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RELATIONSHIP BETWEEN PYRIDOXAL PHOSPHATE AND SOME SYNTHETIC OESTROGENS IN THEIR EFFECT ON KYNURENINE HYDROLASE AND KYNURENINE AMINOTRANSFERASE ENZYMES OF NORMAL MOUSE LIVER

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Abstract The interrelationship between female hormones associated with reproduction and kynurenine metabolism was studied in the whole liver homogenates from male mice. These *in vitro* studies were planned to investigate the possible mechanisms by which β-oestradiol, ethinyloestradiol, mestranol and progesterone may affect the vitamin-B_6-dependent enzymes, kynurenine aminotransferase and kynurenine hydrolase. It was found that the chemical structure of the oestrogenic hormone may be an important factor in determining the occurrence or not of inhibition of these enzymes since mestranol and progesterone have no inhibitory effect on both enzyme systems. Whereas β-oestradiol and ethinyloestradiol inhibit preferably the kynurenine aminotransferase enzyme. The inhibitory effect of β-oestradiol is of the competitive type while that of ethinyloestradiol is a non-competitive one.

RECENT reports have suggested an interrelationship between the female sex hormones associated with reproduction and vitamin B_6 in its role in the metabolism of tryptophan.[1–6] This is because several enzymes along the tryptophan–niacin (kynurenine) pathway e.g. kynurenine hydrolases, aminotransferases and most probably the quinolinic acid decarboxylase, require the participation of vitamin B_6 as coenzyme.[7–11]

It was recently found that the disordered tryptophan metabolism encountered in girls just before menarche, young nonpregnant women in the preovulatory phase, and in women in the post-menopausal age is due to an interaction between vitamin B_6 and female sex hormones associated with reproduction.[12,13] However, the disordered tryptophan metabolism encountered in women in the post-menopausal age is attributed to the trophic hormones excessively secreted from the pituitary gland rather than to the (diminished) secretion of the suprarenal cortical hormones in this phase of life. The increased secretion of the trophic factors from the hyperactive pituitary seem to interfere with the normal activities of the vitamin B_6-dependent enzymes: kynurenine hydrolases and aminotransferases.[12,14,15]

The precise site(s) of action of female sex hormones associated with reproduction upon the tryptophan–nicotinic acid ribonucleotide pathway is not known with certainty. Two proposals have been forwarded to explain the abnormality in tryptophan metabolism produced in the human by oestrogens, whether from endogenous or exogenous sources, and during pregnancy.[3,16,17]

Although many investigators suggested that the abnormal tryptophan metabolism in pregnancy is due to increased tryptophan pyrrolase activity, thus transferring more substrate to tryptophan niacin pathway, this proved difficult to demonstrate. Even after the finding that women treated with oral contraceptive steroids also demonstrated abnormal tryptophan metabolism,[1] investigators reported difficulty demonstrating an ovarian hormone effect upon tryptophan pyrrolase directly.[4-6,18,19] Nevertheless, if the administration of vitamin B_6 is only needed to increase the capacity of the vitamin B_6-dependent enzymes to convert the accumulated metabolites into niacin, then one would expect to get an excretion pattern analogous to that encountered in (either mild, moderate, or severe) absolute B_6 deficiency i.e., a decrease in the excretion levels of *o*-aminohippuric acid, anthranilic acid glucuronide and 3-hydroxyanthranilic acid and an increased level of kynurenine, acetylkynurenine, 3-hydroxykynurenine and xanthurenic acid.[9,20] However, the consistent increase in excretion of kynurenine, *o*-aminohippuric acid, anthranilic acid glucuronide, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid and 3-hydroxyanthranilic acid,[3,12,15,16] seems to refute the proposal that oestrogens cause an induction of tryptophan oxygenase that is mediated via the hypothalamo pituitary adrenal axis and that late in pregnancy, a true vitamin B_6-deficiency is superimposed upon the hormonal effects. In this context, it has been shown that the activity of kynurenine hydrolase enzymes, particularly that concerned with the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid, is reduced much more, and at an earlier stage of vitamin B_6-deficiency than kynurenine aminotransferase activity.[7,21] This would result in an accumulation only of the preceding metabolites including 3-hydroxykynurenine, kynurenine, acetylkynurenine and xanthurenic acid together with the excretion of reduced amounts of *o*-aminohippuric acid and anthranilic acid glucuronide, and this is not the case.

A depressing action of oestrogens on some B_6-dependent enzyme levels was indicated in earlier studies marked sex differences in the kynurenine hydrolase and kynurenine aminotransferase levels in adult rats.[22-24]

Therefore, the present series of studies were carried out to investigate the *in vitro* effects of increasing concentrations of some synthetic female sex hormones, gonadotrophic hormone, and some trophic-released hormones, on the metabolism of kynurenine, which is the central metabolite in the kynurenine pathway of tryptophan metabolism, and its conversion to kynurenic acid and anthranilic acid through the vitamin B_6-dependent enzymes: kynurenine aminotransferase (EC 2.6.1.7) and kynurenine hydrolase (EC 3.7.1.3), respectively. The synthetic female sex hormones investigated in the present study include β-oestradiol, ethinyloestradiol, mestranol and progesterone.

In vitro studies on the effect of these hormones on the above mentioned B_6-dependent enzymes may help in understanding the effects encountered in the *in vivo* experiments. Thus, the present series of studies: (a) exclude the *in vivo* induction by some hormones of tryptophan oxygenase enzyme,[5,25] and (b) throw more light on the mode of action by which these hormones may interfere with these reactions. Moreover, this type of study could initiate research activities directed towards establishing a stronger scientific base for supporting recommendation for supplemental nutritional therapy of contraceptive users i.e., an approach to the study of adverse drug reactions.

MATERIALS AND METHODS

Animals. Adult male albino mice (15–20 g) fed *ad lib.* on a specially prepared diet containing all the necessary factors were used.

Materials. Kynurenine sulphate was purchased from Schuchardt Co. (München, Germany). α-Ketoglutarate was supplied by L. Light and Co. Ltd. (Colnbrook, England). Anthranilic acid (AR) was purchased from Merk AG (Darmstadt, Germany). Pyridoxal phosphate was prepared by using the method described by Beiler and Martin (1947).[26] In some experiments high-quality commercial pyridoxal phosphate (grade "A", Calbiochem, Los Angeles, Calif., U.S.A.) was used. The results obtained with either sample of pyridoxal phosphate were indistinguishable. Kynurenic acid, β-oestradiol 3,17(β)-dihydroxy-$\Delta^{1,3,5(10)}$-estratriene), ethinyloestradiol (17(α)-ethinyl-$\Delta^{1,3,5}$-estratriene-3,17(β)-diol) together with pyridoxal hydrochloride and *N*-(1-naphthyl)-ethylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Lewis, Mo., U.S.A.). Mestranol (17α-ethinyl $\Delta^{1,3,5}$-estratriene-3,17-diol-3-methyl ether) was a gift from Nile Company for Pharmaceutical and Chemical Industry (Cairo, Egypt). Progesterone (17(α)1-ketoethyl-Δ^4-androstene-3-one) was purchased from Ciba Societe Anonyme (Basel, Switzerland) under the proprietary name Lutocycline (M). Redistilled water from an all glass still was used to make solutions.

Preparation of the homogenates. The mice were killed by exsanguination after stunning by a blow on the head. The fresh livers were quickly removed and placed in ice-cold 0·25 M sucrose solution. Tissue homogenates (10 per cent based on the wet weight of the tissue) were prepared in the cold isotonic sucrose solution by using a Potter–Elvehjem homogenizer.

Incubations. Reaction mixtures (final volume, 4 ml) were incubated in 20 ml test tubes shaken in a water bath at 37° with air as the gas phase. At the end of the incubation (3 hr), 1 ml 16% trichloroacetic acid (TCA) was added to each test tube and the mixture transferred to the centrifuge tubes with 1 ml of double distilled water. The precipitate was removed by centrifugation and the supernatants were analyzed. The experimental test tubes were run in quadruplicate and zero time test tubes and a blank made under the same conditions were included in each set of experiments.

The concentration of kynurenine was not rate-limiting for the enzymatic reactions studied, since the maximum amounts of kynurenine utilized after an incubation period of 3 hr, as shown in Results, were not more than 28 per cent of the initial concentration of kynurenine added to the incubation medium (5 μmoles/tube, i.e. 25 μmoles/g liver). Therefore, an incubation period of 3 hr was found adequate for the production of kynurenic acid and anthranilic acid in amounts that facilitate their spectrophotometric determinations. After 3 hr the reaction was not completed, since not more than 28 per cent of the substrate had been utilized (Table 2B). Moreover, our recent kinetic studies have revealed that the production of kynurenic acid and anthranilic acid, measured at different incubation periods up to 180 min, revealed that the relationship between kynurenine utilization and time was linear.*

The concentration of the different materials, when present in the incubation medium (unless otherwise stated) were: DL-kynurenine sulphate, 5·0 μmoles; potassium phosphate buffer (pH 7·4), 0·05 M; α-ketoglutarate, 30·0 μmoles; calcium chloride, 0·005 M; magnesium sulphate, 0·001 M; pyridoxal phosphate, 40·0 μg; 10% whole liver homogenate, 2·0 ml.

* H. Sadek *et al.*, manuscript in preparation.

The water insoluble hormones, β-oestradiol, ethinyloestradiol, mestranol and progesterone, were dissolved in 100% ethanol such that the amount required for addition to the incubation medium was present in 0·1 ml.[41] An alcohol control was always included in each experiment where these insoluble hormones were used.

Quantitative estimation of metabolites. Anthranilic acid and kynurenine were determined by the method of Mason and Berg.[27] Kynurenine and kynurenic acid, and anthranilic acid were also determined by a slight modification in the method used by Miller *et al.*[28] This modification, referred to in our previous publication,[29] was used to overcome the difficulties encountered in using three simultaneous equations with three unknowns.

RESULTS

The effect of increasing concentrations of either β-oestradiol or ethinyloestradiol on the kynurenine aminotransferase and the kynurenine hydrolase, as indicated by the amounts of kynurenic acid and anthranilic acid (in μmoles/g liver) produced respectively, is shown in Table 1A and B. The relative inhibition of both enzyme systems is best illustrated by (a) the ratio of the amount of anthranilic acid to the amount of kynurenic acid produced, and (b) the percentage inhibition, i.e., the percentage difference between the amount of metabolite produced in the control and that produced in the presence of varying concentrations of the hormone with reference to the control value. A concentration of 1×10^{-6} M β-oestradiol or ethinyloestradiol inhibits the kynurenine aminotransferase as evidenced by the reduction in the level of kynurenic acid produced. On the other hand, the kynurenine hydrolase enzyme is activated as shown by the increased production of anthranilic acid (Expt 2). Increasing the concentration of these hormones to 1×10^{-5} M (Expt 3) and to 1×10^{-4} M (Expt 4) induces more pronounced inhibition of the kynurenine aminotransferase and more activation of the kynurenine hydrolase enzyme (Table 1).

The effect of increasing concentrations of mestranol and progesterone on the kynurenine aminotransferase and the kynurenine hydrolase enzyme systems are shown in Table 2A and B. In the presence of 1×10^{-6} M mestranol, no demonstrable effect could be detected on both enzyme systems since the amounts of kynurenic acid and anthranilic acid are equivalent to those produced in its absence (Expt 2, Table 2A). Increasing this hormone concentration to 1×10^{-5} M and 1×10^{-4} M fails to induce any demonstrable effect on the kynurenine aminotransferase enzyme, whereas the kynurenine hydrolase enzyme is slightly activated. On the other hand, in the presence of 1×10^{-6} M progesterone, no significant effect on both enzyme systems is detected. Increasing this hormone concentration from 1×10^{-5} M to 1×10^{-4} M, results, however, in a more pronounced activation of the kynurenine aminotransferase enzyme system only. This is evidenced by the relative increased production of kynurenic acid to that produced in the control experiment or in the presence, in the medium, of 1×10^{-6} M of the hormone; anthranilic acid production is not significantly increased (Table 2B).

The effect of increasing concentrations of pyridoxal-5′-phosphate (Plp) on the action of β-oestradiol, ethinyloestradiol, mestranol, or progesterone on both enzyme systems was investigated and the results are shown in Tables 3A and B, and 4A and B. The results indicate that in the absence of these hormones, the addition of Plp

TABLE 1. EFFECT OF INCREASING CONCENTRATIONS OF β-OESTRADIOL OR ETHINYLOESTRADIOL ON THE METABOLISM OF KYNURENINE BY THE NORMAL MOUSE LIVER HOMOGENATES*

Expt no.	Hormone concn (M)	Metabolites determined (μmole g liver)† Kynurenine utilized‡ KYN	Kynurenic acid KA	Anthranilic acid AA	Ratio AA/KA	% Inhibition KA	% Activation AA
			(A) β-Oestradiol				
1	0·0	4·41	3·06	0·53	0·17	0·0	0·0
2	1×10^{-6}	4·16	2·70	0·54	0·20	12	3
3	1×10^{-5}	4·03	2·10	0·66	0·31	31	26
4	1×10^{-4}	3·91	1·56	0·69	0·44	49	31
			(B) Ethinyloestradiol				
1	0·0	4·41	3·06	0·53	0·17	0·0	0·0
2	1×10^{-6}	4·91	2·76	0·69	0·25	10	31
3	1×10^{-5}	4·91	2·34	0·81	0·35	24	54
4	1×10^{-4}	4·91	1·92	1·86	0·97	37	250

* The incubation medium (4 ml) contained 5 μmoles DL-kynurenine sulphate, 30 μmoles α-ketoglutrate, 40 μg pyridoxal phosphate, 0·005 M calcium chloride, 0·001 M magnesium sulphate and 10 per cent whole liver homogenate (2 ml) in 0·05 M potassium phosphate buffer, pH 7·4. Incubations were carried out for 3 hr at 37°.

† Average values of four experiments.

‡ These values represent the difference between the kynurenine recovered and that originally present in the medium.

Table 2. Effect of increasing concentrations of mestranol or progesterone on the metabolism of kynurenine by the normal mouse liver homogenates*

Expt No.	Hormone conen (M)	Metabolites determined (μmole g liver)† Kynurenine utilized‡ KYN	Kynurenic acid KA	Anthranilic acid AA	Ratio AA/KA	% Activation KA	% Activation AA
			(A) Mestranol				
1	0·0	5·04	3·00	0·51	0·17	0·0	0·0
2	1×10^{-6}	5·17	3·00	0·53	0·18	0·0	3
3	1×10^{-5}	5·04	2·94	0·56	0·19	0·0	9
4	1×10^{-4}	4·91	3·06	0·71	0·23	0·0	38
			(B) Progesterone				
1	0·0	5·17	3·18	0·56	0·17	0·0	0·0
2	1×10^{-6}	5·42	3·36	0·63	0·19	6	14
3	1×10^{-5}	7·18	6·00	0·69	0·11	89	24
4	1×10^{-4}	7·18	7·60	0·74	0·10	139	32

* The incubation medium (4 ml) contained 5 μmoles DL-kynurenine sulphate, 30 μmoles α-ketoglutrate, 40 μg pyridoxal phosphate, 0·005 M calcium chloride, 0·001 M magnesium sulphate and 10 per cent whole liver homogenate (2 ml) in 0·05 M potassium phosphate buffer, pH 7·4. Incubations were carried out for 3 hr at 37°.

† Average values of four experiments.

‡ These values represent the difference between the kynurenine recovered and that originally present in the medium.

TABLE 3. EFFECT OF INCREASING CONCENTRATIONS OF PYRIDOXAL PHOSPHATE ON THE ACTION OF β-OESTRADIOL OR ETHINYLOESTRADIOL ON THE METABOLISM OF KYNURENINE BY THE NORMAL MOUSE LIVER HOMOGENATES*

Expt No.	Pyridoxal phosphate (μg/g liver)	Molar concn of the hormone†											
		0·0			1×10^{-6}			1×10^{-5}			1×10^{-4}		
		KYN‡§¶	KA‡§	AA‡§	KYN‡§¶	KA‡§	AA‡§	KYN‡§¶	KA‡§	AA‡§	KYN‡§¶	KA‡§	AA‡§
		(A) β-Oestradiol											
1	0·0	1·39	0·96	0·32	1·39	0·78	0·33	1·13	0·00	0·41	1·13	0·00	0·50
2	50	2·90	1·50	0·36	2·77	1·02	0·44	2·14	0·60	0·53	1·89	0·00	0·56
3	100	3·53	1·86	0·42	3·15	1·68	0·47	2·39	1·26	0·56	2·39	0·78	0·59
4	150	3·90	2·46	0·45	3·53	2·22	0·48	3·40	1·80	0·63	3·40	1·14	0·63
5	200	4·41	3·06	0·53	4·16	2·70	0·54	4·03	2·10	0·66	3·91	1·56	0·69
		(B) Ethinyloestradiol											
1	0·0	1·39	0·96	0·32	1·13	0·66	0·41	1·13	0·54	0·48	1·13	0·42	0·90
2	50	2·90	1·50	0·36	3·15	1·14	0·53	3·15	0·90	0·71	3·15	0·72	1·14
3	100	3·53	1·86	0·42	3·91	1·80	0·57	3·78	1·44	0·74	3·78	1·14	1·37
4	150	3·90	2·46	0·45	4·41	2·28	0·68	4·41	1·98	0·75	4·28	1·50	1·80
5	200	4·41	3·06	0·53	4·91	2·76	0·69	4·91	2·34	0·81	4·91	1·92	1·86

* The incubation medium (4 ml) contained 5 μmoles DL-kynurenine sulphate, 30 μmoles α-ketoglutrate, 0·005 M calcium chloride, 0·001 M magnesium sulphate, 10 per cent whole liver homogenates (2 ml) in 0·05 M potassium phosphate buffer, pH 7·4. Incubations were carried out for 3 hr at 37°.

† The determined metabolites are expressed in μmole/g liver.

‡ Average values of four experiments.

§ The abbreviations KYN, KA and AA represent kynurenine utilized, kynurenic acid and anthranilic acid produced, respectively.

¶ These values represent the difference between the kynurenine recovered and that originally present in the medium.

Table 4. Effect of increasing concentrations of pyridoxal phosphate on the action of mestranol or progesterone on the metabolism of kynurenine by the normal mouse liver homogenates*

Expt No.	Pyridoxal phosphate (μg/g liver)	Molar concn of the hormone†											
		0·0			1×10^{-6}			1×10^{-5}			1×10^{-4}		
		KYN‡§¶	KA‡§	AA‡§	KYN‡§¶	KA‡§	AA‡§	KYN‡§¶	KA‡§	AA‡§	KYN‡§¶	KA‡§	AA‡§
						(A) Mestranol							
1	0·0	1·26	0·90	0·35	1·39	0·90	0·38	1·39	0·84	0·41	1·26	0·78	0·48
2	50	2·77	1·20	0·42	3·40	1·14	0·42	2·39	1·44	0·48	2·27	1·20	0·51
3	100	3·78	1·80	0·44	4·28	1·80	0·45	4·16	1·80	0·53	3·78	1·92	0·59
4	150	4·66	2·34	0·45	4·91	2·34	0·50	4·66	2·16	0·54	4·28	2·34	0·63
5	200	5·04	3·00	0·51	5·17	3·00	0·53	5·04	2·94	0·56	4·91	3·06	0·71
						(B) Progesterone							
1	0·0	2·27	1·02	0·35	2·27	1·32	0·51	2·27	1·56	0·57	3·02	2·16	0·62
2	50	3·02	1·56	0·45	3·28	1·74	0·54	3·28	4·38	0·62	4·28	4·26	0·65
3	100	4·28	2·28	0·50	4·41	2·64	0·59	5·42	4·92	0·65	5·92	6·06	0·71
4	150	4·66	2·64	0·53	4·91	3·00	0·60	5·92	5·76	0·66	6·30	7·08	0·72
5	200	5·17	3·18	0·56	5·42	3·36	0·63	7·18	6·00	0·69	7·18	7·62	0·74

* The incubation medium (4 ml) contained 5 μmoles DL-kynurenine sulphate, 30 μmoles α-ketoglutrate, 0·005 M calcium chloride, 0·001 M magnesium sulphate, 10 per cent whole liver homogenates (2 ml) in 0·05 M potassium phosphate buffer, pH 7·4. Incubations were carried out for 3 hr at 37°.

† The determined metabolites are expressed in μmole/g liver.

‡ Average values of four experiments.

§ The abbreviations KYN, KA and AA represent kynurenine utilized, kynurenic acid and anthranilic acid produced, respectively.

¶ These values represent the difference between the kynurenine recovered and that originally present in the medium.

stimulates the production of both kynurenic acid and anthranilic acid. In the presence of varying concentrations of β-oestradiol (Table 3A) or ethinyloestradiol (Table 3B), increasing the concentration of Plp stimulates fuller activation of kynurenine hydrolase enzyme as evidenced by the increased production of anthranilic acid. However, increasing the concentration of Plp is associated with a partial reversal of the inhibition encountered on the kynurenine aminotransferase enzyme to an extent dependent upon the amount of β-oestradiol or ethinyloestradiol present in the medium. Furthermore, the addition of Plp up to a concentration of 200 μg/g liver in the presence of varying concentrations of these hormones, fails to result in the production of kynurenic acid in amounts equivalent to those produced in its absence. The difference becomes more pronounced when higher concentrations of these hormones are present in the incubation medium (Table 3).

It is also found that more kynurenine is utilized than that converted to both metabolites in the presence of β-oestradiol or ethinyloestradiol (Table 3A and B). The difference might reflect hydroxylation of kynurenine to 3-hydroxykynurenine or to further metabolism of either of the products.

In the presence of increasing concentrations of mestranol, increasing the concentration of Plp results in the production of kynurenic acid in amounts equivalent to those obtained in its absence (Table 4A). In the latter case, however, the amounts of anthranilic acid produced are slightly higher than those produced in its absence. The amounts of kynurenine utilized are always more than the amounts converted to kynurenic acid and anthranilic acid. The difference might reflect hydroxylation of kynurenine to 3-hydroxykynurenine and further metabolism of the latter metabolites to other products along the kynurenine pathway.

On the other hand, it could be seen from Table 4B that in the absence and presence of a constant concentration of progesterone, increasing the concentration of Plp results in increased production especially of kynurenic acid and of the amounts of kynurenine utilized; the amounts of anthranilic acid produced seem constant, especially in the presence of 1×10^{-5} M and 1×10^{-4} M of the hormone and independent of whether the amounts of Plp added are 100, 150 or 200 μg/g liver. Furthermore, the amounts of kynurenine utilized are found to be constant and nearly equivalent to those converted to both products irrespective of whether the concentration of the hormone present is 1×10^{-5} M or 1×10^{-4} M and independent of whether the amounts of Plp added are 100, 150 or 200 μg/g liver (Table 4B).

Although Plp is not a substrate for either kynurenine hydrolase or kynurenine aminotransferase, simulated lineweaver–Burk double reciprocal plots are constructed for the kynurenine aminotransferase enzyme in the presence of varying concentrations of β-oestradiol or ethinyloestradiol. The amount of product formed after incubation is taken as an estimate of the velocity of the reaction (V), and Plp concentration is used in place of the substrate concentration (S) since Plp interacts with kynurenine and a polyvalent cation to form a complex. This complex seems to be the intermediate involved in the action of B_6-dependent kynurenine hydrolase and kynurenine aminotransferase enzymes.[7,30,31] Moreover, Plp usually acts as a second substrate in these reactions.[32] The constructed graphs are shown in Figs. 1 and 2 for kynurenine aminotransferase using β-oestradiol or ethinyloestradiol in three different concentrations. The negative reciprocal of the intercept of such a plot on the $1/S$ axis is equal to the Michaelis constant (K_m); and its values are expressed in

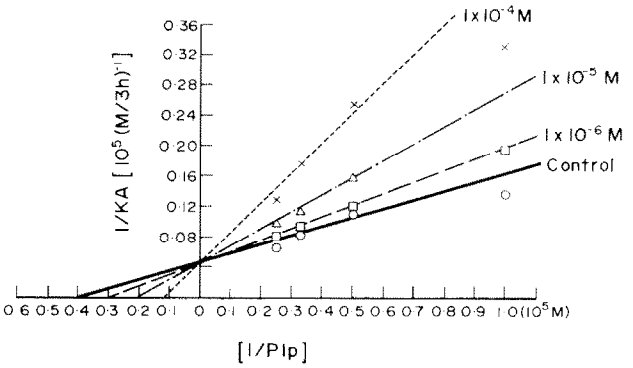

FIG. 1(a). Graphical determination of (K_m) and (V_{max}) for the inhibitory effect of β-oestradiol on Kynurenine aminotransferase enzyme.

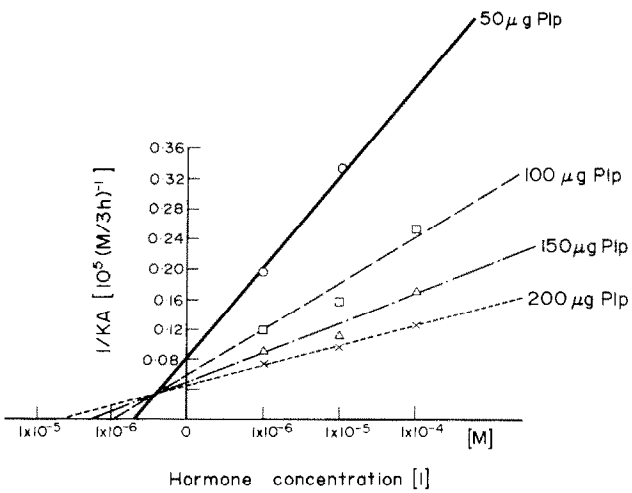

FIG. 1(b). Graphical determination of inhibitor constant (K_i) for the inhibitory effect of β-oestradiol on kynurenine aminotransferase enzyme.

moles/l (M). The reciprocal of the intercept of the same plot on the $1/V$ axis is equal to the maximum velocity (V_{max}) and its values are expressed in (M/3 hr). The graphical method used by Dixon[33] and Dixon and Webb[34] is used for calculating the values for the inhibitor constant (K_i). The kinetic constants of the inhibitory effect of β-oestradiol or ethinyloestradiol on the kynurenine aminotransferase enzyme are included in Table 5.

It can be seen from Fig. 1a that the inhibitory effect of β-oestradiol on the kynurenine aminotransferase enzyme is of the competitive type since the maximum velocity (V_{max}) is a constant value, independent of the amounts of the hormone added. The values of the Michaelis constant (K_m) and the slope are increased by increasing the hormone concentration. The dissociation constant of inhibitor enzyme complex (K_i) is found to be $0{\cdot}46 \times 10^{-6}$ M (Table 5A). On the other hand, the non-competitive nature of the relationship between the Plp substrate complex and ethinyloestradiol (Fig. 2a) on the kynurenine aminotransferase enzyme, may indicate that ethinyloestradiol binds to a different region on the kynurenine aminotransferase enzyme,

TABLE 5. THE KINETIC CONSTANTS OF THE INHIBITORY EFFECTS OF β-OESTRADIOL OR ETHINYLOESTRADIOL ON THE KYNURENINE AMINOTRANSFERASE ENZYME (FIGS. 1 AND 2)

Expt. No.	Hormone concn (M)	Slope K_m/V_{max}	Intercepts: Ordinate $1/V_{max} \times 10^5$	Intercepts: Abscissa $-1/K_m \times 10^5$	V_{max} $\times 10^{-5}$ M/3 hr	K_m $\times 10^{-5}$ M	K_i $\times 10^{-6}$ M
			(A) β-Oestradiol				
1	0·0	0·11	0·04	0·40	22·73	2·52	0·0
2	1×10^{-6}	0·15	0·04	0·30	22·73	3·30	0·46
3	1×10^{-5}	0·22	0·04	0·20	22·73	5·50	0·46
4	1×10^{-4}	0·36	0·04	0·12	22·73	8·27	0·46
			(B) Ethinyloestradiol				
1	0·0	0·13	0·03	0·24	31·25	4·17	0·00
2	1×10^{-6}	0·15	0·04	0·24	27·78	4·17	24·00
3	1×10^{-5}	0·18	0·04	0·24	22·73	4·17	24·00
4	1×10^{-4}	0·23	0·06	0·24	17·86	4·17	24·00

K_m = Michaelis–Menten constant.
V_{max} = Maximum velocity in Molar/3 hr.
K_i = Dissociation constant of the inhibitor complex.

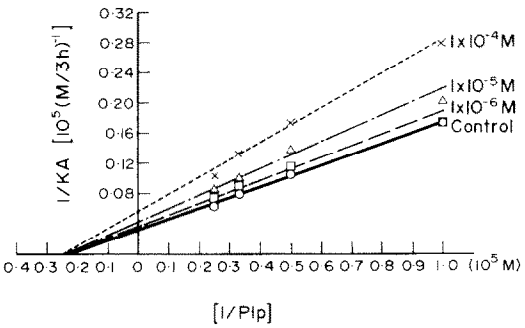

FIG. 2(a). Graphical determination of (K_m) and (V_{max}) for the inhibitory effect of ethinyl oestradiol on kynurenine aminotransferase enzyme.

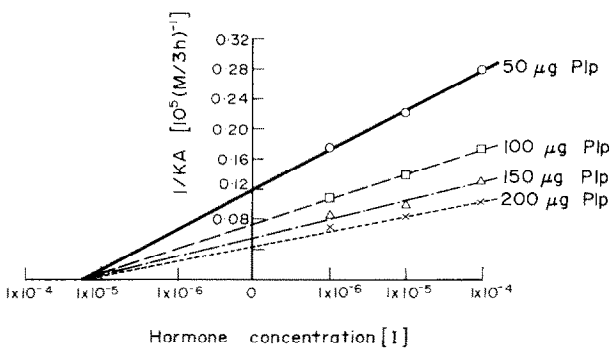

FIG. 2(b). Graphical determination of inhibitor constant (K_i) for the inhibitory effect of ethinyl oestradiol on kynurenine aminotransferase enzyme.

since the values for K_m are constant and independent of the amounts of inhibitor present in the incubation medium. Increasing the hormone concentration results in increased slope values and decreased V_{max} values. The dissociation constant (K_i) of the inhibitor complex is found to be 24×10^{-6} M (Table 5B).

DISCUSSION

It is evident from the present study that β-oestradiol (Table 1A) and its ethinyl derivative (Table 1B) inhibit the vitamin B_6-dependent kynurenine aminotransferase enzyme, whereas the 3-methoxy-17-ethinyl derivative, i.e., mestranol (Table 2A), has no inhibitory effect on both enzymes. It seems that pyridoxal phosphate is not the factor directly responsible for the observed inhibition since increasing concentrations of pyridoxal phosphate are unable to counteract the inhibitory effects of either β-oestradiol (Table 3A) or ethinyloestradiol (Table 3B). The increase in the production of both kynurenic acid and anthranilic acid in response to increasing concentrations of pyridoxal phosphate added, probably reflects stimulation of the uninhibited portion of the enzymes.

In investigating the site of action of β-oestradiol, it is now suggested that this inhibition is brought about by reducing the affinity of the kynurenine aminotransferase enzyme for the pyridoxal phosphate kynurenine complex (Fig. 1a and Table 5A). When both the substrate-complex and β-oestradiol are present, they compete for the

same binding site(s) on the enzyme surface. Thus the degree of association of the kynurenine aminotransferase enzyme with pyridoxal phosphate–substrate complex is depressed. However, no competition occurs between the substrate-complex and β-oestradiol for the same binding sites on the kynurenine hydrolase enzyme; instead, the kynurenine hydrolase enzyme is activated with the consequent overflow of kynurenine metabolism in the direction of anthranilic acid formation (Tables 1A and 2A).

It is noteworthy that ethinyloestradiol inhibits the kynurenine aminotransferase enzyme in a different manner to that induced by β-oestradiol on the same enzyme. (Fig. 2a). The mechanism by which ethinyloestradiol inhibits the kynurenine aminotransferase enzyme seems to be of the non-competitive type (Fig. 2a, Table 5B). The reasons for the different mechanisms by which β-oestradiol and ethinyloestradiol inhibit the kynurenine aminotransferase enzyme are still obscure. However, the competitive mechanism by which β-oestradiol acts on the catalytic site(s) of the kynurenine aminotransferase enzyme may be cautiously interpreted by considering β-oestradiol as antimetabolite, the chemical structure of which resembles that of the substrate required for this particular enzymatic reaction. Moreover, the finding that β-oestradiol affects only the kynurenine aminotransferase activity is not an unexpected observation since a similar difference in the response of these two enzymes, i.e., kynurenine aminotransferase and kynurenine hydrolase, to pyridoxine antimetabolites was shown earlier.[7,8,20,35–37] On the other hand, the non-competitive mechanism by which ethinyloestradiol inhibits the kynurenine aminotransferase enzyme may be attributed to the presence of the 17α-ethinyl group and/or the 3-OH group. Such chemical configuration may help in the binding of ethinyloestradiol to a different region on the enzyme. Therefore, it may be suggested that the inhibitory effect of ethinyloestradiol on the kynurenine aminotransferase enzyme is brought about by an irreversible non-competitive mechanism since increasing concentrations of pyridoxal phosphate are unable to counteract the inhibitory effect of this hormone (Table 3B). Inhibition patterns which are apparently non-competitive are frequently encountered with irreversible inhibitors since the decrease in V_{max} (Table 5B) simply reflects the fact that some enzyme has been removed from the system.

It could be concluded therefore, that the chemical structure of the oestrogenic hormones, from either endogenous or exogenous source, i.e., natural or synthetic and in particular the nature of the substituent group in position 3 on the steroid molecule, may be an important factor in determining the occurrence or not of inhibition of these vitamin B_6-dependent enzymes since mestranol has no inhibitory effect (Table 2A), whereas β-oestradiol and ethinyloestradiol inhibit preferably the kynurenine aminotransferase enzyme (Table 1). The finding that progesterone does not inhibit both enzyme systems (Table 2B) further substantiates this interpretation.

While oestradiol disulphate is a powerful inhibitor of the kynurenine aminotransferase of rat kidney,[17,22,38] the administration of oestradiol benzoate to male rats reduced the activity of hepatic kynurenine hydrolase to that seen in normal females, but it did not influence the activity of hepatic kynurenine aminotransferase.[24] Diethylstilbestrol disulphate inhibits the rat kidney kynurenine aminotransferase at a low concentration. Estrone sulphate inhibits this enzyme at much higher concentrations, whereas several nonanionic steroids such as oestradiol, diethylstilbestrol and estrone are not inhibitory even in saturated solutions.[22] Moreover, high concentrations of

ethinyloestradiol inhibits tryptophan pyrrolase, but its 3-methyl ether derivative, i.e., mestranol, is without effect *in vitro* though it is unlikely that ethinyloestradiol and mestranol have opposite effects on tryptophan pyrrolase activity *in vivo*.[3,39 41]

It is interesting to note that the synthetic and natural oestrogens differ in their *in vivo* effects upon the enzymes concerned in tryptophan metabolism. It seems that the ovarian oestradiol interferes with the normal functioning of the probably vitamin B_6-dependent quinolinic acid decarboxylase. This effect could be overcome by the presence of the naturally occurring ovarian progesterone as well as by the administration, for a short period, of synthetic ethinyloestradiol.[12] In the latter case, ethinyloestradiol has no inhibitory effect on the vitamin B_6-dependent enzymes; the kynurenine hydrolase and the kynurenine aminotransferase. However, in the present *in vitro* experiments; ethinyloestradiol inhibits the vitamin B_6-dependent kynurenine aminotransferase enzyme in mice liver homogenates. The difference between the *in vivo* and the *in vitro* experiments could be attributed to differences in the metabolism of the *in vivo* administered hormone. Of possible relevance to this interpretation is the suggestion of Rose and Brown[24] that the *in vivo* action of oestradiol benzoate on hepatic kynurenine hydrolase could be attributed to the subsequent formation of its bisulphate ester by the liver. Species difference in the response of vitamin B_6-dependent enzymes to vitamin B_6-deficiency and antimetabolites are widely known.[7,8,42 44] However, it seems that the naturally occurring and the synthetic progesterone do not differ in their *in vitro* and *in vivo* effects on these two vitamin B_6-dependent enzymes. Thus, the naturally occurring progesterone antagonizes the *in vivo* effect of the naturally occurring β-oestradiol.[12] Moreover, the synthetic progesterone does not inhibit the B_6-dependent kynurenine hydrolase and kynurenine aminotransferase enzymes in the present *in vitro* studies.

Of particular interest is the finding, in the present work, that β-oestradiol (Tables 1A and 3A), ethinyloestradiol (Tables 1B and 3B) and mestranol (Tables 2A and 4A) do not affect the vitamin B_6-independent kynurenine hydroxylase enzyme since the amounts of kynurenine utilized are more than those converted to kynurenic acid and anthranilic acid. However, there is evidence that the conversion of kynurenine to other metabolites viz., 3-hydroxykynurenine, may be inhibited in the presence of increasing concentrations of progesterone (Tables 2B and 4B) since the amounts of kynurenine utilized are almost equivalent to those converted to both metabolites. This finding further substantiates the recent reports that the female sex hormones, natural or synthetic, may affect other enzyme systems, which are B_6-independent viz., tryptophan oxygenase enzyme.[3,24,39 41,45] However, further investigations are urgently needed to study the possible role of these hormones on the other vitamin B_6-independent enzymatic reactions especially on kynurenine hydroxylase enzyme. Hayaishi[46] has stated that he does not know of any specific inhibitors of this enzyme.

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