Biochemical Pharmacology, Vol. 25, pp. 2411–2413. Pergamon Press, 1976. Printed in Great Britain.

In vitro Trials to counteract the inhibitory effect of β-oestradiol and ethynyloestradiol on the B_6-dependent kynurenine aminotransferase enzyme

(*Received* 3 *March* 1976; *accepted* 9 *June* 1976)

The action of pyridoxine (B_6) on tryptophan metabolism is well established [1]. Several enzymes along the tryptophan–niacin pathway e.g., kynurenine hydrolase and kynurenine aminotransferase require the participation of vitamin B_6 as a coenzyme [2–5].

The discovery that metal ions catalyze non-enzymatic reactions of pyridoxal with amino acids [6, 7] suggested that metal ions might participate in the action of pyridoxal phosphate-dependent enzymes [8]. Previous studies revealed that Zn^{2+} and Mn^{2+} ions activate kynurenine aminotransferase [9].

In a study concerning the interrelation between female hormones associated with reproduction and kynurenine metabolism—the central metabolite in the tryptophan–niacin pathway—it was found that progesterone activates the kynurenine aminotransferase, whereas β-oestradiol and ethynyloestradiol inhibit this enzyme preferably [10].

In these present trials use was made of the activating properties of progesterone [10], metal ions (Zn^{2+} and Mn^{2+}) [9] to counteract the inhibitory effect of β-oestradiol and ethynyloestradiol on the metabolism of kynurenine and its conversion to kynurenic acid (KA) through the vitamin B_6-dependent kynurenine aminotransferase enzyme.

The effect of pyridoxal phosphate (PLP) on the combined effect of either β-oestradiol and the metal ions (Zn^{2+} and Mn^{2+}) or ethynyloestradiol and the metal ions is also studied. This approach may explain the possible role of PLP, as a coenzyme, in counteracting the inhibitory effect of these hormones in the presence of high concentrations of manganese and zinc ions.

MATERIALS AND METHODS

Animals. Adult albino mice weighing 15–20 g, fed on a specially prepared diet containing all the necessary factors, were used. Male mice were used in this study, since oestrogen treatment of males depressed the aminotransferase levels to those typical of females whereas oestrogen treatment of females did not further depress these levels [11].

Materials. Ethynyloestradiol, progesterone and β-oestradiol were purchased from Sigma Chemical Company. These water-insoluble hormones were used as a solution in absolute alcohol [12]. A semi pure preparation of the enzyme [13] and incubation medium was used, and the period of incubation and the quantitative determination of kynurenic acid were carried out as previously described [9, 10]. Manganese chloride and zinc sulphate were purchased from Veb-Labor-Chemie, Apolda, Germany.

RESULTS

The results of these trials to counteract the inhibitory effect of β-oestradiol and ethynyloestradiol on the kynurenine aminotransferase are illustrated in Tables 1 and 2. This counteracting effect is indicated by the amount of kynurenic acid produced. This is best illustrated by the percentage activation or inhibition, i.e., the percentage difference between the amount of the metabolite produced in the control experiment and that produced at varying concentration of the activator added.

It is evident from the results obtained that, at a progesterone of concentration 10^{-5} M, *i.e.* when the concentration of progesterone is equal to that of β-oestradiol and ethynyloestradiol, no inhibitory effect is encountered on kynurenine aminotransferase. When an excess amount of β-oestradiol is present in the incubation medium, this enzyme is powerfully inhibited (84 per cent). When the concentration of progesterone (10^{-4} M) [10] exceeds that of the inhibiting hormone (10^{-5} M) the enzyme is activated in the presence of β-oestradiol and inhibited with ethynyloestradiol (Table 1).

In the second two trials using metal ions to counteract the inhibitory effect of these hormones on the kynurenine aminotransferase, it is found that at equimolar concentrations of the metal ions and the hormones the inhibitory effect is minimum, and less than that produced by the hormone alone. The percentage inhibition produced by β-oestradiol and ethynyloestradiol on this enzyme was found to be 31 per cent and 24 per cent, respectively [10]. Moreover, the enzyme is found to be powerfully inhibited by increasing the concentration of the metal ions from 10^{-3} to 10^{-2} M.

This last observation led to the study of the effect of increasing the concentration of PLP (coenzyme) to the incubation medium containing both excess metal ions (10^{-3} M) and the hormones. The scientific background of this trial is based on the fact that kynurenine (substrate), metal ions and pyridoxal phosphate form a complex and that this complex may be either the proper or the improper one which acts with the active centers located on the B_6-dependent apoenzyme [2]. Moreover, there still exists the possibility that pyridoxal phosphate may saturate the apoenzyme and hence the enzyme activity may increase.

The inhibitory effects induced by the presence of both the hormone and Zn^{2+} ions (10^{-3} M) is found to be decreased by the addition of (PLP), while low concentration only of PLP (200 μg) decrease the inhibitory effect induced by the hormone and Mn^{2+}. Increasing the concentration of PLP increases this inhibitory effect.

DISCUSSION

From the results obtained from these trials it is clear that at equimolar concentrations of the activator (progesterone or the metal ions) and the inhibiting hormone, a counteracting effect is obtained, i.e., progesterone antagonises the inhibitory effect of either β-oestradiol or ethynyloestradiol. This antagonistic effect could be attributed to the different affinities of progesterone, β-oestradiol or ethynyloestradiol for the active centers on the apoenzyme. Progesterone seems to have higher affinity for these active centers. The finding that kynurenine aminotransferase enzyme is more powerfully inhibited in the simultaneous

Table 1. Effect of increasing concentrations of progesterone, Zn^{2+} ions and Mn^{2+} ions on the inhibitory effect of β-oestradiol and ethynyloestradiol on the kynurenine aminotransferase enzyme

No.	Concn of activator added (M)	KA* produced (μmole/g liver)	Activation (%)	Inhibition (%)	KA* produced	Activation (%)	Inhibition (%)
			(A) Progesterone				
		β-oestradiol (10^{-5} M)			Ethynyloestradiol (10^{-5} M)		
1	0.0†	2.94	0.0	0.0	2.94	0.0	0.0
2	10^{-6}	0.84	—	84	3.18	8	—
3	10^{-5}	2.94	0.0	0.0	3.90	33	—
4	10^{-4}	3.60	22	—	2.28	—	22
			(B) Zn^{2+} ions				
		β-oestradiol (10^{-4} M)			Ethynyloestradiol (10^{-4} M)		
1	0.0†	3.00		0.0	4.70		0.0
2	10^{-4}	2.80		7	4.50		4
3	10^{-3}	1.48		51	2.04		57
4	10^{-2}	0.0		100	0.20		96
			(C) Mn^{2+} ions				
		β-oestradiol (10^{-4} M)			Ethynyloestradiol (10^{-4} M)		
1	0.0†	3.00		0.0	4.70		0.0
2	10^{-4}	2.90		3	5.40		—
3	10^{-3}	2.75		8	4.18		11
4	10^{-2}	0.10		97	0.08		98

* Average value of four experiments.
† Control experiment, i.e., in the absence of either the hormones or the activator added.

presence of β-oestradiol (10^{-5} M) and progesterone (10^{-6} M), than in the presence of β-oestradiol alone (31 per cent) [10], could be attributed to the different affinities of the two hormones to interact with the enzyme substrate (kynurenine)–PLP–metal ion complex [14]. β-Oestradiol seems to have a higher affinity for the enzyme substrate under the latter experimental conditions.

The finding that equimolar concentrations of the metal ion and the inhibiting hormone induce no inhibitory effect on this enzyme system could be attributed to the probability that two metal ion complexes are formed under these experimental conditions: kynurenine–PLP–metal ion complex and hormone–PLP–metal ion complex. The former complex seems to be the proper one required for kynurenine aminotransferase enzyme, since metal ions alone activate this enzyme [9]. The existence of a low inhibitory effect under this experimental condition could be attributed to the competition between the proper and the improper complex for the active centers on the enzyme [10].

Moreover, the results of the studies carried out to investigate the effect of increasing concentrations of pyridoxal phosphate on the combined effect of either β-oestradiol or ethynyloestradiol and Mn^{2+} ions or Zn^{2+} ions revealed

Table 2. Effect of increasing concentrations of pyridoxal phosphate (PLP) on the combined effect of β-oestradiol or ethynyloestradiol and the metal ions on kynurenine aminotransferase enzyme

No.	Concn of PLP (μg/g liver)	KA* produced (μmole/g liver)	Inhibition (%)	KA* produced (μmole/g liver)	Inhibition (%)
		(A) Zn^{2+} ions (10^{-3} M)			
		β-oestradiol (10^{-4} M)		Ethynyloestradiol (10^{-4} M)	
1	200†	3.33	0.0	4.05	0.0
2	200	2.70	19	3.45	15
3	300	2.70	19	3.45	15
4	400	2.20	34	3.08	24
		(B) Mn^{2+} ions (10^{-3} M)			
		β-oestradiol (10^{-4} M)		Ethynyloestradiol (10^{-4} M)	
1	200†	3.33	0.0	3.33	0.0
2	200	3.20	4	3.08	8
3	300	2.90	13	2.93	12
4	400	2.40	28	2.45	26

* Average value of four experiments.
† Control experiment, in the absence of the hormone and the metal ions in the incubation medium.

that the role of PLP is the formation of the proper complex. These studies concerning the counteracting effect of progesterone on the inhibitory effect of β-oestradiol or ethynyloestradiol could be one of the future *in vivo* trials to the approach of studying adverse drug reactions in contraceptive users.

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Biochemical Pharmacology, Vol. 25, pp. 2413–2415. Pergamon Press, 1976. Printed in Great Britain.

Inhibition of purine nucleotide biosynthesis by 3-deazaguanine, its nucleoside and 5′-nucleotide

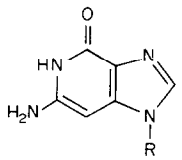
(*Received* 7 *November* 1975; *accepted* 26 *March* 1976)

The synthesis of 3-deazaguanine, 3-deazaguanosine, and 3-deazaguanylic acid (Fig. 1) was recently realized in our laboratories [1] as part of a continuing program to develop chemotherapeutically useful analogs of the naturally occurring purines with particular reference to broad spectrum antiviral agents. Previous studies reporting the antibacterial [2,3] antitumor [4,5] and antiviral [6,7] activity of certain 1- and 3-deazapurines have established the potential chemotherapeutic importance of such compounds. Indeed, 3-deazaguanine, together with the nucleoside and 5′-nucleotide, has demonstrated potent antiviral activity *in vitro* against a variety of DNA and RNA viruses [8] as well as activity *in vivo* against L1210 leukemia and adenocarcinoma 755 in mice [9].

A procedure has been developed by Snyder *et al.* [10], and utilized in our laboratories, in which the apparent activities of eight enzymes of purine nucleotide biosynthesis and interconversion can be calculated when Ehrlich ascites tumor cells are incubated *in vitro* with hypoxanthine [^{14}C]. Previous studies have linked the antagonism of this pathway with a number of compounds possessing antiviral and antitumor activity [11–13]. In particular, the enzyme IMP dehydrogenase, which occupies a key position in this pathway, is a common site of inhibition by many of these compounds. It is, therefore, the purpose of this communication to report the effects of 3-deazaguanine and its derivatives on purine nucleotide biosynthesis in Ehrlich ascites tumor cells.

The synthesis of 3-deazaguanine, 3-deazaguanosine and 3-deazaguanylic acid, as well as the 7-ribosyl derivative of 3-deazaguanine, has been reported [1]. Hypoxanthine [^{14}C] (51 mCi/m-mole, 0.1 mCi/ml) was obtained from ICN Pharmaceuticals, Isotope and Nuclear Division, Irvine, Calif. Ribonucleotide markers were obtained from Sigma Chemical Co., St. Louis, Mo., and PEI-cellulose chromatography plates from Brinkman Instruments Inc., Los Angeles, Calif. Ehrlich ascites tumor cells were the generous gift of Dr. J. Frank Henderson, University of Alberta, Edmonton, Alberta, Canada. Procedures for the maintenance, preparation and incubation of the cells have been described [10,14]. The apparent activity of each of the enzymes in the pathway is determined as the sum of the radioactive substrate (hypoxanthine [^{14}C]) in all metabolites further along in the pathway. Table 1 gives the arithmetic sums denoting each of the reactions together with typical control values in parentheses. The incorporation of radioactivity into NAD was not included in these calculations because of the low amount (less than 100 counts/min) found in this product. By comparison of the control and drug-treated cells, the per cent inhibition of each of the reactions can be calculated after a correction is made for inhibition of the reactions prior to the one under consideration. A complete analysis of these computations can be found in Ref. 10.

IMP dehydrogenase (IMP:NAD-oxidoreductase, EC 1.2.1.14) was partially purified from *E. coli. B.* and assayed



(a) R = H
(b) R = β-D-ribose
(c) R = β-D-ribose-5′-phosphate

Fig. 1. Structures of (a) 3-deazaguanine, (b) 3-deazaguanosine and (c) 3- deazaguanylic acid.