9. THE INACTIVATION OF OESTRONE

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THE work of a large number of investigators has demonstrated quite conclusively that the administration of large amounts of oestrogens does not lead to the excretion of appreciable amounts of these substances in the urine or faeces. The actual amounts that can be recovered vary from 3 to 20% of the quantity administered, and depend upon the hydrolysis of the conjugated oestrogens before the extraction and testing of the urine. The maximum excretion of the oestrogen occurs within the first 24 hr. of its administration, and practically ceases after 48–96 hr. irrespective of the amount administered [Mazer & Israel, 1937]. The failure of the administered oestrogen to be excreted is not due to its storage in the body [Zondek, 1934; Dingemanse & Laqueur, 1937; Frank *et al.* 1932].

From these and similar studies there is no doubt that only a small portion of the oestrogen is excreted, and that the remainder is rapidly destroyed or altered (beyond simple conjugation) so that it can no longer be detected by the usual means. It is generally believed that this inactivation is brought about by the liver [Zondek, 1934; Israel *et al.* 1937].

The nature of this change is unknown, and the closely related compounds that are known have failed to indicate the course of this reaction. The presence of the more unsaturated oestrogens, equilin and equilenin, in the urine of the pregnant mare suggests these compounds as possible metabolites in this species, but it is improbable that equilenin occurs in human urine [Marrian, 1938; Marx & Sobotka, 1938]. Similarly, the presence of oestriol in human pregnancy urine might be construed as indicating a metabolic pathway for oestrone, but the inability of the animal to convert oestrone into oestriol except under the influence of progesterone [Smith *et al.* 1938] would eliminate this change as an important pathway for inactivation. The completely saturated hexahydro-oestradiols, discovered in the urine of non-pregnant females [Marker *et al.* 1938, 1, 2], are undoubtedly derived from the natural oestrogens, and it has been suggested that they are formed by non-enzymic reductive processes in the course of the utilization of oestrone as a hormone. The exact role of these derivatives in the "inactivation" process, if any, is unknown.

It is significant that all the natural oestrogens are phenols, and the presence of an active hydroxyl group in such a molecule would present a likely point of attack for oxidation by the body. The first stage in such an oxidation would be the introduction of a second hydroxyl group in the *o*- or *p*-position. The belief that this is the method utilized by the body for the inactivation of the oestrogens is supported by the following: (1) phenols in general are metabolized in this manner; (2) H_2O_2 will readily inactivate the oestrogens, and its characteristic action on monohydric phenols is the oxidation of these substances to *o*- or *p*-dihydric phenols; (3) the enzyme tyrosinase is capable of oxidizing many

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monohydric phenols to *o*-dihydric phenols (and subsequently to *o*-quinones), and is also capable of inactivating the oestrogens.

A few studies of the polycyclic compounds have indicated that like benzene and the simple phenols they too may be converted into mono- and di-hydric phenols. Naphthalene is oxidized by the body in a manner similar to that of benzene inasmuch as it is partially converted into α -naphthol [Rossbach, 1884], and this in turn is oxidized to a dihydroxy-naphthol [Lesnik & Nencki, 1886]. An excretion product of anthracene has been identified [Boyland & Levi, 1935; 1936] as a dihydroxy-dihydroanthracene. A phenanthrol glycuronic acid of unknown constitution was found in the urine after the administration of phenanthrene [Bergell & Pschorr, 1903].

The introduction of one or more hydroxyl groups into the benzene nucleus by the body seems to be a well-defined metabolic pathway. This is true not only of benzene, naphthalene, anthracene, phenanthrene and phenylalanine as indicated above, but is characteristic of the synthetic oestrogen stilbene [Stroud, 1939] and all aromatic substances in general [Dakin, 1922]. It is not certain that an aromatic character is essential for this reaction, for practically nothing is known of the metabolism of the more saturated ring compounds. The production of mono- and di-hydroxy compounds from decahydronaphthalene and tetrahydronaphthalene [Pohl & Rawicz, 1919; Bernhard, 1939] would indicate that it is not necessary, but the point needs further study. Its importance in a study of the metabolism of the steroids is obvious.

Since all of the natural oestrogens are phenols, it is quite conceivable that they are metabolized by the body in a similar manner, and that the resulting dihydroxy compounds are physiologically inactive.

Metabolism of oestrone methyl ether

An attempt was made to prevent the inactivation of oestrone by the body by blocking the phenolic group. Recovery experiments were carried out with oestrone methyl ether by A. Palmer¹ [personal communication], who found that the inactivation was not prevented and that the substance was probably demethylated by the body into free oestrone. The following method was employed. 1.0 mg. of the methyl ether of oestrone (M.P. 170–171°) was injected daily for 7 days into a castrated monkey, and the corresponding urine samples were collected. These were pooled and extracted for the free and combined oestrogens; each extract was further separated into neutral and phenolic fractions, and then assayed in spayed mice. The total activity recovered was equivalent to about 100 μ g. oestrone, of which one-third was free and two-thirds combined. Nearly all of the activity, whether free or combined, was found in the phenolic fraction. This suggests that the compound was demethylated by the body, possibly as a prerequisite to its oestrogenic action.

Action of H_2O_2 on phenols

Dakin & Herter [1907] have emphasized the similarity between the reactions carried out by the body and those effected through oxidation with H_2O_2 . In the case of the phenols this is borne out to a striking degree, especially in so far as the introduction of a second hydroxyl group is concerned.

Benzene has been oxidized with H_2O_2 to phenol [Leeds, 1881], and that in turn oxidized to quinol and catechol [Martinon, 1885; Henderson & Boyd, 1910]. The cresols are oxidized to the corresponding diphenols [Henderson & Boyd,

¹ The author wishes to express his thanks to Dr Allen Palmer for permission to quote these results.

1910], i.e. *o*-cresol to toluquinol (and some toluquinone), *m*-cresol to toluquinonorcin, and *p*-cresol to homocatechol. 3:5-Diethylphenol yields 3:5-diethyl *p*-benzoquinone. Thymol is converted into thymoquinone and tetrahydroxycymol and carvacrol are oxidized like thymol [Henderson & Boyd, 1910].

The ability of H_2O_2 to introduce hydroxyl groups into the aromatic nucleus has also been demonstrated for benzoic acid [Dakin & Herter, 1907]. The first product formed is the *o*-, *m*- or *p*-hydroxy benzoic acid, and further oxidation leads to the introduction of a second hydroxyl group in the *o*-position to the first, i.e. 2:3- or 3:4-dihydroxybenzoic acid.

The oxidation of phenylalanine by H_2O_2 is identical with the first steps in its oxidation by the body. Thus it gives rise to tyrosine and then to 3:4-dihydroxy-phenylalanine [Raper, 1932]. Similarly tyramine yields 3:4-dihydroxyphenylethylamine.

The oxidation of cholesterol with benzoyl peroxide or selenium dioxide results in the formation of Δ^5 -cholestene-diol-3:4 [Rosenheim & Starling, 1933]. This "oxycholesterol" was studied extensively by Lifschutz, who believed it to be a normal metabolic product of cholesterol. 2:3-Dihydroxyandrostane derivatives have been prepared from Δ^2 -androstene compounds by treatment with H_2O_2 , and the same type of reaction is obtained with Δ^2 -cholestene [Marker & Plambeck, 1939].

Action of H_2O_2 on oestrone

The fact that H_2O_2 destroys the activity of the oestrogens has been known for some time [Thayer *et al.* 1928], but the nature of the reaction involved has never been determined. In view of the preceding studies it is likely that the course of this oxidation is similar to that of these other well-defined cases. Some evidence has been obtained to indicate that the attack is on the phenolic portion of the molecule, and the isolation of the inactivated products is now in progress.

Oestrone is not readily inactivated by H_2O_2 in a neutral solution. 100 mg. oestrone were dissolved in 20 ml. acetone and treated with a large excess of H_2O_2 by successive additions over a period of 1 month. The solution was refluxed after some of the additions and allowed to stand at room temperature after other additions, but at the termination of the experiment over 50 % of the oestrone was recovered unchanged and as active as it was originally. On the other hand, when the oestrone was dissolved in NaOH it was rapidly and completely inactivated by H_2O_2 at room temperature.

The inactivation by peroxide in alkaline solution was prevented by blocking the phenolic group. 5 mg. of the methyl ether of oestrone (m.p. 170–171°) were dissolved in 100 ml. alcohol, and the solution was divided into two equal portions, one of which served as a control. To the other, 40 ml. of H_2O_2 (2400 mg.) and 10 ml. of 20 % NaOH were added, and the solution was allowed to stand at room temperature for 5 days. Under these conditions a much larger quantity of free oestrone would have been completely destroyed, but the activity of the methyl ether was practically unaffected. Excess peroxide was removed by catalase, and the oestrogen was recovered by extraction with ether. Both the control and experimental solutions were taken up in oil and assayed simultaneously in spayed mice. There was no appreciable difference between the activities of the two solutions, each containing about 800 mouse units. This is similar to earlier experience in which blocking the phenolic group prevented oxidation by benzoyl peroxide [Butenandt *et al.* 1932].

Action of tyrosinase on phenols

The general action of tyrosinase on monohydric phenols is to introduce a second hydroxyl group into the *o*-position to the one already present and then to oxidize the dihydric phenol to an *o*-quinone [Raper, 1928]. The fact that *o*-quinones are produced from phenols was pointed out by Szent-Györgyi [1925], and has been confirmed by the isolation of their anilino derivatives [Pugh & Raper, 1927]. The anilino derivative of *o*-benzoquinone has been obtained from phenol and catechol, and the corresponding derivative of homoquinone has been obtained from *m*-cresol, *p*-cresol and homocatechol. The *m*- and *p*-methoxyhydroxybenzenes act like the corresponding cresols in producing *o*-quinones, and these have been isolated as the anilino-quinones. The same type of oxidation is produced in the initial stages of the formation of melanin from tyrosine [Raper, 1928].

Although *o*-quinones are undoubtedly produced in the oxidation of phenols, they are probably not the end products of the reaction [Pugh & Raper, 1927; Nobutani, 1936]. Wagreich & Nelson [1936] have suggested that another hydroxyl group is introduced into the *o*-quinone.

The action of tyrosinase on phenols is specific to the extent that it generally attacks those phenols that give rise to catechol derivatives and quinones, but has no action on those substances that form quinols [Pugh & Raper, 1927; Onslow & Robinson, 1925; 1926; 1928]. It will catalyse the oxidation of a large number of monophenols, as tyrosine, phenol, *m*- and *p*-cresol, *m*- and *p*-methoxyhydroxybenzene, *p*-hydroxyphenylethyl alcohol, *p*-aminophenol, *p*-chlorophenol etc., to *o*-dihydric phenols. In each case this is the first step in the oxidation regardless of the course of further changes.

Action of tyrosinase on the oestrogens

The experimental portion of this report has shown that tyrosinase also attacks the oestrogens, rendering them inactive. From the preceding discussion it is relatively certain that the first stage in this oxidation would be the production of an *o*-dihydric phenol, though further oxidation is also likely.

The inactivation by tyrosinase appears to be a true enzymic action and not merely an artefact. The enzymic effect is abolished by preliminary boiling of the tyrosinase, and it cannot be duplicated by a system of catechol oxidase plus catechol. The inactivation is not due to secondary production of H_2O_2 because it also takes place in the presence of catalase.

EXPERIMENTAL

Each oestrogen was made up in a phosphate buffer by dissolving it in the correct amount of NaOH (14–16 ml. of 20% diluted to 300 ml., the exact amount being determined beforehand), warming and adding 13.622 g. KH_2PO_4 . The salt was dissolved with an additional quantity of water, and the solution was then cooled and diluted to 1000 ml. The amount of NaOH added was arranged to give the final solution a pH of 7.35 so that it could also be used in tissue respiration studies. The solution was stable, and lost none of its activity upon standing. As a routine it was filtered before using, and its activity determined by assay.

The tyrosinase extract was made up for each experiment from a stable preparation of mushrooms. This preparation was obtained by mincing the mushrooms into ice-cold alcohol, squeezing the alcohol through muslin, and

washing several times with fresh portions of cold alcohol. This extraction with alcohol had the two-fold objective of dehydration and removal of most of the naturally occurring phenols. The remaining alcohol was removed by pressure as far as possible from the mushroom residue, and the residue was rapidly dried *in vacuo*. It was then ground to a fine powder. The tyrosinase extract was prepared from this powder by extracting each gram with 20 ml. of 0.1% NH_4OH . The solution was squeezed through muslin and centrifuged, and the supernatant fluid was decanted and tested for its activity on *p*-cresol before applying it to the oestrogen solution.

The inactivation of the oestrogen was carried out by treating 50 ml. of the solution with 15 ml. of the tyrosinase extract. After adjusting to pH 8 (approx.) with dilute NaOH, it was incubated for 5 hr. at 38°. During the incubation it was shaken vigorously to keep the solution adequately aerated. The enzymic action was stopped by placing the solution in boiling water for $\frac{1}{2}$ hr.; sufficient NaOH was added to make the pH 9, and the solution was diluted to a known volume for assay in spayed rats or mice by the technique of Kahnt & Doisy [1928].

The inactivation was carried out at pH 8 because the greatest destruction of tyrosine by tyrosinase occurs at this pH [Evans & Raper, 1937]. The results are summarized in Table 1. Both oestrone and oestradiol lose at least 75% of their

Table 1. *The effect of treatment with various enzymes, etc., on the activity of the oestrogens*

Oestrogen	Nature of treatment: incubation with	Activity of solution	
		Before treatment	After treatment
Oestrone	Tyrosinase	450 m.u.	100 m.u.
	Boiled tyrosinase	450	450
	Fe	450	450
	Cu	450	450
	Catechol oxidase	450	375
	Catechol oxidase plus catechol	450	375
	H_2O_2	450	<100
	Catalase	450	400
	Catalase plus H_2O_2	450	400
Oestradiol	Catalase plus tyrosinase	450	150
	Tyrosinase	250 r.u.	<60 r.u.
	Boiled tyrosinase	250	250
	Fe	250	250
	Cu	250	250
	H_2O_2	250	<60
	Catalase plus H_2O_2	450	400
Diethylstilboestrol	Catalase plus tyrosinase	450	100
	Tyrosinase	600 r.u.	100 r.u.

original activity by this treatment with tyrosinase; the synthetic oestrogen diethylstilboestrol is inactivated to an even greater degree. In 7 out of 15 such experiments with oestrone there was no sign of oestrogenic activity in any one of a group of 8 to 25 mice when the final solution was tested at the level of 100 mouse units; in these cases the inactivation must have been much greater than 75% since both the original and control solutions contained 450 mouse units. In the other 8 experiments with oestrone the final solutions contained very nearly 100 mouse units. A few experiments carried out at pH values between 7 and 8 indicated that the inactivation was practically as great at any point in this range.

A control on the enzymic nature of this reaction was made by testing a boiled extract of tyrosinase. Both the experimental and control tests were

carried out simultaneously and identically except that in the latter case the 15. ml. portion of the tyrosinase extract was placed in boiling water for $\frac{1}{2}$ hr. before being added to the oestrogen solution. The controls were assayed simultaneously with the experimental solutions, and showed no loss of activity.

Several tests on the effect of inorganic salts were also made. 50 mg. of Fe or Cu, as FeCl_3 or CuSO_4 , were added to the oestrogen solution and incubated in the usual way. Assays showed there was no loss of activity in either case, but the results were somewhat complicated by the precipitation of these ions in the buffer. The oxidation of monophenols is generally not catalysed by inorganic ions, but catechol derivatives may be affected [Bhagvat & Richter, 1938].

Onslow & Robinson [1928] have suggested that the oxidation of monophenols by tyrosinase is not a straightforward enzymic action, but is a secondary reaction caused by the *o*-quinone, H_2O_2 , or organic peroxide produced by the action of a polyphenolase on a catechol derivative present in the crude extract. The change from catechol to *o*-quinone would be reversible, and hence only a small amount of a catechol derivate in the extract would be necessary for this secondary catalysis. Catechol derivatives occur naturally in most of the tyrosinase sources, and it is known that *o*-quinones are capable of effecting some secondary oxidations.

The essence of this theory has been supported by Richter [1934], but the idea that the *o*-quinone was solely responsible for the secondary oxidations was abandoned [Onslow, 1931]. The recent isolation [Dalton & Nelson, 1938; 1939] of a crystalline tyrosinase capable of catalysing the oxidation of both mono- and di-hydric phenols seems to have rendered this theory superfluous. Nevertheless it was possible that the inactivation of the oestrogens by a crude tyrosinase extract could have been accounted for on such a basis.

This point was tested by determining the effect of catechol oxidase plus catechol on oestrone under the same conditions in which the oestrone had been inactivated by tyrosinase. The preparation of catechol oxidase¹ was approximately 20% pure and was intensely active towards catechol but practically inert with *p*-cresol. The catechol oxidase itself had very little effect upon the activity of the oestrone solution, and there was no greater effect when 25 mg. of catechol were added to the reaction mixture (Table 1). The oxidation of the catechol by the enzyme was indicated by the immediate and intense colour produced, but the oxidation products of the catechol were not capable of secondarily oxidizing the oestrone.

A certain amount of evidence has been obtained to indicate that H_2O_2 or an organic peroxide is produced in the reaction between tyrosinase and a phenol. It has been rather difficult to demonstrate conclusively that H_2O_2 is produced, and though it has been questioned [Nobutani, 1936; Sutter, 1936] it remains a possibility [Onslow & Robinson, 1926; Evans & Raper, 1937]. The peroxide that is produced would be a by-product of the reaction, and while the enzyme peroxidase may [Evans & Raper, 1937] or may not [Richter, 1934; Szent-Györgyi, 1925] be involved in the subsequent dissipation of the peroxide, it could possibly effect some secondary oxidations.

Thus, if the extract of tyrosinase used in these experiments contained any naturally occurring phenols (a point guarded against by the extraction of the mushrooms with alcohol), it might also contain some H_2O_2 , and the peroxide would inactivate the oestrogen directly. Under the conditions of these experiments H_2O_2

¹ The author wishes to express his thanks to Dr Keilin for supplying (through Mrs Philpot) the catechol oxidase.

will completely inactivate the oestrogens. This was confirmed by adding 15 mg. H_2O_2 to 50 ml. of the oestrogen solution at pH 8, incubating and treating the solution in the same way that the tyrosinase experiments were conducted.

The inactivation of the oestrogens by H_2O_2 could be prevented by adding catalase to the oestrogen solution before introducing the peroxide. A highly active catalase, prepared from liver by the method of Zeile [1931], itself had no effect on the activity of the oestrogen solution under these standardized conditions, and when added together with 60 mg. H_2O_2 it destroyed the peroxide before the latter could inactivate the oestrogen (Table 1).

The addition of catalase to the oestrogen solution before its incubation with tyrosinase did not prevent the enzymic inactivation of the oestrogen. The experiments were conducted as before and any H_2O_2 present in the extract or produced during the incubation would have been destroyed. The inactivation which took place under these conditions must have been a genuine enzymic effect, and while it was not quite as extensive as that which occurred in the absence of catalase, there was no doubt as to the essential feature of the reaction.

DISCUSSION

The ultimate proof of the method whereby the oestrogens are inactivated by the body lies in the isolation of the inactive products from the excreta of a suitable subject. In the absence of this more conclusive proof the evidence herein presented can do little more than suggest a likely pathway. But it is significant that each of the analogies has indicated the same pathway.

The introduction into the oestrone molecule of another hydroxyl group in the *o*- or *p*-position could theoretically produce three compounds; *o*-substitution would give rise to either 2:3- or 3:4-dihydroxy-17-keto-oestratriene; *p*-substitution would yield 10-hydroxy-3:17-diketo-oestradiene(1:4). These derivatives of oestrone are unknown at present. In the case of the synthetic oestrogens the substitution of any group directly into the phenolic nucleus greatly decreases its activity and may inactivate it entirely. Similar derivatives of the natural oestrogens have not been described, except for the halogen derivatives which are relatively inactive [Thayer *et al.* 1931].

SUMMARY

1. Attention is called to the fact that the oestrogens may be metabolized by the body similarly to other phenols, which are oxidized to *o*- or *p*-dihydric phenols. The same type of oxidation of phenols is brought about *in vitro* by H_2O_2 , and *o*-dihydric phenols are also produced as the first step in the oxidation of monophenols by tyrosinase.

2. Oestrone was readily inactivated by H_2O_2 in alkaline solution but not in neutral solution; the methyl ether of oestrone was not inactivated by H_2O_2 in alkaline solution.

3. Oestrone, oestradiol, and diethylstilboestrol were inactivated by incubation with tyrosinase; a boiled tyrosinase extract had no action.

4. Incubation with catechol oxidase or a combination of catechol oxidase plus catechol did not produce any appreciable inactivation.

5. Complete inactivation resulted from the addition of H_2O_2 , but could be prevented by the simultaneous addition of catalase; however, the addition of catalase did not prevent the inactivation by tyrosinase.

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