135. INHIBITION OF THE SUCCINOXIDASE SYSTEM BY EXTRACTS OF TUMOUR AND NORMAL TISSUES

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THE "succinoxidase" activity of certain tumour tissues was found by Elliott *et al.* [1935] and Elliott & Greig [1938] to be very low. It seemed possible that the low activity might be due to the presence in the tumours of an inhibitor of the system as well as to deficiency of the enzymes and carriers responsible for succinate oxidation.

In testing this possibility it was found that the oxidation of succinate by liver suspensions was rapidly and almost completely inhibited by the presence of suspensions or extracts of Walker 256 carcinoma and Philadelphia No. 1 sarcoma, and less actively by Jensen sarcoma. Inhibitory effects were also shown by a number of normal tissues, especially pancreas, which have low succinoxidase activity. Since heart, liver, and kidney, are extremely active in succinoxidase, it was not possible to determine whether they also contain some of the inhibitor. However, incubation of a concentrated liver suspension at 38° for 30 min. before diluting it and determining its activity, caused no change in its activity, which indicated that there was little inhibitor present.

When testing the inhibitory activity of tissue preparations which themselves show succinoxidase activity, it was assumed that their succinoxidase activity was not affected by the presence of small amounts of liver. If *A* was the O₂ uptake due to liver alone, *B* that due to the other tissue preparation alone, and *C* the O₂ uptake with both together, the percentage inhibition of liver activity by the other tissue preparation was taken as $\frac{A+B-C}{A} \times 100$. Most of the work on the inhibitor was done with suspensions and extracts of Walker 256 carcinoma since this tissue was conveniently available and contained a very active inhibitor.

The inhibitor acted on the succinoxidase in a progressive manner, the inhibition increasing with time, and it appeared that its action involved a destruction of catalytic components of the succinoxidase system. The inhibition of succinate oxidation was more pronounced from the start if liver suspension was first incubated with tumour extract or suspension before adding the succinate.

The inhibitor could be obtained in an opalescent solution by centrifuging phosphate buffer suspensions of homogenized or sand-ground tissues. After dialysis overnight in narrow cellophane bags against distilled water, none of the inhibitor seemed to be lost. Heating to 100° for 10 min. caused considerable delay in the inhibitory activity of the extracts but did not abolish it. When tumour slices instead of suspension or extract were used, the inhibitory effect, as might be expected, was initially quite low but increased steadily as the inhibitor diffused out into the medium.

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Pancreas extracts were very strongly inhibitory and the inhibitory activity of tissue extracts could be imitated with dilute solutions of commercial trypsin (Difco). It seems likely that the inhibitor is a proteolytic enzyme which destroys components of the succinate oxidizing system.

Results showing the above effects are given in Tables 1 and 3.

Table 1. *Summary of effects of tissue suspensions and extracts on the rate of succinate oxidation by other tissue suspensions*

Tissue A	Moist wt. mg.	Tissue B	Moist wt. mg.	% inhibition of A by B (assuming no effect of B on A)		
				0-30 min.	30-60 min. (30-45 min.)*	60-90 min.
Liver suspension (rat)	50	Walker 256 carc. suspension	200	87	97	—
" (rabbit)	50	" carc. suspension	200	90	99	—
" (rat)	50	" carc. suspension	190	63	(89)	—
" "	50	" extract from	190	72	(94)	—
" "	50	" extract dialysed from	190	63	(90)	—
" "	50	" extract heated 6 min. at 100°	190	35	(61)	—
" "	50	" suspension	200	85	96	98
" "	50	" suspension	100	78	93	97
" "	50	" suspension	50	63	86	93
" "	50	" suspension	50	—	—	—
(Incubated separately and together 30 min. before adding succinate)				—	82	91 95
Liver suspension	50	Walker 256 slices	350	35	66	88
"	25	Philadelphia No. 1 sarc. suspension	200	78	87	—
"	25	" extract dialysed from	200	86	91	—
"	25	" extract heated 10 min. at 100°	200	9	40	—
"	50	Jensen sarc. suspension	200	36	66	—
"	25	" suspension	200	25	80	—
"	25	" extract dialysed from	180	79	88	—
"	50	Pancreas suspension	200	92	97	—
"	50	Spleen suspension	200	67	75	71
"	50	" "	200	—	—	—
(Incubated separately and together 30 min. before adding succinate)				200	72	70 72
Liver suspension	25	Spleen extract	200	32	80	86
"	25	Muscle suspension	200	16	(20)	—
"	25	Brain suspension	200	29	54	—
"	25	Brain extract dialysed from	180	5	9	—
"	50	Testis suspension	200	18	26	—
"	50	Thymus suspension	200	39	56	—
"	50	Lung suspension	200	29	71	—
"	50	Blood	200	-11†	-19†	—
"	50	Trypsin (Difco) solution	30	82	95	—
"	50	" "	9	57	88	—
Muscle suspension	200	Walker 256 suspension	190	70	(73)	—
Brain suspension	200	Philadelphia No. 1 suspension	200	20	47	—
Heart suspension	25	Walker 256 extract from	200	9	6	4
Kidney suspension	25	Walker 256 extract from	200	89	96	—

* Figures in brackets for 15 min. only, after first 30 min.

† Acceleration.

It is now generally accepted that the succinoxidase system consists of at least three catalysts, succinic dehydrogenase, cytochrome, and cytochrome oxidase. The dehydrogenase causes succinate to reduce cytochrome, and the reduced cytochrome is reoxidized by cytochrome oxidase. It has recently become apparent that an extra catalyst is interposed between the dehydrogenase and

cytochrome [Hopkins *et al.* 1939; Horecker *et al.* 1939; Stern & Melnick, 1939; Euler & Hellström, 1939].

Attempts were made to discover which particular component of the system was specially inhibited. The activity of liver succinic dehydrogenase, determined by methylene blue reduction, was considerably inhibited (50–60 %, Table 2) by the presence of tumour extract, but the inhibition was not as complete as was the case with the aerobic succinate oxidation, and previous incubation of the liver suspension with the tumour extract did not increase the inhibition.

When cytochrome *c* was added to the liver suspension the rate of O₂ uptake with succinate was considerably increased. Tumour extract caused considerable inhibition of this O₂ uptake rate (Table 3). However, the rate in the presence of tumour extract was considerably greater when cytochrome *c* was added than when no cytochrome was added, indicating again that the effect of the inhibitor on the original succinoxidase activity was not due solely or mainly to action on the dehydrogenase. The % inhibition was less in the presence of added cytochrome *c* than in its absence.

Table 2. *Effect of extract of Walker No. 256 sarcoma on liver succinic dehydrogenase*

	Reduction time		Activity 600 $\frac{t-t_{\text{succ.}}}{t.t_{\text{succ.}}}$ ($\mu\text{l. O}_2$ equiv./hr.)	Inhibition of liver activity by tumour extract
	No succ. <i>t</i>	With succ. <i>t</i> _{succ.}		
Liver suspension, 35 mg.	∞	12	50	
Tumour extract, equiv. 200 mg.	196	100	2.8	
Liver + tumour extract	43	17	21.4	c. 63 %
After incubation of liver suspension, tumour extract, and the two mixed, for 60 min. at 38°.				
Liver suspension	∞	19	31.6	
Tumour extract	450 $\pm$	260 $\pm$	1.0	
Liver + tumour extract	95	25.5	17.0	c. 49 %

The same extract with the same liver suspension in the same amounts and at the same time, in an O₂ uptake experiment caused 53 % inhibition in the first 20 min., 75 % after 30 min., and 83 % after 60 min.

* Elliott & Greig [1938].

The activity of the liver cytochrome oxidase was determined by measuring the rate of O₂ uptake with *p*-phenylenediamine in the presence and absence of added cytochrome *c*. *p*-Phenylenediamine reduces oxidized cytochrome and the activity of the cytochrome oxidase-cytochrome system can be determined by measuring O₂ uptake in the presence of excess of *p*-phenylenediamine; when excess of cytochrome *c* as well as the diamine is added, a measure of the cytochrome oxidase itself is obtained. Tumour extracts themselves caused fairly rapid oxidation of *p*-phenylenediamine, especially when cytochrome *c* was added. As in the determination of the inhibition of succinate oxidation, it was assumed that the liver tissue did not affect the activity of the inhibitory extract.

Tumour extract inhibited the oxidation of *p*-phenylenediamine by liver considerably, but the extent of inhibition and the rate of increase of inhibition were less than with the succinate oxidation. In the presence of added cytochrome, the rate of *p*-phenylenediamine oxidation by liver suspension was much increased and tumour extract caused some inhibition which did not increase greatly with time. The rate of *p*-phenylenediamine oxidation by liver suspension with added cytochrome, even in the presence of tumour extract, was much greater than the rate without cytochrome, even in the absence of inhibitor. The

Table 3. *Effects of tissue extracts on succinate oxidation by liver and other tissue suspensions in the presence and absence of added cytochrome c*

Tissue A	Moist wt. mg.	Tissue B	Moist wt. mg.	O ₂ uptake (μl.)						% inhibition of A by B (assuming B unaffected by A)					
				0-30 min.	30-60 min.	60-90 min.	0-30 min.	30-60 min.	60-90 min.	0-30 min.	30-60 min.	60-90 min.	0-30 min.	30-60 min.	60-90 min.
				Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.
Liver suspension	50	Walker 256 extract from	200	401	537	289	397	—	—	—	—	—	—	—	—
		Both together		52	234	11	123	—	—	87	58	96	70	—	—
Liver suspension	25	Trypsin solution	15	140	307	122	260	93	206	—	—	—	—	—	—
		Both together		80	77	22	17	3	8	43	75	82	94	97	96
Liver suspension	25	Spleen extract from	200	183	309	143	260	123	212	—	—	—	—	—	—
		Both together		125	344	29	284	17	232	32	13	80	19	86	22
Liver suspension	25	Pancreas extract from	100	175	320	151	271	125	223	—	—	—	—	—	—
		Both together		12	23	3	2	0	3	93	93	98	100	100	99
Liver suspension	25	Pancreas extract from	25	74	204	61	179	—	—	—	—	—	—	—	—
		Both together		20	26	2	4	—	—	73	88	97	98	—	—
Heart suspension	25	Walker 256 extract from	180	192	267	162	208	142	190	—	—	—	—	—	—
		Both together		174	443	152	360	136	293	9	-46*	6	-51*	4	-32*
Kidney suspension	20	Walker 256 extract from	190	128	343	97	293	—	—	—	—	—	—	—	—
		Both together		14	110	4	70	—	—	89	73	96	79	—	—
Walker 256 suspension	200			15	119	9	96	7	76	—	—	—	—	—	—
Walker 256 slices	350			31	—	15	—	8	—	—	—	—	—	—	—
Walker 256 slices	300			—	63	—	56	—	54	—	—	—	—	—	—

* Acceleration.

rate of *p*-phenylenediamine oxidation depends upon both the concentration of oxidase and of cytochrome *c* [Elliott & Greig, 1938] and, while the extract evidently inhibited some of the oxidase, the active remainder of the oxidase could give a larger O₂ uptake when excess of cytochrome was added. But since the inhibition of cytochrome oxidase was less extensive than the inhibition of the "succinoxidase", cytochrome oxidase inhibition could not fully account for the strong and rapidly progressive inhibition of the oxidation of succinate.

Cytochrome *c* did not appear to be affected at all by tumour extract; the intensity of its absorption bands, after reduction, and its catalytic activity were not affected by incubation with the extracts.

Since the intense progressive inhibition of the succinoxidase system of liver cannot be fully accounted for by destruction of succinic dehydrogenase, cytochrome *c*, or cytochrome oxidase, it seems that some other factor concerned in the succinoxidase system in liver must be strongly affected by the inhibitor. This can scarcely be the intermediate, discovered by Hopkins *et al.* [1939], which is necessary for cytochrome *c* reduction by succinate with succinic dehydrogenase, since addition of cytochrome *c* considerably increased succinate oxidation even in the presence of the inhibitory extract. It is possible that the factor affected is cytochrome *b*. It is known [Keilin, 1929; Ball, 1938] that succinate with its dehydrogenase can reduce cytochromes *a* and *b* as well as *c*, presumably through the intermediate of Hopkins *et al.* It is believed by many that these three cytochromes act in series, H being transferred from one to the next in the order *b*, *c*, *a*. However, the H from the succinic dehydrogenase system may reduce any cytochrome directly. If liver suspension is rich in cytochrome *b* but low in *c*, the effect of the inhibitor may be to destroy cytochrome *b*, thus cutting down the O₂ uptake, but the addition of cytochrome *c* may provide an alternative mediator of H transport. The same argument might suggest that cytochrome *a* is affected, but it now seems probable that cytochrome *a*, or at least one form of it (*a*₃), can be identified with cytochrome oxidase [Keilin & Hartree, 1939], and, as mentioned above, destruction of the oxidase cannot account for the whole effect of the inhibitor on liver succinoxidase.

The effects of spleen extract on the activity of liver enzymes were similar to, but less marked than, those of tumour extract. Pancreas extract was extremely active; small amounts of extract strongly inhibited all the activities measured. Trypsin in small amounts also inhibited all the activities though, as with the tumour inhibitor, the effect on cytochrome oxidase was not as great or progressive as the effect on succinate oxidation.

Tumour suspension inhibited the succinoxidase activity of rabbit liver quite as strongly as that of rat liver. The succinoxidase activities of rat brain and skeletal muscle suspension were appreciably inhibited by tumour suspensions. Rat kidney suspensions were strongly inhibited, the inhibition of *p*-phenylenediamine oxidation being about as strong as that of succinate oxidation.

The succinoxidase activity of heart suspensions was scarcely at all inhibited, and, in the presence of added cytochrome *c*, tumour extracts actually caused considerable acceleration (40–55%), allowance being made for the activity of the extracts themselves. The *p*-phenylenediamine oxidation by heart was inhibited appreciably in the absence of added cytochrome *c*, scarcely at all in its presence. It would seem that succinate oxidation in heart tissue is not mediated by the factor which is specially susceptible to the inhibitor. The extra acceleration of succinate oxidation by the tumour extract when cytochrome *c* was added is difficult to account for; it may possibly be due to a better dispersion of heart tissue by proteolytic enzymes in the extract. The inhibitor evidently

affects some part of the cytochrome system of heart since, in the absence of added cytochrome *c*, the oxidation of *p*-phenylenediamine is considerably inhibited.

It is possible that the low succinoxidase activity found in some tumours and other tissues can be partly accounted for by the action of the inhibitor liberated under the experimental conditions. The rate of succinate oxidation by tumour suspensions and slices falls off very rapidly to a low value, but addition of cytochrome *c* increases the rate considerably, indicating that dehydrogenase and oxidase are present in fair amount all the time but that an intermediary is lacking (see bottom of Table 3).

This work emphasizes the need for caution in drawing conclusions as to the quantitative distribution of enzyme systems and their biochemical role from work on "surviving" whole tissue preparations. The nature and distribution of the inhibitor, and the nature of the particular mediator of succinate oxidation which seems to be most strongly affected, require further study.

In the paper of Elliott & Greig [1938], it was mentioned that, while the succinate-oxidizing activities of suspensions of a number of tissues were increased by adding cytochrome *c*, the activities of liver and kidney were not so affected. In the present work, rather low values for the succinoxidase activity of these two tissues were found without added cytochrome, and a considerable acceleration on adding cytochrome *c*. It is not understood why this was not previously observed.

METHODS

The methods used were the same as those of Elliott & Greig [1938]. The fresh, weighed tissue was homogenized in *M*/20 phosphate buffer, pH 7.4, using the apparatus of Potter & Elvehjem [1936]. Occasionally the suspensions were prepared by grinding with sand. The extracts were obtained by centrifuging the suspensions clear, each extract being made up to the original volume of the suspension with a little buffer with which the sediment had been washed. Rat tissues were used throughout except for one experiment (in Table 1) with rabbit liver.

For the manometric experiments, the Barcroft manometer vessels contained air and a total of 3 ml. of fluid made up of the tissue suspensions, or extracts, substrate solution, and water, with a final concentration of *M*/30 phosphate buffer. The concentration of succinate used was 9 mg./ml., of *p*-phenylenediamine 10 mg./ml. When cytochrome *c* was added, 0.2 ml. of a solution containing 26 μ g. bound Fe/ml. in 1 % NaCl were used, the solution being obtained by the method of Keilin & Hartree [1937]. In succinate experiments the two manometer flasks received the same additions except that succinate was put only in the right-hand flask. In *p*-phenylenediamine experiments, the left-hand flask contained only water; O₂ uptakes were corrected for the small, slightly autocatalytic oxidation of *p*-phenylenediamine which occurred in the absence of tissue and which was slightly increased in the presence of cytochrome solution.

The methylene blue reduction experiments were made as described by Elliott & Greig [1938] using bent Thunberg tubes shaken in a water bath. The 3 ml. of fluid contained liver suspension, tumour extract, 30 mg. neutralized succinic acid, and 0.5 ml. of 0.0703 %¹ methylene blue, and the reduction time was taken as the period required for the colour to match that in a tube containing the same materials, aerated, with 1/20 the amount of dye. The rate of dye reduction by succinic dehydrogenase was calculated by the formula used by Elliott & Greig which allows for the reduction time in the absence of succinate.

¹ In the paper of Elliott & Greig [1938] the concentration of methylene blue was incorrectly given as 0.703 %.

The succinic dehydrogenase activities of the liver suspension, tumour extract and the two mixed, were determined simultaneously. The inhibition of liver activity by tumour extract was calculated on the assumption that the liver material did not affect the activity of the tumour extract. Only extracts and not suspensions of tumour tissue could be used for these experiments since strong suspensions reduce methylene blue rapidly without succinate.

SUMMARY

1. The aerobic oxidation of succinate with liver suspensions is strongly inhibited by suspensions or extracts of various tissues, especially of certain tumours, pancreas and spleen. The inhibitor is non-dialysable and, to some extent at least, thermolabile. Commercial trypsin in small amounts is also inhibitory.

2. The succinic dehydrogenase (anaerobic reduction of methylene blue with succinate) and the cytochrome oxidase (oxidation of *p*-phenylenediamine in the presence of added cytochrome *c*) are both inhibited by tumour extracts, but not strongly enough to account for the main effect of the inhibitor on the complete succinoxidase system of liver. Cytochrome *c* appears not to be affected. It is concluded that the inhibitor acts particularly strongly on some other component of the succinoxidase system of liver, possibly cytochrome *b*.

3. Kidney suspensions are affected similarly to liver suspensions. The succinoxidase activities of brain and muscle suspensions are also inhibited. But the succinoxidase activity of heart suspensions is scarcely at all affected and tumour extracts actually provoke an acceleration of succinate oxidation with heart suspension in the presence of added cytochrome *c*. The oxidation of *p*-phenylenediamine with heart is considerably inhibited by tumour extract in the absence of added cytochrome *c*, but not in its presence.

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