercaptopurine riboside	80	5
	160	15
	320	20
6-Azauracil	800	5
	1600	5
Trichloropurine	200	10
	800	25
Testosterone	80	30
	150	45
	300	55
Oestradiol-17 β	80	10
	160	25
	330*	30
Stilboestrol	40	20
	80*	30
Methotrexate + oestradiol-17 β	25 + 40	20
	50 + 80	30
	75 + 120	35
	100 + 160	40
Methotrexate + testosterone	25 + 40	20
	50 + 80	40
	75 + 120	50
	100 + 160	60

Antimetabolites of the antifolate, antipurine and antipyrimidine groups all caused measurable inhibition, as did folic acid itself.

Both 6-mercaptopurine and its riboside are inhibitory, although the riboside is considerably less active. 2,6,8-Trichloropurine is also inhibitory, but only marginal inhibition is observed with high concentrations of 6-azauracil. Testosterone, oestradiol-17 β and stilboestrol also caused inhibition under the same conditions. Experiments with stilboestrol, and to a smaller extent with oestradiol-17 β , were limited by the low solubility of these substances at pH 8.4.

Combined effect of folic acid and folic acid analogues. Fig. 2 shows that the inhibitory effects of both aminopterin and methotrexate are almost linear with respect to concentration up to 100 μ M. Within this limit folic acid exerts a higher inhibition than either analogue, although the response is not linear with concentration. At concentrations above 100 μ M these analogues are more inhibitory than folic acid. The inhibitory effects of mixtures of folic acid and methotrexate or of folic acid and aminopterin are greater than those produced by these constituents separately.

Effect of steroid hormones on inhibition by folic acid derivatives. Both oestradiol-17 β and testosterone inhibit the NAD-linked 3 α -hydroxy steroid dehydrogenase, but the addition of methotrexate causes an almost additive increase in inhibition of dehydrogenase activity (Table 2). A similar result was obtained with the corresponding NADP-linked 3 α -hydroxy steroid dehydrogenase.

Fig. 2. Inhibition of NAD-linked 3 α -hydroxy steroid dehydrogenase by folic acid and its antimetabolites singly and combined: O, methotrexate; Δ , aminopterin; $\square$, folic acid; $\blacktriangle$, aminopterin + folic acid; $\bullet$, methotrexate + folic acid. Inhibition is expressed as a percentage of the dehydrogenase activity in a test solution containing no inhibitor.

DISCUSSION

The results presented follow a pattern similar to those previously reported for the steroid-activated NAD(P)-transhydrogenating systems obtained respectively from rat liver and human placenta (Frearson & Williams, 1964; Mainwaring & Williams, 1965). Talalay *et al.* (1958) believe that steroid-activated transhydrogenation can be explained in terms of coupled steroid dehydrogenases that are nucleotide-specific, whereas Hagerman & Vilee (1959) regard at least some of the transhydrogenases as specific enzymes. The parallel between the inhibition of transhydrogenase and dehydrogenase by antimetabolites appears to favour the first mechanism for the 3α -hydroxy steroid systems obtained from rat liver, since inhibition of either NAD- or NADP-linked dehydrogenase would necessitate an inhibition of transhydrogenase. On the other hand, a specific transhydrogenase might also be sensitive to the same inhibitors. The pH for optimum inhibition is the same for transhydrogenation although the pH optima for the two systems are different.

The inhibitory effect of antimetabolites on the 3α -hydroxy steroid dehydrogenase appears to bear some relationship to the effectiveness of these compounds against a variety of experimental rodent neoplasms. This relationship was also observed in our experiments with the steroid-activated transhydrogenase. Oestradiol- 17β and testosterone have also been used for the treatment of endocrine tumours, especially mammary tumours, and stilboestrol is a most effective drug in the treatment of prostatic cancer. Folic acid also inhibits the steroid-dependent transhydrogenating system obtained from rat liver (Mainwaring & Williams, 1965) as well as glutamate dehydrogenase, lactate dehydrogenase, malate dehydrogenase and glucose 6-phosphate dehydrogenase (Vogel, Snyder & Schulman, 1963).

Combination of folic acid with either methotrexate or aminopterin gave additive inhibition of steroid dehydrogenase. This finding could be of importance in medical practice, since folic acid could be used to limit the toxicity of its antimetabolite without preventing any inhibitory effect on the dehydrogenase system of the organism. A similar effect has been shown with other dehydrogenase systems (Vogel & Schulman, 1962; Vogel *et al.*

1963). Di Paolo (1963) has also demonstrated a correlation between growth inhibition and the inhibition of dehydrogenase activity in Ehrlich carcinoma cells. These results, together with those of Johnson, Corte & Jasmin (1958), Kaufman & Levenberg (1959) and Vogel *et al.* (1963, 1964), suggest that folic acid analogues are not merely specific inhibitors of folate reductases but have a wider physiological importance.

Assuming that some degree of hormonal control can be exerted through steroid dehydrogenation in hormone-dependent tissues, then the interaction between antimetabolites and steroid hormones would be of interest, especially in fast-growing tissues. Many tumours arising from endocrine-controlled organs are themselves steroid-sensitive, and can often be inhibited by 'antagonistic steroids'. The present work shows that the inhibition of 3α -hydroxy steroid dehydrogenase by such steroid hormones and by stilboestrol is additive with that produced by folic acid and its analogues.

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