The Effect of 2,4-Dinitrophenol and of Fluoride on Oxidations in Normal and Tumor Tissues

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INTRODUCTION

Earlier work from this laboratory (9, 11) has shown that the addition of fluoride permits isotonic homogenates of the Walker 256 carcinosarcomas and the Flexner-Jobling carcinoma to oxidize to an appreciable extent the various intermediates of the tricarboxylic acid cycle when supplemented with adenosinetriphosphate (ATP).

From data on phosphate balance (11, 12), it has been suggested that the oxidative level is affected by the balance between high-energy phosphate breakdown and the uptake of inorganic phosphate into organic form. Thus, in the tumors where phosphate breakdown is very high, fluoride acts to preserve the respiratory rate by inhibiting the breakdown of high-energy phosphate (9, 11). Since the homogenates of several normal tissues do not need the addition of fluoride to demonstrate oxidation (8, 11, 12), it was thought worthwhile to investigate other tumors than those mentioned to observe whether the behavior in the presence of fluoride is characteristic of all tumor tissue.

Since it has been shown that 2,4-dinitrophenol (DNP) acts to stimulate the breakdown of high-energy phosphate (2, 4, 13), the present work was concerned with whether DNP further decreases the low oxidation rate in those tissue homogenates in which there is a stimulation by fluoride and whether it increases the oxidation of those tissue homogenates in which there is no effect by fluoride or where fluoride is inhibitory. In general, DNP and fluoride have been found to act somewhat antagonistically with respect to their effects on oxidation. Thus, the behavior of a homogenate of a tissue in the presence of DNP and of fluoride will roughly characterize that tissue as to its ability to break down high-energy phosphate and as to the ability of its oxidative phosphorylation mechanism to keep pace with this breakdown.

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4 × 10^{-3} \mu M cytochrome c; 8 \mu M ATP; 10 \mu M fumarate; and 90 \mu M pyruvate; enough solid sucrose to make the final mixture isotonic; and enough isotonic sucrose solution to compensate for variations in the amount of homogenate or mitochondria added and give a final volume of 8.0 ml. The tissue homogenates or mitochondria were always prepared in isotonic sucrose. When fluoride was added, the final concentration was usually 0.01 M, and the flasks without fluoride contained sodium chloride at the same molarity (0.01 M). The concentration of DNP was varied over a wide range, but the molarity was always so low that the controls contained equivalent amounts of water. Inorganic phosphate was measured by a modification (18) of the Lowry-Lopez method (7). Oxygen uptake was measured by means of the conventional Warburg apparatus and incubations were at 38° C. unless otherwise noted.

RESULTS

In rat liver homogenates, DNP stimulated respiration while fluoride inhibited it (Chart 1). These effects are probably due to a DNP-stimulated breakdown of ATP to give phosphate acceptor in the form of adenosinediphosphate (ADP) and to an inhibition of this breakdown by fluoride (18, 19). The addition of ADP also stimulated respiration to about the same extent as did the addition of DNP. Chart 2 shows that additions of DNP, from a final concentration of 3 × 10^{-5} M, progressively increased the respiration rate; 0.03 M fluoride had a quantitative effect equal to 0.01 M fluoride in inhibiting the rate. Thus, liver homogenates have a rate of ATP breakdown which is inadequate to saturate the oxidative capacity. This imbalance is even more pronounced in the mitochondria, since the respiratory rate of liver mitochondria can be stimulated three- to sevenfold by the addition of 3 × 10^{-6} M DNP (cf. 4, 19), an even greater extent than in the case of the whole homogenate. It would therefore appear that the other constituents of the liver cell—nuclei (cf. 12), microsomes (cf. 14), and perhaps supernatant fluid contain factors for the breakdown of ATP, but that these additional “ATPase” factors do not supply enough phosphate acceptor to saturate the oxidative system in liver homogenates. The addition of fluoride might be expected to have a greater effect on liver ho-

The pyruvic acid was vacuum-distilled and stored at 0-3° C. as a 1 M solution, which was neutralized with dilute KOH just before use.

Cytochrome c was obtained from E. R. Squibb and Sons through the courtesy of Dr. Asger Langlykke; DPN was obtained as the free acid from D. Groth and Dr. G. A. LePage; a-ketoglutaric acid was prepared by Dr. W. Ackermann; hexokinase was prepared from bakers’ yeast, by an unpublished method of W. F. Loomis; other ingredients were commercial products used without further purification and converted to potassium salts if necessary.
Previously, it had been found that final DNP concentrations of from $6 \times 10^{-4}$ to $6 \times 10^{-6}$M slightly increased the oxidative rates during the first 30 minutes, but that after this time the rate with $6 \times 10^{-6}$M DNP dropped very sharply, while the rates with $6 \times 10^{-4}$M, $1.5 \times 10^{-4}$M, and $3 \times 10^{-6}$M DNP declined more slowly. However, after 60 min. all rates were less than that of the control without added DNP (Chart 2). DNP at a concentration of $1.2 \times 10^{-4}$M decreased the rate 45 per cent in the first 10 min., and by 40 min. the respiration had been completely suppressed. Fluoride at a concentration of 0.03 M decreased the rate even more than 0.01 M fluoride. These results with DNP and with fluoride confirm earlier data of Pardee and Potter (8). The rates of phosphate breakdown and phosphate uptake are thus more nearly balanced in kidney than in liver homogenates, in that phosphate breakdown seems to be adequate enough to supply phosphate acceptor for the respiration (cf. 8). However, in kidney mitochondria $3 \times 10^{-6}$M DNP gave a threefold initial stimulation in the respiratory rate, with only slight decline in the rate after 90 min., and 0.01 M fluoride did not affect the rate at all. Thus, the other components of the kidney cell (cf. 8) seem to be more influential than the corresponding components of the liver cell in providing factors for bringing phosphate breakdown into balance with phosphate uptake.

Charts 4–10 show the great dissimilarity of various tumors in their response to DNP and fluoride. For example, the Flexner-Jobling carcinoma (Chart 4) and the human fibrosarcoma (Chart 5) yield homogenates in which the rate of phosphate breakdown is much too great relative to the capacity of the tumor to regenerate high-energy phosphate oxidatively. Concentrations of DNP from $7.5 \times 10^{-4}$M to $6 \times 10^{-4}$M DNP greatly decreased the already low oxidative rate of the Flexner-Jobling tumor (Chart 2), while 0.01 and 0.03
m fluoride increased the rate to the same extent. When the Flexner-Jobling tumor was homogenized in the presence of 0.01 m fluoride, not only was the subsequent oxidative rate increased (cf. Charts 4 and 6), but the addition of DNP had no effect in accelerating phosphate breakdown. That the rate of phosphate breakdown is excessive relative to oxidation in the mitochondria from the Flexner-Jobling tumor was shown by experiments in which concentrations of DNP from $7.5 \times 10^{-5}$ to $3 \times 10^{-4}$ M decreased the already low mitochondrial respiratory rate. Also, 0.01 M and 0.08 M fluoride were as effective in increasing the rate as they were in the case of the whole homogenate. The high rate of ATP breakdown by Flexner-Jobling carcinoma mitochondria is also shown in Table 1; these data show that the addition of Flexner-Jobling carcinoma mitochondria stimulates the respiratory rate of liver mitochondria (cf. 8). This stimulation is presumably caused by an activation of ATP breakdown to provide enough phosphate acceptor for the oxidative system of the liver mitochondria, since this synergistic effect of the tumor mitochondria is greatly reduced by the addition of fluoride and since the effect can be reproduced by DNP.

Between the 8th and 11th days after transplantation of the Flexner-Jobling carcinoma, the tumors approximately doubled their weight every 36 hours. In experiments on homogenates from pairs of 8-, 9-, 10-, and 11-day-old tumors that developed from matched implants, there was no detectable change in the effects of DNP and of fluoride on the respiration. Also, in this series of experiments there was no clear-cut effect of added di-phosphopyridine nucleotide (DPN) (0.002 M) on

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**Chart 7.** The effect of DNP and fluoride on oxidation by homogenate of mouse 98/15 hepatoma. Each flask contained the standard medium, except that 6 μM ATP were added instead of 3 μM ATP, and 100 mg. of the mouse 98/15 hepatoma homogenate. When added, DNP was at a final concentration of $3 \times 10^{-4}$ M.

**Chart 8.** Effect of DNP and fluoride on oxidation by mouse adrenal cortex tumor homogenate. Each flask contained the standard medium with cytochrome c omitted and 100 mg. of the mouse adrenal cortex tumor homogenate. When added, DNP was at a final concentration of $3 \times 10^{-4}$ M.

**Chart 9.** Effect of DNP and fluoride on oxidation by Novikoff hepatoma homogenate. Each flask contained 150 mg. of the Novikoff rat hepatoma homogenate and the standard medium with cytochrome c omitted and with 6 μM ATP added instead of the 3 μM ATP. When added, DNP was at a final concentration of $3 \times 10^{-4}$ M.

**Chart 10.** Effect of DNP and fluoride on oxidation by homogenate of C3H mouse mammary carcinoma. Each flask contained the standard medium, except that the substrates were 5 μM fumarate and 10 μM pyruvate, and 125 mg. of the C3H mouse mammary carcinoma homogenate. When added, DNP was at a final concentration of $3 \times 10^{-4}$ M.

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the respiratory rate of these tumor homogenates. In most instances there was an inhibition of the already low respiratory rate (Chart 4), and the addition of fluoride did not relieve the inhibition by DPN. The oxidative rate of the mitochondria from this tumor was also inhibited by DPN. In only one instance was there an appreciable stimulation of the respiratory rate of the homogenate by DPN. It is concluded that the effects observed are independent of the age of the tumors within the limits noted and are not due to necrosis.

In contrast, three other tumors, the mouse hepatoma 98/15 (Chart 7), the adrenal cortical tumor (Chart 8), and, to a lesser extent, the Novikoff hepatoma (Chart 9) resemble kidney in their responses to DNP and to fluoride. All these tumor homogenates had a respiratory rate that was much higher than that of the Flexner-Jobling tumor, that was inhibited by fluoride, and that showed a response to DNP very unlike that of the Flexner-Jobling tumor and more similar to that of kidney. DNP at a concentration of \(7.5 \times 10^{-4}\) M had very little inhibitory effect on the high oxidative rate of the adrenal cortical tumor homogenate, but higher concentrations of DNP (up to \(6 \times 10^{-4}\) M) had increasingly larger inhibitory effects on the respiration (Chart 2).

The CSH mouse mammary carcinoma (Chart 10) gave a unique response, since both fluoride and, to a much lesser extent, DNP stimulated the respiration; \(1.5 \times 10^{-4}\) M DNP gave approximately the same small increase in rate as did \(8 \times 10^{-4}\) M DNP, while \(6 \times 10^{-4}\) M DNP had no effect (Chart 2). Also, with this tumor, DPN stimulated the respiration of the homogenate, not so much as fluoride, but more than DNP. The significance of these effects is lessened by the low magnitude of oxygen uptake in all conditions.

Table 2 shows that most metabolites involved in the tricarboxylic acid cycle, with the exception of succinate, are not oxidized very well by the Flexner-Jobling carcinoma or the CSH mouse mammary tumor homogenates. However, all the substrates except citrate and cis-aconitate were oxidized to a much greater extent in the presence of fluoride.

### Table 1

**Effect of Flexner-Jobling Tumor Mitochondria upon Respiration of Liver Mitochondria**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Substrate</th>
<th>Plus F</th>
<th>Minus F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver mitochondria</td>
<td>822</td>
<td>221</td>
<td>681</td>
</tr>
<tr>
<td>Flexner-Jobling mitochondria</td>
<td>40</td>
<td>38</td>
<td>14</td>
</tr>
<tr>
<td>Liver mitochondria plus Flexner-Jobling mitochondria</td>
<td>214</td>
<td>144</td>
<td>570</td>
</tr>
<tr>
<td>Theoretical</td>
<td>151</td>
<td>130</td>
<td>347</td>
</tr>
</tbody>
</table>

### Table 2

**Effect of Substrate on Oxidation of Flexner-Jobling and CSH Mouse Mammary Tumor Homogenates**

Each flask contained the standard medium except for the substrate and either 150 mg. Flexner-Jobling tumor homogenate or 125 mg. of the CSH mouse mammary carcinoma homogenate. All substrates were added in \(15 \mu\) M quantities except pyruvate (\(10 \mu\) M) and fumarate (\(5 \mu\) M).

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Substrate</th>
<th>Plus F</th>
<th>Minus F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flexner-Jobling</td>
<td>Succinate</td>
<td>95</td>
<td>72</td>
</tr>
<tr>
<td>Flexner-Jobling</td>
<td>Alpha-ketoglutarate</td>
<td>57</td>
<td>17</td>
</tr>
<tr>
<td>Flexner-Jobling</td>
<td>Oxalacetate</td>
<td>49</td>
<td>17</td>
</tr>
<tr>
<td>CSH mouse</td>
<td>Pyruvate + fumarate</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>CSH mouse</td>
<td>Citrate</td>
<td>104</td>
<td>35</td>
</tr>
</tbody>
</table>

### Table 3

**Oxidative Phosphorylation in the Flexner-Jobling Homogenate**

Each flask contained 125 mg. Flexner-Jobling tumor homogenate and the standard medium with cytochrome c omitted and with \(6 \mu\) M AMP substituted for the ATP. Incubated for 10 min. at 30°C.

<table>
<thead>
<tr>
<th>Additions</th>
<th>(O_2) (\mu) atoms</th>
<th>(P) (\mu) atoms</th>
<th>(P/O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-2.3</td>
<td>0.60</td>
<td>0.95</td>
</tr>
<tr>
<td>0.01 M NaF</td>
<td>-2.2</td>
<td>-4.27</td>
<td>0.45</td>
</tr>
<tr>
<td>Hexokinase, glucose (11 (\mu)M)</td>
<td>-1.6</td>
<td>-0.14</td>
<td>1.3</td>
</tr>
<tr>
<td>0.01 M NaF, hexokinase, glucose</td>
<td>-1.65</td>
<td>-4.27</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Corrected for period of equilibration.

Tables 3 and 4 show that, under appropriate conditions, tumors as different as the Flexner-Jobling carcinoma, which has a very low oxidative rate in the absence of fluoride, and the 98/15 mouse hepatoma, which has a high oxidative rate that is inhibited slightly by fluoride, can give a net phosphate uptake with a \(P/O\) ratio which is lower than that obtained with normal tissue under similar conditions. In both cases, the incubation temperature was lowered to 30°C to reduce the effects of side reactions draining off high-energy phosphate.
The incubations were limited to short time periods, so that, as can be seen in Table 4, the coupling of phosphorylation to oxidation would not be disrupted by the longer time periods. In the case of the Flexner-Jobling tumor homogenate, it can be seen that only in the presence of fluoride was there a net esterification of inorganic phosphate. Although the 98/15 hepatoma homogenate had a high oxidative rate in the absence of fluoride, the addition of fluoride increased the already high phosphate uptake, indicating that there is some phosphate loss even with this tumor. It is interesting that with the addition of glucose without hexokinase to the tumor homogenate there was a greater net phosphate uptake, with or without fluoride, without any increase in respiration, indicating hexokinase activity (cf. 6). These data show the differences between the 98/15 hepatoma and the Flexner-Jobling carcinoma are supported by earlier findings in the literature. R. Kielley (3) found that, in the absence of fluoride, the mitochondria of the 98/15 hepatoma would esterify inorganic phosphate in the presence of hexokinase with a P/O ratio of approximately 2 when α-ketoglutarate was oxidized, while Williams-Ashman and Kennedy (27) found that, in the presence of fluoride and hexokinase, the particulate matter of the Flexner-Jobling tumor would esterify inorganic phosphate when succinate was the substrate, with a P/O ratio of 0.7.

DISCUSSION

It is clear from the experimental data that the six tumors tested gave different patterns of oxidative response to DNP and to fluoride. There seems to be no correlation between any gross observations on the rates of growth of the various tumors and the oxidative responses of their homogenates in the presence of DNP or fluoride.

The possibility of such correlations may well be overshadowed by the influence of factors such as a residual resemblance to the tissue of origin and the species of animal used. Thus, there may be an alteration common to all these tumors, but it may fail to result in a consistent pattern of response to DNP and fluoride because of the differences in function and in enzymatic constitution of the tissues of origin of these tumors.

It might be argued (21) that, in the process of homogenizing the various tumors, the stability of the bound complexes is reduced more in one tumor than in another and that the responses to DNP and to fluoride reflect only a secondary effect resulting from the disruption of the organization of the cell. However, this possible differential disruption by homogenization would still reflect a difference among the various tumors in the stability of the oxidative phosphorylative system, and would also reflect a difference between most normal tissues (except spleen and thymus) and tumors in this respect (cf. 8). In addition, the great differences in oxidative response among the tumors tested here must be emphasized. The Flexner-Jobling carcinoma and the Novikoff hepatoma, which under our conditions have approximately the same high growth rate and thus synthesize approximately equal amounts of tissue per unit time, exhibit marked differences in the oxidative responses to fluoride and to DNP. Another difference among tumors is illustrated by the difference in the P/O ratios of the Flexner-Jobling carcinoma and the mouse 98/15 hepatoma when fluoride was added (cf. Tables 3 and 4) and in the great variation among P/O ratios obtained with the particles of various tumors by Williams-Ashman and Kennedy (27) when hexokinase was used as a trapping agent for esterified phosphate.

As a corollary to the difference in stability of the oxidative phosphorylative system between various tumors and normal tissues is the DPN requirement exhibited in a number of tumors as noted by Weinhouse and his group (21, 25, 26), by Williams-Ashman and Kennedy (27), and by R. Kielley (8). This requirement is not exhibited by kidney or liver under the same conditions (3, 21, 26). In nearly all these tumors the leakage of DPN...
or its disruption from a bound site can be repaired by the addition of DPN, but in the Flexner-Jobling carcinoma the addition had no effect on homogenate or mitochondrial oxidation, although it has been reported (27) that even in this tumor the addition of DPN stimulated the oxidation of glutamate.

It has been reported that the individual enzymes necessary for the complete oxidation of any member of the tricarboxylic acid cycle were contained in all of the tumors tested (21–24). Also, it is clear that many of these tumors can oxidize various substrates of the tricarboxylic acid cycle to an appreciable extent if DPN is added (3, 24–26). However, it has been found that a-ketoglutarate dehydrogenase and aconitase activities were lower in tumor tissue than in normal tissue (24), that both succinoxidase and cytochrome oxidase were lower in mouse hepatoma than in mouse liver (17), and that 98/15 hepatoma has a much lower DPNH dehydrogenase activity than does normal mouse liver (5).

It has been stated (25, 26) that in those tumors where oxidation was low in comparison to normal tissue the oxidative activities of these tumor mitochondria on a per milligram N basis were of the same magnitude as those of three normal tissues studied. In contrast is a comparison between the 98/15 mouse hepatoma and mouse liver. Schneider and Hogeboom (17) state that the total succinoxidase and cytochrome oxidase activities of the liver mitochondria were over 5 times that of hepatoma mitochondria and that the specific activity of these enzymes (on a per milligram N basis) of the liver mitochondrion was 2.5 times that of the tumor mitochondrion. Therefore, the decreased amount of mitochondrial N/mg wet weight of tissue in the hepatoma, as compared to the normal liver, still does not account for the decreased activity in the hepatoma. Thus, while it would appear that in some tumors the decreased amount of mitochondrial-bound enzyme per unit of cellular N can be attributed to the lowered content of mitochondria (25, 26), in other tumors there is an absolute decrease in some of the enzymes associated with mitochondria (17). At any rate, in both these cases the amount of enzyme per unit of tumor cell N, or, in other words, the enzymatic capacity of the cell, is reduced to a fraction of what it is in homologous normal tissues.

The capability of a tissue to be stimulated by DNP or by fluoride is also a measure, in a sense, of the oxidative capacity of the tissue. For example, the stimulation of the oxidation of some tumors by the addition of fluoride or by the addition of DPN may mean that the oxidative capacity of the tumor is not sufficient to regenerate the lost co-factors, such as DPN or ATP. Also, the ability of some tumor oxidations to be inhibited by fluoride and to be unaffected or even stimulated by DNP may mean that in these cases the tissue is operating at a level of oxidation which is sufficient to regenerate the co-factors. Thus, it would appear that the wide variation in oxidative capacity among tumors is but a reflection of the wide variation existing among normal tissues. Perhaps even the greater lability of the phosphorylating mechanism in some tumors and not in others is but a reflection of what a comparison among normal tissues has shown and of the fact that these variations must be ascribed to a residual enzyme pattern derived from the tissue of origin.

From these studies it seems clear that the conclusions reached from metabolic studies on any given tumor cannot be generally assumed to hold for other tumors. Thus, investigators are faced with the task of finding the strategic metabolic steps in the growth process in the presence of a variety of enzymatic patterns that represent in varying degrees the multiplicity of enzyme patterns seen in normal growing and nongrowing tissues. Although we have repeatedly found a lower oxidative capacity in tumor tissues in comparison with the tissues of origin, particularly in the case of liver, the present studies make generalizations on this point inadvisable, largely because of the inability to provide adequate data on the tissues of origin of all the tumors studied. It is equally apparent that the recently advanced opposite generalization that the oxidative enzyme pattern of tumors is no different from that of normal tissues is also untenable, because here the reservation is not based upon a lack of suitable controls, but countered by positive findings in the case of some of the hepatomas.

It would appear that the next step is to study the metabolism of a variety of tumors under conditions of actual growth in the host and to ascertain the balance between as many alternative catabolic and anabolic pathways as possible, on the assumption that uncontrolled growth involves the dominance of certain anabolic pathways over the catabolic pathways for the same substrates. Perhaps these metabolic switches can eventually be found, even though the important reactions may occur in a variety of over-all enzyme patterns. Possible support for this view was obtained in the...
studies with fluoroacetate (10) in which one of the possible interpretations was that tumors do not activate acetate effectively. This interpretation has been supported by subsequent studies with acetate-1-C\textsuperscript{14} (1). In attempting to find a metabolic pathway that might be characteristic of all tumors, studies with glucose-1-C\textsuperscript{14} in vivo have been started in this laboratory (15), and it has been found that derivatives of glucose are extensively used in anabolic reactions. Thus, it may be possible eventually to study the important shunts in glucose metabolism even in the presence of the divergent enzyme balances described in the present report.

SUMMARY

The capacity and the lability of the coupled oxidative phosphorylation systems of liver, kidney, and of seven different tumors have been investigated with the aid of DNP and of fluoride, which act to stimulate and to inhibit, respectively, the net breakdown of high-energy phosphate. No over-all oxidative pattern common to tumor homogenates has been observed. The great difference in oxidative response among various tumors has been considered to represent the contribution of the tissue of origin to the enzyme pattern of the tumor tissues, and to reflect the multiplicity of enzyme patterns seen in normal growing and nongrowing tissues. The significance of the findings was discussed in relation to the problem of finding the balance between anabolic and catabolic pathways that involve the same substrates.

REFERENCES

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