# Biocatalysts in Cancer Tissue

## II. Inhibition of the Succinoxidase System by Tumor Extracts*

H. G. Albaum, Ph.D.,** and V. R. Potter, Ph.D.

*(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wis.)*

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That certain tumor tissues are low in succinic dehydrogenase activity was first reported by Elliott and Greig (3). Elliott (2) later pointed out that the low values may be due to (a) the possibility that the concentration of this enzyme is really low in tumor, or (b) the possibility that a normal concentration of enzyme exists but that it has been partly inactivated by an inhibitor found in certain normal and tumor tissues.

According to Elliott (2) extracts from tumor tissues (Walker carcinosarcoma 256, Philadelphia No. 1 sarcoma, and Jensen sarcoma) and from certain normal tissues (especially pancreas and spleen) when added to liver, kidney, brain, and muscle suspensions inhibit the activity of the succinoxidase system, the greatest measurable effect being exerted upon the succinic dehydrogenase. Elliott reported, furthermore, that the inhibitor acts on the succinoxidase system in a progressive manner, the inhibition increasing with time; and the inhibition is more pronounced from the start if the liver suspension is treated with the tumor extracts before the succinate is added. In addition, according to Elliott, the inhibitor is nondialyzable and to some extent thermostable. The inhibitory action of the pancreas extracts could be imitated with dilute solutions of commercial trypsin. On the basis of all these findings, Elliott concluded that the inhibitor is probably a proteolytic enzyme and may account in part for the low values observed for succinoxidase activity in certain tumor tissues.

If such an inhibitor for an important, normally occurring enzyme system really exists in tumor tissue, additional information concerning its nature is of prime importance. The present investigations were undertaken to learn more about this inhibitor, the conditions under which it occurs, whether it is found in other tumors, whether the inhibitor from tumor is similar to that from pancreas, and, finally, whether valid assays for the succinoxidase system can be made on tumor tissue.

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### EXPERIMENTAL

*Enzyme system.*—The test system used in the experiments to be described was a succinoxidase preparation from liver. Such preparations approximate the high activity of the enzyme system *in vivo* (8, 9). Rat liver homogenates were freshly prepared and kept in ice water until pipetted. The reaction mixtures contained 0.8 ml. of 0.1 *M* sodium phosphate pH 7.4, 0.4 ml. of $10^{-4}$ *M* cytochrome *c*, 0.3 ml. of $4 \times 10^{-3}$ *M* $CaCl_2$, 0.3 ml. of $4 \times 10^{-3}$ *M* $AlCl_3$, 0.3 ml. of 0.5 *M* recrystallized sodium succinate pH 7.4, 0.1 ml. of either a 5 or 10 per cent liver homogenate in either 0.033 *M* sodium phosphate pH 7.4 or distilled water, plus water and pancreas, tumor, or treated liver homogenate to give a final volume of 3.0 ml. About once a week the calcium, aluminum, and cytochrome solutions were made up into one solution of which 1 ml. was pipetted into each flask. During the course of the investigations, crystalline ribonuclease, trypsin, and chymotrypsin were used (secured through the courtesy of Dr. Kunitz, of The Rockefeller Institute) as well as crystalline insulin. The trypsin and chymotrypsin preparations contained about 50 per cent $MgSO_4$ and were controlled appropriately. For use in experiments, the trypsin and chymotrypsin were made up in 0.001 *N* $H_2SO_4$. The rate of oxygen uptake was measured in a conventional Warburg apparatus at 38° C. The enzyme system as set up will not oxidize succinate beyond the fumarate stage. Except when otherwise indicated, the inhibitors were incubated with the liver homogenate for one hour prior to testing.

*Inhibition by pancreas and pancreatic enzymes.*—The data shown in Table I confirm Elliott's (2) observation that extracts from pancreas inhibit the succinoxidase system in liver; they show also, as Elliott pointed out, that the inhibition with pancreas is progressive and that the inhibition is greater if the addition of succinate is delayed. With the delayed addition of succinate, almost complete inhibition of succinoxidase activity was obtained with a ratio of 3 parts of pancreas to 10 parts of liver. The results with crystalline trypsin shown in Table I also confirm Elliott's observations with commercial trypsin.

Chymotrypsin also inhibits to about the same extent as trypsin.

That all the inhibitory activity of the pancreas cannot be due to trypsin or chymotrypsin is also shown by the data in Table I, for even with 10 mgm. of either pure trypsin or chymotrypsin one does not attain as great an inhibition as that obtained with only 3 mgm. of moist pancreas. Some of the inhibition may be due to ribonuclease, which inhibits succinoxidase activity, as can be seen from Table I. It is also of interest to note that crystalline insulin appears to inhibit succinoxidase activity. This inhibition, however, may not be caused by insulin *per se* but by

Table I: Inhibition of Liver Succinoxidase by Pancreas and Pancreatic Constituents

Test system as described in text (10 mgm. liver homogenate)

Inhibition calculated from oxygen consumed per 10 minute interval

| Experiment No. | Inhibitor | Inhibitor concentration, mgm. per flask | Inhibition, per cent: Substrate added at beginning, 10-20 min. | Inhibition, per cent: Substrate added at beginning, 30-40 min. | Inhibition, per cent: Substrate added at 20 min., 10-20 min. | Inhibition, per cent: Substrate added at 20 min., 30-40 min. |
|---|---|---|---|---|---|---|
| 809 | Pancreas | 1.0 | 6 | 13 | 78 | 89 |
| | | 2.0 | 49 | 78 | 84 | 97 |
| | | 3.0 | 74 | 92 | 95 | 95 |
| | | 4.0 | 87 | 93 | 96 | 100 |
| | | 5.0 | 90 | 95 | 95 | 95 |
| 810 | Trypsin | 0.1 | −19 | −18 | | |
| | | 1.0 | 13 | 22 | | |
| | | 5.0 | 40 | 58 | | |
| | | 10.0 | 56 | 72 | | |
| | Chymotrypsin | 0.1 | −4 | −7 | | |
| | | 1.0 | 5 | 14 | | |
| | | 5.0 | 38 | 45 | | |
| | | 10.0 | 58 | 69 | | |
| 1126 | Ribonuclease | 0.3 | | | 33 | 31 * |
| | | 0.9 | | | 58 | 58 |
| 1125 | Insulin † | 1.5 | | | 68 | 55 * |
| | | 3.0 | | | 78 | 70 |
| | | 5.4 | | | 89 | 85 |

* Incubated for 1 hour with liver homogenate prior to tests.

† Test system contained 5 mgm. of liver.

minutes by control, 41.6, 42.0 μl.; by 30 mgm. of tumor, 10.3, 10.7 μl.; by liver plus tumor, 52.0, 51.8, 51.5, 52.5 μl. It is obvious that the total oxygen consumed is the sum of the individual quantities taken up by liver and tumor and that the tumor has exerted no inhibitory effect on the liver succinoxidase system. When the ratio of tumor to liver was increased to 10:1, an inhibition of liver succinoxidase to the extent of only 10 per cent occurred.

Since no inhibitory effects were obtained with healthy tumor tissue, it seemed possible that Elliott's results might have been obtained by the inclusion of some necrotic tissue, which conceivably might inhibit liver succinoxidase. Accordingly, experiments were carried out with younger and older tumors (the younger ones presumably having less necrotic tissue than the older ones), and the healthy tissue from the outside was carefully separated from the inner necrotic tissue. In every case, homogenate equivalent to 60 mgm. of tumor was added to 5 mgm. of liver for 1 hour at 37° C. prior to testing in the Warburg vessels. The results of such a series of experiments are shown in Table II. A number of important conclusions may be drawn from these data. First, the healthy tissue from the outside of the tumor gives little or no inhibition. Second, the only tissue which gives inhibition to any appreciable extent is the necrotic tissue from the inside of the tumor, this inhibition in-

the presence of zinc in the crystalline preparation since succinic dehydrogenase is inhibited in the presence of zinc (10).

*Inhibition by tumor homogenates.*—Whereas the results reported in this paper with pancreas and trypsin completely confirm those of Elliott, the first results obtained with tumor tissue (Walker carcinosarcoma 256, 7 to 10 days old) were completely negative, whether the tissue was incubated with the liver homogenate before addition of the succinate or not. In one typical experiment, with Walker 256 about 7 days old, when the necrotic tissue was carefully scraped away before homogenization and 30 mgm. of tumor were added to 10 mgm. of liver the following results were obtained: average $O_2$ consumed per 10

creasing with the age and, consequently, the necrosis of the tumor.

It appears, therefore, that Elliott's reported inhibition of liver succinoxidase may have been due to the necrotic tissue rather than to the healthy tissue. Incidentally, it should be pointed out that the necrotic tumor tissue in all cases has a lower succinoxidase activity than does the adjacent healthy tumor tissue. This fact may explain the wide variation in the succinate $Q_{O_2}$ values in the tumors studied by Elliott and Greig. Also, it is important to point out that any given tumor of sufficient age may or may not give inhibition depending upon what portion is tested.

TABLE II: INHIBITION OF LIVER SUCCINOXIDASE BY VARIOUS TUMOR HOMOGENATES TREATED AS INDICATED

Test system as described in text (5 mgm. liver homogenate)

Inhibitor concentration per flask 60 mgm.

| Experiment No. | Tumor | Average inhibition, per cent: Outer tissue | Average inhibition, per cent: Inner tissue |
|---|---|---|---|
| 1011 | Walker 256 (13 days old; very little necrosis) | 6 | 17 |
| 1017 | Walker 256 (17 days old; much necrosis) | 1 | 43 |
| | Heated 100° for 10 minutes | 28 | 31 |
| | Dialyzed in distilled water at 4° C. for 24 hours | 38 | 61 |
| | Heated, dialyzed | 43 | 60 |
| | Autolyzed, 37° C. for 24 hours | 68 | 77 |
| 1006 | Flexner-Jobling (14 days old; no necrosis; inner tissue cheesecake in consistency) | 1 | 7 |
| 1026 | Flexner-Jobling (18 days old; some necrosis; beginning liquefaction) | 0 | 17 |
| | Heated 100° for 10 minutes | 11 | 8 |
| | Autolyzed at 37° for 24 hours | 42 | 20 |
| | Dialyzed in distilled water 24 hours | 18 | |
| | Set in ice chest 24 hours | 21 | |
| | Heated, dialyzed | 10 | |
| 1124 | Jensen sarcoma (10 days old; little necrosis; no liquefaction) | 0 | 18 |
| | Heated 100° for 10 minutes | 7 | 13 |
| 1007 | Jensen sarcoma (14 days old; much necrosis; considerable liquefaction) | 8 | 52 |
| | Heated 100° for 15 minutes | 14 | 24 |

When tissue is deprived of an adequate blood supply, as is that in the interior of a tumor, it undergoes necrosis. The reason is not completely clear, but it appears that one of the factors responsible for the tissue breakdown is the release of autolytic enzymes of the cathepsin type. It is conceivable that these enzymes, which would be particularly abundant in a well necrosed tissue, might be the agents mainly responsible for the inhibition of the liver succinoxidase. If such an interpretation is correct, it should be possible to take healthy tumor tissue that does not act as an inhibitor and allow it to undergo autolysis, after which it should inhibit. That such an interpretation is correct is indicated by the experiments (1017 and 1026) with autolyzed tumor included in Table II. Tumor tissue that originally gives no inhibition on autolysis does inhibit the succinoxidase system.

*Inhibition by liver.*—If the inhibition of liver succinoxidase is due in part to the liberation of catheptic enzymes as indicated above, then autolyzed tissue from other sources, including liver itself, should inhibit. That this is true is shown by the data in Table III in which liver succinoxidase is almost completely inhibited by autolyzed liver.

These observations make the results with pancreas, trypsin, and chymotrypsin fit into the same kind of pattern as those with tumor, since in both cases inactivation of the succinoxidase system appears to be associated with proteolytic enzymes.

TABLE III: INHIBITION OF LIVER SUCCINOXIDASE BY LIVER HOMOGENATE TREATED IN VARIOUS WAYS

Test system as described in text (5 mgm. liver homogenate per flask)

Inhibitor concentration per flask 50 mgm.

| Experiment No. | Treatment | Average inhibition, per cent |
|---|---|---|
| 1021 | Untreated | 0 |
| | Autolyzed 24 hours, 37° C. | 98 |
| | Heated 100° for 10 minutes | 26 |
| | Dialyzed 24 hours in distilled water | 14 |
| | Dialyzed 24 hours in buffer, pH 7.4 | 11 |

*Mechanism of inhibition.*—If both the inhibitors from pancreas and the succinic dehydrogenase have the SH structure, it is possible that at least part of the inhibition by pancreas may be caused by the formation of the following type of complex: enzyme · S · SR, where R represents any SH compound present in pancreas since this appears to be the mechanism by which cysteine and glutathione inhibit succinic dehydrogenase (10). Such complex formation would prevent succinate from approaching the enzyme surface and thus produce inhibition. If such an interpretation is correct, it should be possible to inactivate the inhibitors of pancreas, at least in part, by first incubating pancreas with an SH poison, and then adding it to liver homogenate. That this can be done is shown by the results in Fig. 1, in which the $Q_{O_2}$ for successive 10 minute intervals is plotted against time. By this method of plotting, the data in effect show the amount of active succinic dehydrogenase at any given time (10). It will be observed that there is a slow spontaneous decrease in activity in the control, which consisted of the liver succinoxidase test system alone.

When either quinone or pancreas was also present in the test mixture, the rate of inactivation was much greater and was nearly complete after 60 minutes. However, when both were present the inhibition was much less than with either inhibitor alone, indicating reaction between the quinone and pancreas to yield a less toxic preparation. The data in Fig. 1 are typical of those in the tables and show the magnitude of the absolute quantities from which the percentage inhibitions were calculated and reported in the tables.

From the results presented above, it appears that at least part of the inhibition produced by the pancreas may be associated with the formation of —S—S—

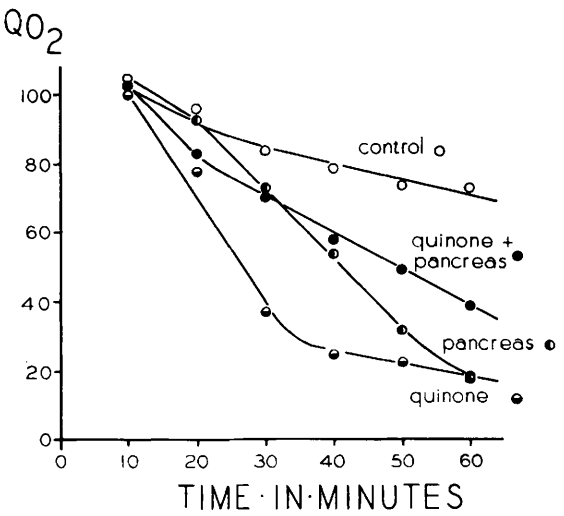


FIG. 1.—Relationship between $Q_{O_2}$ (cu. mm. $O_2$ uptake per mgm. dry liver per hour) and time for liver succinoxidase alone and when treated with quinone, pancreas, and quinone-treated pancreas. The quinone-pancreas was prepared by incubating the quinone with the pancreas for 1 hour at 37° C. prior to testing.

Concentration of pancreas, 2 mgm.; quinone-final, $M/30{,}000$ (10.87); liver, 5 mgm. The control consists of the succinoxidase test system as described in the text, with 0.1 ml. of a 5 per cent liver homogenate as the source of the succinoxidase.

linkages between succinic dehydrogenase and SH compounds occurring in pancreas. Grassmann, Dyckerhoff, and von Schoenebeck (4) report that pancreatic trypsin is inhibited by cysteine. This suggests that trypsin may be an SH enzyme and that the mechanism of inactivation with cysteine may involve the formation of a complex of the type mentioned above: trypsin—S—S—Cy. It is possible that the inactivation of a portion of the inhibitors from pancreas may be due to the inactivation of trypsin, which can no longer function as an inhibitor.

That the inhibition by pancreas involves more than a simple interaction between SH groups, however, is indicated by the fact that heated pancreas produces less inhibition than untreated pancreas (Table IV). Indeed, If Mirsky (7) is correct in pointing out that the denaturation of a protein (such as would occur on heating) unfolds the protein so that SH groups that were not exposed in the native protein become exposed in the denatured protein, one might observe greater inhibition than before, rather than less, if the inhibition were due only to nonspecific sulfhydryl interaction. On the other hand, if part of the inhibition depends upon the integrity of a functional proteolytic enzyme (whether from pancreas or tumor), then one should get less inhibition following heating. The data on heated pancreas show that part of the inhibition of succinoxidase activity by pancreas requires the integrity of pancreatic enzymes that are destroyed by heating. Apparently the initial formation of a succinic dehydrogenase-proteolytic enzyme complex may be dependent upon the presence of SH groups, while the hydrolysis of the enzyme protein depends in addition upon the native proteolytic enzyme. The latter would be expected to be destroyed by heating. This interpretation fits in with the experiments of Bernheim (1), who showed that the succinic dehydrogenase may be inactivated before considerable hydrolysis occurs, suggesting the formation of a complex, followed by proteolysis.

TABLE IV: INHIBITION OF LIVER SUCCINOXIDASE BY PANCREAS HOMOGENATE TREATED IN VARIOUS WAYS

Test system as described in text (10 mgm. liver homogenate)

| Experiment No. | Inhibitor | Inhibitor concentration, mgm. per flask | Average inhibition, per cent |
|---|---|---|---|
| 828 | Pancreas | 4 | 98 |
| | Pancreas, heated 100° for 10 minutes | 4 | 15 |
| 829 | Pancreas | 5 | 95 |
| | Pancreas, heated 100° for 10 minutes | 5 | 16 |
| | Pancreas, dialyzed | 5 | 95 |
| | Pancreas, heated, dialyzed | 5 | 13 |

Results similar to those with pancreas are obtained with the "inhibitors" from tumor (Table II, see especially experiments 1017 and 1026). Heating to 100° C. destroys part of the inhibition but not all. It is suggested that the portion remaining is due to the presence of free SH groups on tissue proteins exposed by heat denaturation. That these SH groups are not on low molecular weight compounds of the glutathione type is also indicated by the data in Tables II and IV, where it is shown that they are not dialyzable after heating. The data (Tables II and III) also show that heating confers on tissues unable to inhibit succinic dehydrogenase in the living state inhibitory powers exactly like those possessed by necrotic tissue after heating.

Dialysis apparently also produces denaturation of protein and thus exposes SH groups that may inactivate the succinic dehydrogenase. In the case of tumor (see Table II, experiments 1017 and 1026) dialysis causes greater inhibition than heating for 10 minutes at 100°. This increase in the inhibitor activity cannot depend upon the liberation of autolytic enzymes since heating to 100° for 10 minutes does not destroy this additional inhibitor. This type of phenomenon does not seem to occur in liver (Table III). That the inhibition produced by heated liver is probably due to SH groups exposed on protein molecules resulting from denaturation is indicated by the work of Stotz and his associates (11), who showed that heating extracts from guinea pig liver increased the number of nondialyzable SH groups.

## DISCUSSION

The results presented above indicate that, with respect to tumor tissues, Elliott's inhibitor [1] is found only in necrotic tumors or in tumor tissue that has been allowed to undergo autolysis. This fact emphasizes the importance of determining succinic dehydrogenase in healthy tumor tissue and suggests that such tests would be valid.[2]

The nature of the inhibitor appears to be complex and to include several factors, which occur in different proportions according to the source of the inhibitor and its treatment. Elliott's statement that the inhibitor is probably a proteolytic enzyme is essentially correct as far as whole tumors are concerned, but this inhibition appears to be negligible in tumors free from necrosis. We have been able to work with much smaller tumors than Elliott used because the sensitivity of the succinoxidase test system has been greatly increased (8, 9) since Elliott's experiments were completed. In necrotic or autolyzed tumors, or in autolyzed liver, the inhibitor activity is probably associated with proteolytic enzymes. The activity remaining when these sources are heated probably depends upon the liberation of fixed SH groups. In pancreas, the inhibitor appears to be due to both proteolytic enzymes and to ribonuclease.[3] In heated pancreas, the inhibitor activity is probably caused by both ribonuclease and fixed SH groups, since ribonuclease is remarkably heat-stable (5). The inhibition observed by Elliott in certain normal tissues may be due in part to ribonuclease, since the only normal tissues that contained appreciable amounts of inhibitor were pancreas and spleen; and these tissues contain much more ribonuclease than other normal tissues, according to unpublished studies by Mr. J. A. Bain and Dr. H. P. Rusch of this Institute.

[1] Elliott reported that heart muscle preparations were not inhibited by the tumor extracts but were, in fact, considerably stimulated; and he suggested that the succinoxidase system in heart muscle differed from that in other tissues. The recent work of Swingle, Axelrod, and Elvehjem (12, Table III) demonstrates that heart muscle is unable to destroy its coenzyme I even in the presence of added calcium ions at a rate sufficient to prevent toxicity to its succinoxidase, when succinate is added at the beginning of the experiment. This is in contrast to liver and brain, which possess sufficient nucleotidase to prevent the inhibition of the succinoxidase. The stimulation observed by Elliott in the case of heart succinoxidase plus tumor extracts is adequately explained by the presence of nucleotidase in the latter, and there is now no necessity to assume that the heart succinoxidase is any different from that in other tissues.

[2] The results of such a survey will be reported at an early date.

[3] Although ribonuclease is believed to act specifically in hydrolyzing ribonucleic acid (5), Loring (6) has shown that it inactivates the tobacco mosaic virus, which is a ribonucleoprotein. It is thus possible that the succinoxidase system may include a ribonucleoprotein. Experimental data bearing on this problem will appear in a separate publication.

## SUMMARY

Experiments have been carried out on an inhibitor from tumor tissues with a succinoxidase preparation from liver as a test system. The results of these experiments may be summarized as follows:—

1. Healthy tumor tissues contain no inhibitor.

2. Necrotic tumor or healthy tumor after autolysis possesses such an inhibitor.

3. Liver, after autolysis, also shows inhibitor activity.

4. Pancreas, as well as crystalline trypsin, chymotrypsin, and ribonuclease, inhibits succinoxidase activity.

5. The inhibitory action is not completely destroyed by heating, and it is suggested that the heat-stable inhibition may be due to SH compounds and to ribonuclease in certain tissues.

6. It is concluded that succinoxidase assays on healthy tumor tissues would not involve inhibitor action and would therefore be completely valid.

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