2,4-Dinitrophenol: Lack of Interaction with High-Energy Intermediates of Oxidative Phosphorylation

Abstract. *If the uncoupling agent 2,4-dinitrophenol is added just prior to addition of the phosphate acceptor adenosine diphosphate to rat liver mitochondria previously incubated with substrate, orthophosphate, and oxygen, steady-state phosphorylation is abolished, whereas the initial phase of rapid phosphorylation, the so-called "adenosine triphosphate jump," is undisturbed. This leads to the conclusion that uncoupling agents operate by interfering with the synthesis of high-energy intermediates, not by hydrolyzing or otherwise inactivating them.*

The mechanism by which free energy is transferred during mitochondrial oxidative phosphorylation from the exergonic electron-transport reaction to the endergonic phosphorylation reaction remains poorly understood. In isolated intact mitochondria, electron transport and phosphorylation are tightly coupled, that is, in the absence of ADP (*1*) and P_i, little oxygen is consumed even though substrate and oxygen are present in excess. The requirement of phosphorylation for rapid electron transport through the respiratory chain is abolished when certain chemicals, so-called uncoupling agents, are added in appropriate concentrations to the mitochondria. We are now reporting on experiments that localize the site of action of the typical uncoupling agent DNP more directly than has hitherto been possible.

Suspensions of rat liver mitochondria are incubated in the presence of β-hydroxybutyrate, $^{32}P_i$, and $MgCl_2$ in isotonic glycylglycine buffer at *p*H 7.0 for 10 minutes at 10°C, as described previously (*2*). Addition of ADP to this mixture elicits the onset of phosphorylation. Before the steady-state phosphorylation becomes established, there is a brief period of more rapid ATP-formation, the so-called ATP-jump (*2*, *3*).

Use of the automatic rapid-sampling apparatus (*4*) permits the two phases of ATP synthesis to be clearly distinguished, as shown in Fig. 1*a*. The transient phase of more rapid phosphorylation, which under our experimental conditions is over in less than 3 seconds, represents the interaction of ADP with accumulated high-energy intermediates (*3*). The subsequent slower rate of phosphorylation (steady-state), which is that normally observed, is limited by the rate at which these high-energy intermediates can be resynthesized from the respiratory chain. The effect of uncoupling agents on the transient reaction, that is, the ATP-jump, was investigated by adding appropriate concentrations of DNP 1½ seconds before addition of ADP. As shown in Fig. 1*b*, DNP did not affect the ATP-jump. In contrast, steady-state phosphorylation was uncoupled by 80 to 90 percent. In control experiments, where DNP was added after steady-state phosphorylation had been established, uncoupling occurred to a similar extent and within less than 2 seconds. The DNP concentration employed (0.01m*M*), is insufficient to stimulate the activity of adenosine triphosphatase in these preparations. This eliminates the possibility that the uncoupling phenomenon is due to a rapid hydrolysis of the newly formed ATP. The persistence of the ATP-jump in presence of uncoupling concentrations of DNP proves that this agent does not interfere with the actual synthesis of the terminal phosphate bond of ATP. Of the two alternative mechanisms of uncoupling— prevention of resynthesis of the high-energy intermediates or their destruction in the nonphosphorylated form— the destruction mechanism has been rendered unlikely by recent investigations of Vignais (*5*) and Butow (*6*) when considered together with the findings reported here. These investigators added $^{32}P_i$ and ADP together to preparations initially incubated with substrate and oxygen in a phosphate-free medium. Vignais used digitonin fragments at 1°C, while Butow used P_i-depleted mitochondria at room temperature, which emphasizes the generality of their observations. With both types of preparations, the ATP-jump proved to be independent of the presence of P_i in the initial incubation medium. This clearly shows that the interaction of P_i with the high-energy intermediates is at least as rapid as the ATP-jump. In addition, Vignais found that adding nonradioactive P_i and ADP simultaneously to preparations initially incubated with trace amounts of $^{32}P_i$ failed to result in preferential incorporation of $^{32}P_i$ into the ATP formed during the ATP-jump (*7*). This practically instantaneous randomization of $^{32}P_i$ with unlabeled P_i demonstrates that phosphorylation of the high-energy intermediates, if it occurs at all, is readily and rapidly reversible. Thus, there is no indication for the formation of stable phosphorylated high-energy intermediates. Hence, the inability of DNP to abolish the ATP-jump implies that the nonphosphorylated high-energy intermediates must be stable to uncoupling concentrations of DNP.

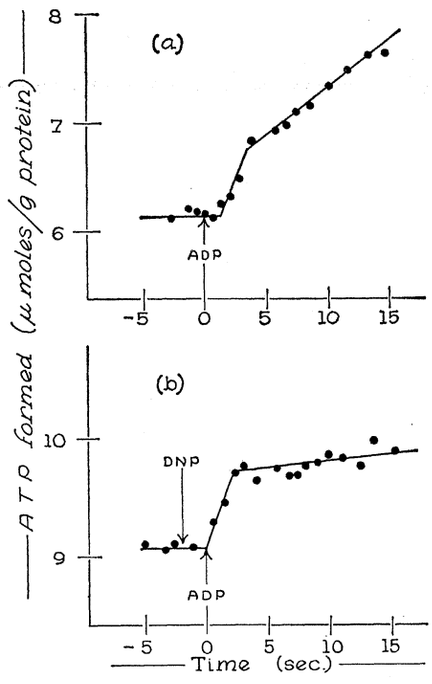

Fig. 1. Effect of DNP on transient and steady-state phases of phosphorylation. Rat liver mitochondria (5 mg of protein per milliliter) were incubated with β-hydroxybutyrate (10m*M*) and $^{32}P_i$ (10m*M*, about 0.1 mc/ml) prior to addition of ADP (0.5m*M*) at zero time. Determination of $AT^{32}P$ was based on the procedure of Hagihara and Lardy (*9*). The gas phase was air; the temperature, 10° ± 0.5°C; and the volume, 12 ml. The liver from one male Sprague-Dawley rat was used in each of these two experiments.

These results lead to the conclusion that DNP uncouples oxidative phosphorylation by interfering with the formation of the high-energy intermediates, rather than by hydrolyzing or otherwise inactivating them. This mode of action permits an explanation of the dilemma caused by otherwise irreconcilable observations reported from a number of different laboratories; this was recently pointed out in a penetrating review by Racker (*8*).

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References and Notes

1. The following abbreviations are used: ADP, adenosine diphosphate; P_i, orthophosphate; DNP, 2,4-dinitrophenol; and ATP, adenosine triphosphate.
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Growth Inhibition of Sarcoma and Carcinoma Cells of Homozygous Origin

Abstract. *Three sarcomas and one carcinoma, originating in homozygous mice, were tested for growth in isologous animals and various semi-isologous F_1 hybrids after inoculation of 10^3 to 10^5 cells. Findings indicated the existence of an "F_1 hybrid effect" for sarcomas and carcinomas, as previously described for lymphomas and normal hematopoietic cells, there being a lower tumor frequency and a longer latency period in the hybrids than in isologous mice.*

In an earlier report I described the differential behavior of transplanted lymphoma lines in genetically compatible homozygous and F_1 hybrid mice (*1*). Isoantigenic variant sublines were studied, isolated from lymphoma LNSF of A × A.SW F_1 hybrid origin by selective passage in one of the parental strains, A or A.SW. Subsequent to such passage, they became specifically compatible with the parental strain of selection and had lost, apparently irreversibly, the H-2 isoantigens specifically derived from the opposite parental strain. When compared with the original LNSF line in the F_1 hybrid type of origin (A × A.SW), it was found that the variants grew more slowly. In the parental strain where they have been selected they grew faster than in the hybrid and behaved in the same way as the unselected line in the F_1 hybrids. Although the mechanism of this phenomenon is not clear at present, it can be attributed to the H-2 antigenic loss characterizing the variant cells and the resulting difference between graft and host when they are inoculated into F_1 hybrids. It is probably analogous to the finding of Snell that homozygous lymphomas grow better in mice of their strain of origin than in various F_1 hybrids (*2, 3*). The possible existence of a similar F_1 hybrid effect with normal hematopoietic cells is indicated from studies by Popp (*4*), McCulloch and Till (*5*), and Cudkowicz and Stimpfling (*6*) on homozygous cells transplanted to lethally irradiated mice of various F_1 hybrid genotypes and to irradiated homozygous mice of the isologous strain. On the other hand, no differences have been found in the survival of skin grafts from homozygous mice transplanted to the same strain and to various genetically compatible F_1 hybrids (*7*). It appeared, therefore, essential to know whether the F_1 effect is restricted to normal cells of the antibody-forming system and their malignant counterparts, or whether it is a general phenomenon; the first alternative would substantiate the suggestion of Snell and Stevens that the F_1 effect found by them with homozygous lymphomas is due to "an abortive graft versus host reaction" (*3*).

In the present work an attempt has been made to demonstrate an F_1 hybrid effect with three methylcholanthrene-induced fibrosarcomas, two of which were of C57 BL/Kl (MC57 G and MC57 S) and one of C57 L/Kl origin (MLG), and with one spontaneous mammary carcinoma of A.CA origin (S2C). The tumors were serially propagated by subcutaneous transfer in mice of their strain of origin. Trypsinized cell suspensions were prepared in a way similar to that for tissue culturing and inoculated subcutaneously in a volume of 0.1 ml containing 10^3, 10^4, or 10^5 eosin-unstained cells into homozygous mice of the strain of tumor origin and to various genetically compatible F_1 hybrids, as shown in Fig. 1. All mice inoculated were of the same weight (16 to 18 g), and approximately the same proportion of males and females were used in the experiments. The mice were inspected every 2nd to 4th day after inoculation, and the time required for the appearance of a tumor was recorded, as well as the growth rates of the tumors.

The pooled results (Fig. 1, *A–D*) demonstrate that all tumors grew better in mice of their strain of origin than in different semi-isologous F_1 hybrids. As already found for lymphoma cells (*1*), different F_1 hybrids varied in their capacity to support tumor growth. The C57 BL sarcomas grew regularly better in A.CA × C57 BL F_1 mice than in A × C57 BL, DBA × C57 BL or C3H × C57 BL F_1 hybrids. The difference between homozygous and F_1 hybrid mice was apparent both with regard to latency period preceding tumor appearance and total tumor frequency, whereas no certain differences were detected in the growth rates of established tumors. As with lymphoma cells, the "F_1 effect" did not increase after prior immunization of the mice (*2, 3, 8*) and the effect remained after irradiation of the mice with 450 roentgens prior to grafting (*1*).

Thus the previously described F_1 effect is not restricted to cells of the antibody-forming system and their malignant counterparts. As a corollary, it is not likely to be caused by an abortive

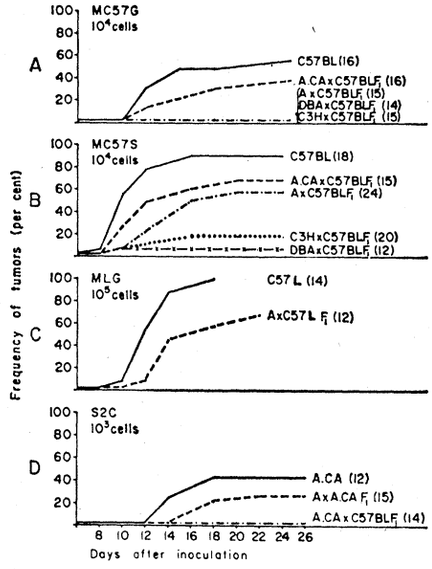

Fig. 1. Inoculation of four different tumor lines, derived from and carried in homozygous mice, to homozygous mice of the strain of tumor origin and various semi-isologous F_1 hybrids. The number of mice inoculated in each group is shown within parentheses. The data are pooled from three separate experiments with each tumor line. *A*, Tests with MC57 G, a methylcholanthrene-induced fibrosarcoma in C57 BL, serially carried during 16 to 18 passages before the tests (10^4 cells were inoculated). *B*, Tests with MC57 G, a methylcholanthrene-induced fibrosarcoma in C57 BL, serially carried during 12 to 16 passages before the tests (10^4 cells inoculated). *C*, Tests with MLG, a methylcholanthrene-induced fibrosarcoma in C57 L, serially carried during 28 to 30 passages before the tests (10^5 cells inoculated). *D*, Tests with S2C, a spontaneous mammary carcinoma in A.CA, serially carried during 39 to 41 passages before the tests (10^3 cells inoculated).