The Influence of some Synthetic Oestrogens and Related Substances upon the Succinoxidase System of Rat Liver

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Practically nothing is known concerning the manner in which oestrogens, either natural or synthetic, bring about their characteristic effects *in vivo*, and the difficulty of correlating oestrogenic activity with chemical structure is notorious. Particular interest, therefore, attaches to the observations of McShan & Meyer (1946) on an apparently specific inhibition of the succinoxidase enzyme complex in rat liver and pituitary tissues, produced by stilboestrol (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene), hexoestrol and dien-oestrol in low concentrations. These workers indicated that the inhibition was exerted not upon the succinic dehydrogenase, but upon the cytochrome oxidase component of the system, being thereby distinguished from inhibitions of the type produced by malonate, from which it also differed in respect of the markedly lower concentrations of oestrogen necessary to inhibit to a given degree. When one or both of the 4:4' phenolic hydroxyl groups were replaced by carboxymethoxy groups it was reported that the inhibitory power of the resulting compounds towards succinoxidase and the oestrogenic potency declined in parallel. These findings, as was pointed out by the authors, suggest that the oestrogenic effect of such compounds is functionally associated with their action upon the succinoxidase system. This possible elucidation of the mode of action of oestrogens acquires an enhanced significance in the light of the relationship existing in some cases between oestrogenicity and carcinogenicity. If the elicitation of an oestrous or neoplastic response in the living organism could be shown to be consistently associated with the ability of the agent to interfere with a specific enzymic reaction, a fundamental contribution to our understanding of these processes would have been made. We have, therefore, amplified the observations of McShan & Meyer (1946) by investigating the action of a wider range of oestrogens, and of substances of related chemical structure whose oestrogenic potency is slighter or in some instances negligible. The hypothesis that oestrogenicity is correlated with the property of inhibiting some component of the succinoxidase system is not supported by our results. Since this work was completed a further communication by McShan, Meyer & Erway (1947) has appeared in which the concept is likewise abandoned. Some of our experiments,

however, throw interesting light upon the question of the constitution of the succinoxidase system. Evidence has been previously adduced that succinate, activated by its dehydrogenase, is not, as was once thought, capable of reacting directly with the cytochrome *c*-cytochrome oxidase complex, nor of reducing cytochrome *c* without the intervention of other intermediates, and the findings reported here support this view.

EXPERIMENTAL

In general the technique of McShan & Meyer (1946) was followed closely. As a source of the succinoxidase system and its components, homogenized rat liver was used. Adult males from the laboratory stock were killed by decapitation and the appropriate amounts of tissue excised, weighed and homogenized immediately in ice-cold glass-distilled water by the method of Potter & Elvehjem (1936). The activity of the homogenates was unaffected by storage for several hours at 0°, and it was found advantageous as a routine to allow them to stand for 2 hr. in the refrigerator, and then to decant the supernatant suspension from the slight sediment of coarse undisintegrated cell debris. This yielded very uniform dispersions which could be accurately pipetted and gave highly reproducible results, replicates commonly agreeing to within 2 or 3%. Preparations which had been kept for 24 hr. or more at 0° usually retained over 80% of their succinoxidase activity, but in view of possible differential deterioration of the constituents of the enzyme system, results obtained with such homogenates are not included. The final homogenate was customarily used in a concentration of 5% (in terms of fresh tissue) for succinoxidase or succinic dehydrogenase assay, and 1 or 0.5% for cytochrome oxidase determination. The equivalent dry weights of tissue were determined by drying samples of homogenate to constant weight at 105–110°.

Cytochrome *c* was prepared from heart muscle of freshly slaughtered horses by the method of Keilin & Hartree (1937), the only modifications being that glass-distilled water was used instead of dilute NaCl for the final dialysis, and that the product was stored in the refrigerator in aqueous solution (10^{-3} M) without chloroform or other preservative. Standardization was carried out spectrophotometrically with the Hilger-Nutting instrument, and the preparations showed no evidence of deterioration over a period of several months.

Sodium succinate was prepared by accurate neutralization of a concentrated aqueous solution of pure succinic acid with NaOH, followed by precipitation and washing with ethanol, filtration and drying.

The substances tested for inhibitory activity are listed in Table 1. We are indebted to Mr W. Lawson of this laboratory for carrying out the synthesis of the majority and for generously presenting us with specimens. The oestrogenic potency was assayed in this laboratory by the usual routine technique, using rats (Wilder Smith & Williams, 1947). The 'oestrogenically active dose' is the total weight of compound which, when administered in 5 or 6 injections over a 3-day period, results in 100% positive response. The method by which solutions of these compounds were obtained was substantially that outlined by McShan & Meyer (1946); they were dissolved in small quantities of 2N-NaOH, which was then partly neutralized with HCl and subsequently diluted with distilled water, the quantities being adjusted so that the resulting solutions were 0.006M with respect to NaOH, 0.002M with respect to NaCl, and of the desired molarity, usually between 0.0005 and 0.002M, with respect to the substance under test. Sometimes poor solubility of the inhibitor necessitated stronger alkali; in all cases appropriate control solutions were made up with NaOH and HCl and added to the control manometric vessels in the experiments. Inconsistencies in preliminary results were traced to the circumstance that the oestrogens or inhibitors were occasionally partially, or wholly, precipitated from their alkaline solutions on being mixed with the rest of the contents of the reaction vessels, which were buffered at a lower pH, i.e. 7.4. Attempts to overcome this difficulty by such expedients as the use of caffeine as a solubilizer (Weil-Malherbe, 1946) were unsuccessful because purines in the concentrations necessary inhibited the succinoxidase system powerfully; the use of more alkaline buffers to prevent precipitation also severely impaired the enzymic activity. Solutions of some of the compounds were supersaturated, and often spontaneously deposited crystals within an hour or two; for this reason they were always made up immediately before use. It was found that the tendency to precipitate in the reaction mixtures was much reduced if the inhibitors were introduced into the Warburg flasks before any of the other constituents of the reaction mixture other than water, and this was made standard routine. In addition, 'pilot' mixtures were set up in which samples of the inhibitor solutions were added to mixtures which imitated the contents of the experimental vessels except that homogenate and cytochrome were omitted; it was then possible to be reasonably certain whether the substances were or were not remaining in solution.

Conventional Warburg manometric technique was employed. When homogenates were used, the procedures for assay of succinoxidase and cytochrome oxidase described by Schneider & Potter (1943) were followed in essentials. For succinoxidase, each vessel contained 0.2 ml. of enzyme preparation; 0.2 ml. of a solution 0.006M with respect to both AlCl_3 and CaCl_2 ; 0.2 ml. of 0.75M-Na succinate; 0.1 ml. of 10^{-3} M-cytochrome *c*; 0.3 ml. of inhibitor or control solution; 1.0 ml. of phosphate buffer (0.1M, pH 7.4); and glass-distilled water to make a total volume of 3.0 ml. In cytochrome oxidase investigations the succinate was replaced by 0.3 ml. of 0.114M-Na ascorbate (freshly prepared by neutralization of ascorbic acid with NaOH); 1.0 ml. of 10^{-3} M-cytochrome *c* was used instead of 0.1 ml.; and the concentration of the homogenate was reduced from 5 to 0.5%. For the measurement of succinic dehydrogenase activity, cytochrome was omitted, and 0.5 ml. of

a 0.5% aqueous solution of brilliant cresyl blue (Weil-Malherbe, 1937; McShan & Meyer, 1946) was included; sometimes cyanide in 10^{-3} M concentration was present also. In all experiments with homogenates the gas phase was air.

Slices and strips of tissue were used in a phosphate-Ringer solution, pH 7.4 (Dickens & Šimer, 1930), to which Na succinate (0.0375M) and dissolved oestrogen (10^{-4} M) or control solution were added. The centre wells contained 0.2 ml. of 10% NaOH, the total fluid volume was 2.2 ml., and the gas phase was O_2 . Flasks were shaken at 96 oscillations/min. in a water bath at 37.5°. Spectroscopic observations were made either with a hand spectroscope or with a Hilger constant deviation wave length spectrometer.

RESULTS

Under the conditions stated the Q_{O_2} for succinate oxidation by the liver homogenates was consistently in the neighbourhood of 70 in the controls, rarely falling below 65 or exceeding 75. This is in fairly good accord with the figures of McShan & Meyer (1946) and other American workers, though it agrees poorly with recent values given by Hoch-Ligeti (1947) in this country, which on the average were of the order of half this magnitude. However, the discrepancy may be largely accounted for by the fact that we used 10 mg. of wet tissue in 3 ml., with the addition of calcium and aluminium ions, whereas Hoch-Ligeti (1947) used 40 mg. of tissue in the absence of Ca^{++} and Al^{+++} . For the oxidation of ascorbic acid by cytochrome oxidase the range of variation in Q_{O_2} was somewhat wider, but the values were always between 300 and 400.

When succinic dehydrogenase activity was measured in the presence of brilliant cresyl blue, with or without cyanide, but in the absence of added cytochrome *c*, the *Q* value was generally reduced to less than half of its normal level, i.e. to about 30. Some specimens of this dye seemed more injurious to the enzymes than others; their use was naturally avoided when possible. Methylene blue proved markedly less efficient as a carrier, and after preliminary trials was not employed in these experiments.

Inhibition of succinoxidase in liver homogenates

Table 1 contains the summarized results showing the overall inhibition of succinoxidase by the whole range of compounds tested, presented in the form of percentage inhibitions in relation to the control values, for three levels of concentration of inhibitor. These inhibitions have been calculated on the basis of the average Q_{O_2} over the first four 10 min. periods; the uptake was almost invariably nearly linear for at least twice that time. Several tests were made with each substance; flasks were set up in duplicate, and habitually gave agreement to within less than 5%, the mean value being taken. The figures in

Table 1 are taken from the protocols of single typical experiments, and are fully representative since discordant or anomalous results were not encountered, except on a few occasions when the inhibitor had precipitated from solution.

almost negligible inhibitory power, while their effectiveness as oestrogens is considerable. The most convincing evidence is perhaps that furnished by bisdehydrodisynolic acid methyl ether and 2-(6'-methoxy-2'-naphthyl)-1:1-dimethyl-*n*-valeric acid,

Table 1. *Structure, oestrogenic potency and inhibitory power towards succinoxidase of compounds tested*

(The compounds are grouped according to their activity on the basis: high oestrogenic activity = oestrogenic dose < 1 mg.; high inhibitory power = > 50% inhibition at 2×10^{-4} M.)

Compound	Oestrogenic dose	Percentage inhibition at molar conc.		
		5×10^{-5}	10^{-4}	2×10^{-4}
A. High oestrogenic and high inhibitory activity				
Stilboestrol (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene)	<0.5 μ g.	60	87	100
Hexoestrol	0.2	51	83	100
Dienoestrol	<0.5	17	78	95
B. High oestrogenic and low inhibitory activity				
Sodium hexoestrol diphosphate	2 μ g.	1	—	3
Sodium hexoestrol monosulphate	10	7	12	20
Dipotassium hexoestrol disulphate	25	0	3	8
Dipotassium stilboestrol disulphate	25	9	10	—
4:4'-Dicarboxymethoxystilboestrol (sodium salt)	200	—	0	5
4:4'-Dicarboxymethoxyhexoestrol (sodium salt)	200	—	18	22
7-Methoxy-2-methyl-1-ethyl-1:2:3:4-tetrahydro-phenanthrene-2-carboxylic acid (bisdehydrodisynolic acid methyl ether)	0.5	1	3	9
2-(6'-Methoxy-2'-naphthyl)-1:1-dimethyl- <i>n</i> -valeric acid	3	5	7	13
C. Low oestrogenic and high inhibitory activity				
<i>iso</i> Hexoestrol	1 mg.	27	57	99
4:4'-Dihydroxystilbene	10	65	90	97
1(4'-Hydroxyphenyl)-2-phenylethane	>100	12	26	52
1:2-Di(4'-hydroxyphenyl)cyclohexane	>10	8	25	82
4-Hydroxybenzylideneacetophenone	100	22	42	60
Benzylidene-4-hydroxyacetophenone	>100	15	30	58
4- <i>tert.</i> Amylphenol	100	15	32	—
4:4'-Dihydroxytriphenylmethane	25	27	53	100
3-Phenyl-3-(4'-hydroxyphenyl)-2:4-dimethylpentane	2	50	92	—
2-Phenylphenol	>100	30	65	—
2-Phenyl-2-(4'-hydroxyphenyl)propane	50	30	55	98
D. Low oestrogenic and low inhibitory activity				
4-Hydroxystilbene	10 mg.	9	26	45
1:2-Di(4'-hydroxyphenyl)ethane	100	7	11	23
4-Hydroxydiphenylmethane	>100	0	20	34
4:4'-Dihydroxydiphenylmethane	100	5	10	23
4:4'-Dihydroxydiphenyl	100	2	4	11
Epoxy-2:3-di(4'-hydroxyphenyl)butane	>10	1	3	9
1:2-Di(4'-hydroxyphenyl)cyclohex-1-ene	>10	8	20	40
3:4-Di(4'-hydroxyphenyl)hexane-1:6-diol	>10	0	1	11
4:4'-Dihydroxydibenzylideneacetone	>100	11	16	19
1-Naphthol	>100	7	—	25

Table 1 shows that there is no systematic correlation between oestrogenic and inhibitory powers. It is true that the potent oestrogens stilboestrol, hexoestrol and dienestrol strongly inhibit succinoxidase, but racemic *isohexoestrol*, whose oestrogenic potency is about 1/5000 that of *mesohexoestrol*, exhibits only slightly less inhibitory capacity. The sodium and potassium salts of the sulphates, phosphates and carboxymethoxy derivatives (so-called oxyacetates or substituted glycollic acids) possess

which are among the most powerful synthetic oestrogens known, and yet exert virtually no inhibition upon the succinoxidase system. Compounds which exert powerful inhibition of succinoxidase, but possess low oestrogenic activity, include 4:4'-dihydroxystilbene, the two benzylideneacetophenones, 4-*tert.* amylphenol, 4:4'-dihydroxytriphenylmethane, 3-phenyl-3-(4'-hydroxyphenyl)-2:4-dimethylpentane, 2-phenyl-2-(4'-hydroxyphenyl)propane and 2-phenylphenol.

The effect of oestrogens on the metabolism of tissue slices and strips

There is no great effect of this group of substances on the respiratory rate of sliced or intact tissues. Fig. 1 shows a slight stimulating effect of hexoestrol and isohexoestrol upon the respiration of liver slices in the absence of added succinate; when succinate is present as substrate, however, the effect is abolished. Inhibitory effects similar to those observed with homogenized tissue were not seen.

Fig. 1. Effect of oestrogens on metabolism of rat-liver slices. Lower three curves, no succinate; upper three curves, succinate present. A, no oestrogen; B, hexoestrol 10^{-4} M; C, isohexoestrol 10^{-4} M. (Succinate + isohexoestrol record does not extend beyond 30 min. owing to experimental mishap.)

Since oestrogenic hormones *in vivo* have a pronounced effect on the uterus and vagina, it was of interest to examine the *in vitro* effect on these organs. The tissues were dissected from freshly killed ovariectomized rats in dioestrus, and were introduced into the Warburg flasks with no treatment beyond being opened up with scissors so that their entire surfaces were freely accessible to the liquid in the vessels. The Q_{O_2} was measured in the usual way, using succinate as added substrate, but no significant differences were detectable between the controls and the systems containing oestrogen (dienoestrol).

Effect of oestrogens on the metabolism of homogenates

These inhibitors might conceivably act by stimulating the oxidative formation from malate of oxaloacetate, which powerfully inhibits succinic dehydrogenase; and it has been shown by Swingle, Axelrod & Elvehjem (1942) that the probable function of Ca ions in accelerating the oxidation of succinate (whence their inclusion in the reaction mixtures employed in the work described in this paper) is that of assisting in the enzymic destruction of coenzyme I, the latter being essential for the formation of oxaloacetate. Oxaloacetate may also be removed by transamination with glutamate, yielding α -keto-glutarate and aspartate, and Swingle *et al.* (1942) found that glutamate reverses the inhibition of the succinoxidase system caused by oxaloacetate, and stimulates the succinoxidase system to an extent comparable with the effect of calcium. An experiment was carried out to test the effect of L-glutamate (Table 2), but no evidence was obtained that its presence affected in any way the inhibition produced by stilboestrol.

McShan & Meyer (1946) considered that the locus of the inhibition was the cytochrome oxidase component of the succinoxidase system, since they found that a concentration of oestrogen which inhibited the complete succinoxidase system to a pronounced degree had only a feeble effect upon succinic dehydrogenase, whereas cytochrome oxidase was markedly affected. Using 10^{-4} M-stilboestrol, their figures show an average inhibition of 76 % for succinoxidase, 52 % for cytochrome oxidase, and 11 % for succinic dehydrogenase. This evidence for attributing to stilboestrol an exclusive effect upon cytochrome oxidase is not entirely convincing; and our own values are even less so, indicating in general a distinctly higher figure, in the region of 30 %, for the inhibition of the dehydrogenase.

In order to investigate this matter further, and especially to ascertain whether the mechanism of interference with the succinoxidase system by these inhibitors is the same irrespective of their oestrogenic potency, we selected from the compounds listed in Table 1 a few showing marked inhibitory power but feeble oestrogenicity, and studied their effects upon the three systems, succinoxidase, cytochrome oxidase and succinic dehydrogenase.

Table 2. Influence of glutamate on the inhibition of succinoxidase due to stilboestrol

(Ca⁺⁺ and Al⁺⁺⁺ omitted from reaction mixtures. Concentration of stilboestrol 10^{-4} M, of L-glutamate 5×10^{-3} M.)

	Control	Stilboestrol	Glutamate	Stilboestrol + glutamate
Q_{O_2}	63.3	18.5	63.9	17.0
Inhibition due to stilboestrol (%)	—	71	—	74

The following were chosen: 2-phenylphenol, 4:4'-dihydroxystilbene, 1(4'-hydroxyphenyl)-2-phenylethane and 4-hydroxybenzylideneacetophenone. The first two in 10^{-4} M concentration and the second two in 2×10^{-4} M concentration inhibit succinoxidase in liver homogenates by 50–100%. Stilboestrol was also included as a representative of the class of strong inhibitors which are powerfully oestrogenic. The results summarized in Table 3 were all obtained with the same preparation of rat-liver homogenate.

Table 3. *Differential inhibition of components of the succinic oxidase system*

Inhibitor (M)	Percentage inhibition of enzyme system (calculated on first 40 min.)		
	Complete succinoxidase system	Succinic dehydrogenase	Cytochrome oxidase
Stilboestrol (10^{-4})	86	33	57
4:4'-Dihydroxystilbene (10^{-4})	78	0	3
2-Phenylphenol (10^{-4})	61	29	0
4-Hydroxybenzylideneacetophenone (2×10^{-4})	56	47	10
1(4'-Hydroxyphenyl)-2-phenylethane (2×10^{-4})	51	43	48

It is apparent that the inhibition of succinoxidase activity cannot be explained adequately in terms of

the succinic dehydrogenase were practically unaffected by this substance (Fig. 2). It is clear that the mechanism of inhibition involves some factor or stage additional to those yet considered.

It was noticed that when cytochrome *c* was added to the buffered flask contents containing 4:4'-dihydroxystilbene, prior to the introduction of the enzyme preparation, a change of colour suggestive of partial reduction of the cytochrome took place within a few minutes. This was confirmed by the appearance of the absorption bands of reduced cytochrome *c* (though not in great intensity) under these conditions. If the inhibition of succinoxidase produced by the stilbene derivative depends upon the partial destruction or immobilization of cytochrome, it should be reversed by large increases in the amount of cytochrome available. The experiment recorded in Fig. 3 was carried out to test this possibility. The lowest concentration of cytochrome *c* employed here, viz. 3×10^{-5} M, is sufficient to saturate the succinic oxidase system with this component. At a concentration of 4:4'-dihydroxystilbene giving a convenient degree of partial inhibition, there were no significant differences in the oxygen uptake with a two- or five-fold increase in cytochrome concentration.

Further observations indicate that the inhibitors may affect either the reduction or the reoxidation of cytochrome *c*. In controls without oestrogen, the cytochrome *c* at the end of an experiment is always in the oxidized state; it becomes reduced in a few minutes on standing and can be reoxidized by shaking with air. The process is freely reversible. If complete or nearly complete inhibition of succinoxidase has been caused by stilboestrol, hexoestrol,

Fig. 2. Effect of 4:4'-dihydroxystilbene (2×10^{-4} M) upon cytochrome oxidase (upper pair of continuous curves), succinic dehydrogenase (lower pair of continuous curves) and the complete succinoxidase system (discontinuous curves). Minus sign, without inhibitor; plus sign, with inhibitor. The ordinates indicated on the right-hand side apply to cytochrome oxidase only. Inhibitions (%): succinoxidase, 93; succinic dehydrogenase, 7; cytochrome oxidase, 2.

that of either cytochrome oxidase or succinic dehydrogenase; furthermore, inhibition of the former

isohexaestrol or *dienoestrol*, the cytochrome *c* is seen to be irreversibly reduced. If only partial inhibition has been produced by one of these compounds the cytochrome is oxidized at the end of an experiment and becomes reduced on standing, as in the controls, but at a slower rate depending upon the concentration of oestrogen. Thus, while increasing concentrations of oestrogen progressively retard the reduction of cytochrome *c*, once this has been accomplished the oestrogen, if in high enough concentration, has the opposite effect of completely preventing its reoxidation. Clearly, therefore, more factors than one are affected by these compounds and the situation is more complex than McShan & Meyer's (1946) explanation would imply.

Fig. 3. Effect of varying concentration of cytochrome *c* upon the inhibition of succinoxidase produced by 5×10^{-6} M 4:4'-dihydroxystilbene. Cytochrome concentration: A, 3×10^{-6} M; B, 6×10^{-6} M; C, 1.5×10^{-4} M. Upper group of curves: no inhibitor. Lower group of curves: inhibitor present.

The behaviour of 4:4'-dihydroxystilbene is different. This completely inhibits in about the same concentration as does stilboestrol, but in such cases the cytochrome *c* in the reaction mixtures is invariably in the completely oxidized state, and cannot be reduced by prolonged standing or anaerobic conditions. Since it has been shown that the succinic dehydrogenase is unimpaired it follows that some factor which links that enzyme to cytochrome *c* has been put out of action.

DISCUSSION

The hypothesis originally put forward by McShan & Meyer (1946), that a relatively simple and specific biochemical property, that of inhibiting a known

enzyme, could be ascribed to all oestrogenic substances, has been shown to be fallacious. On the one hand, there are oestrogens which do not inhibit succinoxidase; and on the other, compounds structurally closely allied, but, nevertheless, non-oestrogenic or nearly so, which inhibit it strongly. As already mentioned, McShan *et al.* (1947) have also come to this conclusion. They too encountered slightly oestrogenic and non-oestrogenic substances which were highly effective inhibitors, although, because they studied a rather limited series of oestrogens, they did not demonstrate the converse. The fact that they found such substances as thyroxine and hydroquinone to be inhibitors strengthened their opinion that the presence of phenolic groups in the molecule is associated with the property in question. This is probably so, although our results (Table 1) do not support the implication of the American authors that the number of phenolic groups in the molecule bears a direct relation to the ability to inhibit succinoxidase.

We cannot yet explain the absence of inhibitory effect of oestrogens on tissue slices and strips, but considerations of permeability of intact cells or precipitation, destruction or inactivation of the oestrogen by the tissue might all be involved. The appearance of the liver slices after an experiment was sometimes suggestive of a layer of insoluble material. There is evidence in the literature that oestrogens are inactivated by liver *in vivo* and *in vitro*. For example, Heller (1940) demonstrated oxidative inactivation of natural oestrogens by liver slices; Zondek, Sulman & Sklow (1943) extended this observation to stilboestrol, and Zimmerberg (1946) reported that stilboestrol could be inactivated either by conjugation or oxidation. Levy (1947) produced evidence that cytochrome *c* and cytochrome oxidase are implicated in the inactivation of oestradiol by liver.

The main interest of the work that we have reported lies in the question of the precise point or points of attack at which the inhibitors prevent the succinoxidase system in homogenates from functioning normally. Many of them apparently interfere with more than one stage of the enzyme complex, but the existence of a factor linking succinic dehydrogenase to cytochrome *c* is indicated by the inhibition exerted specifically at that point by 4:4'-dihydroxystilbene. Such a suggestion of course has been made frequently during the past decade, and there is little doubt that the classification of succinic dehydrogenase as a 'cytochrome-reducing dehydrogenase' (Green & Brosteaux, 1936) is hardly valid without modification. Hopkins, Lutwak-Mann & Morgan (1939) and Stern & Melnick (1939) indicated the probable necessity to postulate a factor able to link succinic dehydrogenase to the cytochrome system. Dixon & Zervas (1939) put forward a similar

claim for yeast lactic dehydrogenase, another so-called 'cytochrome-reducing' enzyme; Bach, Dixon & Zerfas (1946) adduced further evidence that a new factor designated as cytochrome b_2 forms an essential part of this enzyme system, and gave reasons for believing that an additional factor is necessary to gear it to cytochrome c . Keilin & Hartree (1940) referred to the possible existence of an intermediate factor in mammalian succinoxidase, while not excluding the view that integrity of colloidal structures might be the consideration involved. Elliott (1940) believed that a succinoxidase inhibitor which he extracted from tumours and other tissues, and regarded as a proteolytic enzyme, acted upon some entity additional to succinic dehydrogenase and cytochrome oxidase. Straub (1941) was able to sever the linkage between the dehydrogenase and the cytochrome system, and then to remedy the deficiency by means of a heart preparation, thereby reconstituting the whole succinoxidase system. More recently, Stoppani (1947) has shown that a soluble factor can be obtained from liver which is able to link succinic dehydrogenase with cytochrome c . One of the most interesting contributions in relation to the work described in the present paper is that of Ball, Anfinson & Cooper (1947), who studied the inhibition of a succinoxidase preparation from heart muscle by a series of 2-hydroxy-3-alkylnaphthoquinones. These proved to be extremely potent inhibitors of succinoxidase at even lower concentrations than those requisite with our inhibitors, and were without action upon cytochrome oxidase. However, succinic dehydrogenase, though not the sole or main seat of the effect, was considerably inhibited when functioning with methylene blue; so that although the naphthoquinones used by Ball *et*

al. (1947) are undeniably highly useful tools for the elucidation of the sequence of events in succinate oxidation, it may be that 4:4'-dihydroxystilbene, and possibly other compounds of the same type, are capable of yielding clearer results by virtue of their narrower specificity. Very recently Slater (1948) has published a preliminary report of evidence, based on a study of the inhibition of succinoxidase by 2:3-dimercaptopropanol (BAL), which leads to conclusions substantially identical with those expressed here.

SUMMARY

1. The oestrogenic potency of synthetic oestrogens is not correlated with their efficacy as inhibitors of the succinoxidase system of rat liver. Some powerful oestrogens have little or no inhibitory capacity, and the converse is true of many compounds closely related chemically to the oestrogens.

2. The point in the array of respiratory enzymes at which the inhibition takes effect varies from one compound to another. The relative extents to which succinic dehydrogenase and cytochrome c oxidase are inhibited are not the same for different substances.

3. 4:4'-Dihydroxystilbene, which possesses low oestrogenic activity, is especially noteworthy because it exerts a powerful inhibitory effect upon the succinoxidase system which is not due to interference either with succinic dehydrogenase or with cytochrome oxidase. The substance is likely, therefore, to be valuable in the further investigation of the respiratory enzyme complex.

4. Further evidence is adduced in favour of the hypothesis that the succinoxidase system involves hitherto uncharacterized factors.

REFERENCES

- Bach, S. J., Dixon, M. & Zerfas, L. G. (1946). *Biochem. J.* **40**, 229.
 Ball, E. G., Anfinson, C. B. & Cooper, O. (1947). *J. biol. Chem.* **168**, 257.
 Dickens, F. & Šimer, F. (1930). *Biochem. J.* **24**, 905.
 Dixon, M. & Zerfas, L. G. (1939). *Nature, Lond.*, **143**, 557.
 Elliott, K. A. C. (1940). *Biochem. J.* **34**, 1134.
 Green, D. E. & Brosteaux, J. (1936). *Biochem. J.* **30**, 1489.
 Heller, C. G. (1940). *Endocrinology*, **26**, 619.
 Hoch-Ligeti, C. (1947). *Cancer Res.* **7**, 148.
 Hopkins, F. G., Lutwak-Mann, C. & Morgan, E. J. (1939). *Nature, Lond.*, **143**, 556.
 Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc. B*, **122**, 298.
 Keilin, D. & Hartree, E. F. (1940). *Proc. Roy. Soc. B*, **129**, 277.
 Levy, H. (1947). *Arch. Biochem.* **14**, 325.
 McShan, W. H. & Meyer, R. K. (1946). *Arch. Biochem.* **9**, 165.
 McShan, W. H., Meyer, R. K. & Erway, W. F. (1947). *Arch. Biochem.* **15**, 99.
 Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **164**, 495.
 Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.
 Slater, E. C. (1948). *Nature, Lond.*, **161**, 405.
 Stern, K. G. & Melnick, J. L. (1939). *Nature, Lond.*, **144**, 330.
 Stoppani, A. O. M. (1947). *Nature, Lond.*, **160**, 52.
 Straub, F. B. (1941). *Hoppe-Seyl. Z.* **272**, 219.
 Swingle, K. F., Axelrod, A. E. & Elvehjem, C. A. (1942). *J. biol. Chem.* **145**, 581.
 Weil-Malherbe, H. (1937). *Biochem. J.* **31**, 299.
 Weil-Malherbe, H. (1946). *Biochem. J.* **40**, 351.
 Wilder Smith, A. E. & Williams, P. C. (1947). *J. Endocrinol.* **5**, 152.
 Zimmerberg, H. (1946). *J. biol. Chem.* **166**, 97.
 Zondek, B., Sulman, F. & Sklow, J. (1943). *Endocrinology*, **33**, 333.