Effects shown by hepatocarcinogens on isolated liver mitochondria

KIELLEY[1] has recently reported that certain carcinogens of the aminofluorene series inhibited the oxidation of glutamate in a phosphorylating system of rat liver mitochondria.

We have shown[2] that N-2-fluorenylacetamide (2-FAA) exerted a still greater inhibition on the oxidation of L-glutamate by mouse liver mitochondria than that reported by KIELLEY for rat liver mitochondria. In the course of this investigation it was found that the inhibition of glutamate oxidation in a phosphorylating system of mouse liver mitochondria was completely prevented when the suspensions were supplemented with diphosphopyridine nucleotide (DPN). KIELLEY has lately reported[3] that N-2-fluorenyl-diacetamide behaves also as a powerful competitor of the DPN of the glutamate dehydrogenase.

As already briefly mentioned[2] the aminofluorenes are by no means the only compounds which show this behaviour. Table I illustrates that the hepatocarcinogens *o*-aminoazotoluene (*o*-AAT) and *p*-monomethylaminoazobenzene (*p*-MAB) and the "non"-carcinogen *p*-aminoazobenzene (*p*-AB)* are similarly active. Experiments with *p*-dimethylaminoazobenzene (*p*-DAB) were inconclusive owing to the insolubility of this carcinogenic azo dye. The inhibition displayed by the compounds on the glutamate oxidation of rat liver mitochondria was somewhat less than on that of mouse liver mitochondria.

TABLE I

INHIBITION OF THE GLUTAMATE OXIDATION OF MOUSE LIVER MITOCHONDRIA BY *o*-AMINOAZOTOLUENE, *p*-MONOMETHYLAMINOAZOBENZENE AND *p*-AMINOAZOBENZENE, AND THE COUNTERACTION BY DPN

Experimental conditions as described[1,2]. In these and the following experiments, 0.02 ml of an alcoholic solution of the carcinogen ($1.2 \cdot 10^{-2}$ *M*) was added to the flasks. Total fluid volume of flasks, 1.6 ml; final concentration of carcinogen, $1.5 \cdot 10^{-4}$ *M*. The controls always received 0.02 ml 95% ethanol. Incubation during 20 min with sucrose mitochondria from 250 mg fresh liver at 27° C.

Additions	*Oxygen uptake µatoms*	*P:O*
None	8.9	1.5
o-AAT	4.4	1.9
o-AAT + DPN	8.9	1.6
p-MAB	3.4	2.1
p-MAB + DPN	8.3	1.6
p-AB	4.9	1.7
p-AB + DPN	9.0	1.4

TABLE II

OXIDATION OF L-GLUTAMATE AND ACCOMPANYING PHOSPHORYLATION BY SUCROSE AND SUCROSE-EDTA MITOCHONDRIA FROM MOUSE LIVER IN THE PRESENCE OF *o*-AMINOAZOTOLUENE WITHOUT OR WITH DPN ADDED

For experimental conditions see Table I. Mitochondria were isolated in 0.25 *M* sucrose and in 0.25 *M* sucrose containing 0.001 *M* EDTA.

Additions	*Mitochondria isolated in:*			
	Sucrose		*Sucrose-EDTA*	
	Oxygen uptake (µatoms)	*P:O*	*Oxygen uptake (µatoms)*	*P:O*
None (control)	7.7	1.6	5.6	2.0
o-AAT	3.0	1.8	5.5	1.5
o-AAT + DPN	7.0	1.6	5.7	1.5
None (control)	7.3	1.4	4.0	2.1
o-AAT	4.3	1.7	3.0	1.4
o-AAT + DPN	7.3	1.5	4.1	1.8

*This compound is listed as very weakly carcinogenic by KIRBY AND PEACOCK[4].

In the above experiments mitochondria were isolated in 0.25 *M* sucrose. We had found earlier[5] that the inclusion of ethylenediaminetetraacetate (EDTA) in the medium used for preparing mitochondria from spontaneous mouse hepatomas, protected against the loss of DPN from the particles, since, in contrast to the sucrose mitochondria, the sucrose-EDTA mitochondria from these tumors oxidized DL-β-hydroxybutyrate in the absence of added DPN. Therefore, experiments were also carried out to investigate the effect of *o*-AAT on liver mitochondria isolated in 0.25 *M* sucrose containing 0.001 *M* EDTA. Table II shows that the sucrose-EDTA mitochondria (controls) oxidize L-glutamate at a slower rate than the sucrose mitochondria, but that the P:O ratios of the former are higher than those obtained with the latter (fluoride, hexokinase and glucose were not present). However, whereas *o*-AAT inhibited the oxidation of the sucrose mitochondria and always enhanced the P:O ratio somewhat (compare also Table I), it had little if any effect on the oxidation of the sucrose-EDTA mitochondria and consistently depressed the oxidative phosphorylation by five- to seven-tenths of a unit. It appears that the extra phosphorylation brought about by the use of EDTA was uncoupled in the presence of *o*-AAT.

Subsequent experiments revealed that *o*-AAT, *p*-MAB, *p*-AB and 2-FAA ($1.5 \cdot 10^{-4}$ *M*) activated the latent "ATPase" of the mitochondria from mouse liver. Sucrose mitochondria incubated with ATP for 5 min at 27° C in tris(hydroxymethyl)aminomethane, pH 7.2, liberated 7–13 times as much inorganic phosphate in the presence of the carcinogens as in their absence. Isolation of the mitochondria in sucrose-EDTA gave the same ATPase activation by *o*-AAT. When the determinations were carried out in histidine buffer at pH 6.4 it was found that *o*-AAT stimulated phosphate release about 10-fold, *p*-MAB was almost half as active, but the effects of 2-FAA and *p*-AB were small. At pH 7.2 roughly the same pattern of activation was found except that *p*-MAB was somewhat less and *p*-AB somewhat more active.

Liver mitochondria incubated in the presence of carcinogen and DPN did not split the latter compound. The carcinogens thus do not provoke a DPNase activity in the mitochondria, which is in accordance with our earlier observation[2] that nicotinamide did not counteract the inhibition produced by 2-FAA on the oxidation of glutamate.

LEHNINGER and his associates[6] have shown that the uncoupling agent 2,4-dinitrophenol stabilizes the morphology of isolated rat liver mitochondria by inhibiting their spontaneous swelling. Thyroxine, on the other hand, induced a very marked swelling in these mitochondria. Since we have shown[7] that mitochondria isolated from primary hepatomas of the mouse (strain CBA, *o*-AAT) and the rat (strain R-Amsterdam, *p*-DAB) did not show the latter response, it was thought interesting to study the effect of the carcinogens alone and in combination with thyroxine on the morphology of mouse liver mitochondria. It was found that addition of *o*-AAT or *p*-MAB ($1.5 \cdot 10^{-4}$ *M*) caused the mitochondria to swell. Although a $3 \cdot 10^{-5}$ *M* concentration of thyroxine was twice as active as the carcinogens*, the combination carcinogen plus thyroxine was not more active than the carcinogen alone. Thus in the presence of carcinogen the complete swelling ordinarily induced by thyroxine is prevented. In this connection it is of interest to compare the results of previous experiments[7] in which it was found that liver mitochondria, deprived of ATP and DPN through the action of tumor mitochondria, did not respond any more to thyroxine in terms of swelling.

In conclusion it may be stated that the hepatocarcinogens studied belong to the class of compounds that labilizes the biochemical structure of liver mitochondria *in vitro*.

Tentatively the following series of events is suggested: the carcinogens interact with and partly eliminate a factor governing the structural integrity of the mitochondria, resulting in swelling of the particles, dislocation of the DPN and activation of the latent ATPase. This interpretation would also fit in with the failure to observe a prominent effect of N-2-fluorenyldiacetamide on crystalline glutamic dehydrogenase[3].

Finally we want to stress that the present observations do not necessarily imply that the mitochondria are the prime targets on which the carcinogens are acting while inducing a neoplastic response in the living cell.

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Received March 5th, 1957

* Unpublished experiments suggest that thyroxine may have an even stronger effect than the carcinogens in dislocating the bound DPN from the mitochondrial structures.