Preliminary Notes

The formation *in vitro* of protein-bound derivatives of aminoazo dyes by rat liver and its enhancement by benzpyrene pretreatment*

Previous studies have demonstrated protein-bound dye in the livers of rats fed various dyes related to the hepatocarcinogen 4-dimethylaminoazobenzene (DAB) and have indicated that these metabolites have a role in this carcinogenic process[1,2]. This communication reports on the enzymic formation *in vitro* of protein-bound derivatives of aminoazo dyes, principally 3'-methyl-4-monomethylaminoazobenzene (3'-methyl-MAB). Slices from normal adult rat liver incubated with 3'-methyl-MAB formed twice as much protein-bound dye as slices or homogenates from the livers of normal 3-week old rats. Liver slices or homogenates from 3-week old rats injected 20 h previously with 1 mg 3,4-benzpyrene bound about 5 times as much dye as the untreated controls (Table I).

TABLE I

FORMATION OF PROTEIN-BOUND DYE IN RAT-LIVER PREPARATIONS

Liver preparation	*Benzpyrene pretreatment*	*Cofactors added*	*Protein-bound dye, extinction/50 mg protein*
Homogenate	—	DPN, TPN	0.04
	+	DPN, TPN	0.24
	+	—	0.03
Microsome	+	DPN, TPN	0.19
	+	DPNH, TPNH	0.33

Each flask contained preparations from 500 mg of liver from 3-week old rats in a final volume of 5 ml of 0.154 *M* Krebs-Ringer bicarbonate buffer, pH 7.4. Where indicated, 2.5 μmoles each of the pyridine nucleotides were added together with 120 μmoles nicotinamide. 2 μmoles 3'-methyl-MAB were added last in 0.2 ml acetone; the flasks were incubated 3 h at 37° under 95% O_2–5% CO_2. The reaction was stopped by the addition of 3 vol. acetone. The proteins were extracted exhaustively and analyzed[1]; the extinction of the dye in 1.5 ml of ethanolic HCl was determined at 515 mμ. 3,4-Benzpyrene (1 mg) was injected intraperitoneally in 0.25 ml corn oil 20 h before assay.

This pretreatment with hydrocarbon has been shown to induce the synthesis of the azo dye N-demethylase and reductase systems of rat liver[3]. No activity was found when N_2 replaced the O_2 in the gas phase or when the liver preparations were heated for 5 min at 100°. Homogenates required diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) for maximum activity. Microsomes had higher activity when fortified with TPNH and DPNH than with TPN and DPN.

With homogenates the amount of bound dye formed was almost as great with 3'-methyl-4-aminoazobenzene (3'-methyl-AB) as with 3'-methyl-MAB; 3'-methyl-DAB yielded only one-half as much. 60 to 70% as much protein-bound dye was formed from MAB and AB as from their 3'-methyl derivatives. No bound dye was formed from 3-hydroxy-AB. The absorption maximum in ethanolic HCl of the bound dye formed *in vitro* from 3'-methyl-MAB is 505 mμ as compared to 513 mμ for the parent dye and 524 mμ for the bound dye formed *in vivo*[4]. Each of these dyes is yellow in neutral or alkaline media. Further comparative studies of the protein-bound dyes formed *in vivo* and *in vitro* are in progress.

HULTIN[5] recently reported the binding of ^{14}C to protein in liver homogenates or microsomes incubated with MAB labeled with ^{14}C in the aniline ring. Two of the requirements for binding in Hultin's system (microsomes and TPNH) are also necessary for the reduction of the azo linkage[6] and the *para*hydroxylation of the aniline formed[7,8]. Thus, binding of aniline or a derivative thereof may have occurred in these preparations. Therefore, we incubated labeled aniline, MAB, or DAB[9] with liver microsomes. The amount of protein-bound ^{14}C from aniline was two to three times that obtained with either dye (Table II). Since less than one-half of the dye was reduced, more aniline should be bound if it is a closer precursor. The formation of protein-bound ^{14}C under these conditions thus cannot be equated with the formation of protein-bound aminoazo dye. In contrast, the above experiments based on the characteristic pink color of aminoazo dyes in

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TABLE II

THE INCORPORATION INTO RAT-LIVER MICROSOMES OF ^{14}C FROM LABELED DAB, MAB, AND ANILINE

Labeled compound	Specific activity (counts/min/mg protein)	
	Unincubated flasks	20 min incubation
[2′:3′:5′:6′-$^{14}C_4$] DAB	0	11
[2′:3′:5′:6′-$^{14}C_4$] MAB	0	20
[2:3:5:6-$^{14}C_4$] Aniline	3	40

Each flask contained the microsomes from 240 mg of normal adult rat liver, 3 μmoles TPNH, and 0.25 μmole of labeled compound ($4.6 \cdot 10^5$ counts/min/flask). Other flask additions and treatments were those described by HULTIN[5].

acid solution[4] unequivocally demonstrate the enzymic formation of protein-bound dye by rat liver preparations.

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Beeinflussung der Tumorglykolyse durch Mitochondrien verschiedenen Ursprungs

Bei der Untersuchung von Stoffwechselvorgängen an Embryonen unter anaeroben Bedingungen zogen wir zum Vergleich auch den Ehrlich-Ascitestumor der Maus heran. Dieser Tumor hat wie andere Tumoren nach WARBURG u. Mit.[1] eine grosse aerobe und anaerobe Glykolyse. Embryonen bilden dagegen nur unter anaeroben Bedingungen grössere Mengen Milchsäure[2,3].

In weiteren Versuchen wurde die Glykolyse verschiedener Zellfraktionen aus Ascitestumor, 3½ Tage alten Hühnerembryonen und Mäuseleber gemessen. Nach dem Homogenisieren der Gewebe im Potter-Elvehjem-Homogenisator mit isotoner Saccharoselösung wurden durch fraktioniertes Zentrifugieren aus dem Homogenat die Mitochondrien und nach dem Abzentrifugieren aller Strukturbestandteile die Überstände gewonnen. Die Ausbeute an Mitochondrien war bei den einzelnen Geweben verschieden. Bei Leber war sie etwa 4 × grösser als beim Ascitestumor und bei Hühnerembryonen. — Die Fraktionen wurden einzeln oder in verschiedenen Kombinationen für die Untersuchung verwendet. Die Versuchsansätze enthielten vom Tumorüberstand die ursprünglich in 100 mg intakter Zellen enthaltene Menge (= 0.1 g-Äquivalent), von den Mitochondrien die in 100–600 mg enthaltene Menge (= 0.1–0.6 g-Äquivalente)*. Ausserdem wurden $3 \cdot 10^{-3}$ *M* $MgCl_2$, $1 \cdot 10^{-3}$ *M* ATP**, 0.6–$1.0 \cdot 10^{-3}$ *M* DPN**, $2 \cdot 10^{-3}$ *M* Phosphat, $0.5 \cdot 10^{-4}$–$1.0 \cdot 10^{-2}$ *M* Nicotinsäureamid, $2.5 \cdot 10^{-2}$ *M* $KHCO_3$ (für Atmungsversuche KCl) zugefügt und die Lösung durch Zugabe von Saccharose isoton gemacht. Die Versuche wurden unter aeroben Bedingungen ausgeführt. (Gasraum 5 % CO_2/Luft). Das Substrat war entweder Glucose ($1 \cdot 10^{-2} M$) oder K-Hexosediphosphat ($1 \cdot 10^{-2} M$). Gesamtvolumen 2.2 ml.

Bei allen Versuchen wurden die Druckänderungen im Warburg-Apparat gemessen und die Reaktion dann durch Kühlen abgestoppt. Nach Abzentrifugieren der Mitochondrien und Ent-

* 0.1 g-Äquivalent = Menge an Überstand bzw. an Mitochondrien enthalten in 100 mg intakter Zellen.

** ATP = Adenosintriphosphat; DPN = Diphosphopyridinnucleotid; ADH = Alkoholdehydrogenase.