The mechanism underlying the inhibition of the liver mitochondrial oxidation of glutamate by the carcinogen N-2-fluorenylacetamide

KIELLEY[1] has recently studied the inhibitory effect of several isomeric fluorenylacetamides on the oxidation of a number of substrates in a phosphorylating system of rat liver mitochondria. Of the isomers and substrates tested, N-2-fluorenyldiacetamide had the greatest effect on glutamate oxidation, whereas N-2-fluorenylacetamide (2-FAA) exhibited approximately half the inhibition shown by the former compound.

We have now found that 2-FAA may exert a powerful inhibition on L-glutamate oxidation in a phosphorylating system of *mouse* liver mitochondria and that this inhibition can be completely prevented by the simultaneous addition of diphosphopyridine nucleotide (DPN). As shown in Table I, 2-FAA caused about 65% inhibition of the glutamate oxidation. It appeared also that the rate of oxygen consumption of the control suspensions, incubated in the absence of 2-FAA, was stimulated to some degree by the addition of DPN. In a number of experiments, not presented here, in which the latter stimulation was not found, the inhibition produced by 2-FAA was less than that illustrated in Table I. This was usually found when the amount of mitochondria added to the respirometers was higher than that present in the experiment shown in the table. Since the "intactness" of the mitochondrial structure occasionally varies somewhat, being definitely less the smaller the amount of mitochondria present, it is evident that the inhibition produced by 2-FAA is inversely related to the mitochondrial integrity involving DPN.

TABLE I

THE COUNTERACTION BY DPN OF THE N-2-FLUORENYLACETAMIDE-INDUCED INHIBITION OF GLUTAMATE OXIDATION IN A PHOSPHORYLATING SYSTEM OF MOUSE LIVER MITOCHONDRIA

Mitochondria were isolated from the livers of ♂ mice ($BC_3H_e \times BC_3H_e$) in 0.25 *M* sucrose (10 min 5,000 × *g* at 0° C, after removal of the nuclear fraction), washed twice and used immediately in the experiments. Incubation was carried out with particles from 250 mg fresh liver tissue in each flask. The medium was that used by KIELLEY[1] except that the concentration of inorganic phosphate was lowered to 0.013 *M*. L-Glutamate (0.035 *M*) was added from the side-arm after temperature equilibration had been reached, 2-FAA and DPN were present in a final concentration of $1.5 \cdot 10^{-4}$ *M* and 10^{-3} *M* respectively. Total volume 1.6 ml. Incubation at 27° C during 20 min with shaking in air. Oxygen uptake by standard Warburg technique. Inorganic phosphate was determined by the method of Fiske-Subbarow. The data listed are those obtained in the presence of glutamate after correction for the respective control values found in the absence of amino acid.

Additions	*Oxygen uptake* μ*atoms*	*Phosphate esterified* μ*moles*	*P:O*
None	7.2	11.5	1.6
2-FAA	2.6	4.8	1.8
2-FAA + DPN	7.8	12.3	1.5
DPN	9.5	11.7	1.2

It may thus be concluded from the present experiments that 2-FAA caused a loss of DPN from the mouse liver mitochondria. The carcinogen might have been active either by releasing the coenzyme from the mitochondrial structure or by developing a latent DPN-splitting enzyme. The latter could not have been the nicotinamide-sensitive DPNase, since nicotinamide ($5 \cdot 10^{-3}$ *M*) did not counteract the inhibition produced by 2-FAA. It was therefore concluded that the carcinogen induced a partial release of DPN from its bound form. This is, however, not an exclusive feature of this compound since many other substances[2,3,4] are similarly active. The last observation should serve as a warning against postulating a mechanism for the carcinogenic action of fluorene derivatives merely on account of KIELLEY's experiments and the present *in vitro* ones.

Furthermore, any generalization as to one primary mechanism connected with the carcinogenic process in terms of a DPN deficiency is not warranted, since CARRUTHERS *et al.*[5] have shown that the DPN content of cellular fractions, isolated from livers of rats fed *p*-dimethylaminoazobenzene up to 8 months, is normal. Moreover, not all isolated tumour mitochondria are deficient in DPN[6,7,8].

A limited number of experiments were carried out with mitochondria prepared from the livers of rats of the inbred strain R-Amsterdam. Prolonged feeding of 2-FAA did not produce liver tumours in these rats[9]. The mitochondria were resistant to the action of 2-FAA (P:O ratio's obtained with L-glutamate: 2.2).

It was found, in accordance with KIELLEY's results[1], that the inhibitory effect of 2-FAA on the oxidation of DL-β-hydroxybutyrate (BHB) by mouse liver mitochondria was less than that on L-glutamate.

Summarizing, the following conclusions may be drawn:

(1) 2-FAA is active in releasing the bound DPN of the L-glutamate dehydrogenase from isolated mouse liver mitochondria, but is less active towards the bound DPN of the BHB-dehydrogenases. This may mean that DPN is more loosely bound to the one apoenzyme than to the other, or that the enzymes are located differently. A similar conclusion with regard to the DPN of the BHB-dehydrogenases as compared with the DPN of the pyruvate oxidase system has been reached along other lines[7].

(2) Certain preparations of mouse liver mitochondria are more sensitive to the action of 2-FAA than others, indicating that, *in vitro*, the DPN is bound more tightly to the latter than to the former mitochondria. Species and strain differences may also exist in this respect.

(3) Since DPN is involved in the inhibition produced by 2-FAA, oxidations not dependent upon this coenzyme, *e.g.* succinate oxidation, are not affected by 2-FAA.

Experiments on the DPN content of mitochondria isolated from animals receiving 2-FAA are being carried out at the moment.

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[1] R. K. KIELLEY, *Biochim. Biophys. Acta*, 21 (1956) 574.
[2] L. ERNSTER, *Exptl. Cell Research*, 10 (1956) 704.
[3] R. ZETTERSTRÖM AND L. ERNSTER, *Nature*, 178 (1956) 1335.
[4] F. E. HUNTER, Jr., J. DAVIS AND L. CARLAT, *Biochim. Biophys. Acta*, 20 (1956) 237.
[5] C. CARRUTHERS, V. SUNTZEFF AND P. N. HARRIS, *Cancer Research*, 14 (1954) 845.
[6] P. EMMELOT AND P. J. BROMBACHER, *Biochim. Biophys. Acta*, 22 (1956) 487.
[7] P. EMMELOT AND C. J. BOS, *Enzymologia* (in the press).
[8] C. E. WENNER AND S. WEINHOUSE, *Cancer Research*, 13 (1953) 21.
[9] R. VAN NIE, personal communication.

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Note added: The liver carcinogens *o*-aminoazotoluene (mouse) and *p*-monomethylaminoazobenzene (rat), and the non-carcinogen *p*-aminoazobenzene have recently been found to behave similarly to 2-FAA in a phosphorylating system of mouse liver mitochondria. The resulting inhibition of the oxidation of glutamate was also completely counteracted in the presence of DPN. The powerful liver carcinogen (rat) *p*-dimethylaminoazobenzene was inactive, but this might have been due to its insolubility under the conditions of the present experiments.

While this note was in the press, KIELLEY, *Biochim. Biophys. Acta*, 23 (1957) 447, reported also that 2-FAA behaves as a powerful competitor of the DPN of the glutamate dehydrogenase.