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INHIBITION BY 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE OF *IN VITRO* CELL-FREE PROTEIN SYNTHESIS: POSSIBLE INVOLVEMENT OF AN ELECTROPHILIC METABOLITE

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Abstract—*In vitro* addition of the hepatocarcinogen 3′-Me-DAB‡ to a cell-free protein synthesizing system using PMS from rat liver resulted in a marked inhibition of [^{14}C]leucine incorporation. The inhibitory effect was dependent on the carcinogen being preincubated in the system in the presence of NADP$^+$. NADP$^+$ alone caused a marked stimulation of [^{14}C]leucine incorporation. Both xenobiotic metabolism and the 3′-Me-DAB inhibitory effect were decreased by Triton X-100 treatment of the PMS and increased by phenobarbitone pretreatment of the rats. The NADP$^+$ stimulatory effect on protein synthesis was doubled by Triton X-100 treatment and decreased by phenobarbitone pretreatment. The results indicate that the effects of 3′-Me-DAB and NADP$^+$ on protein synthesis may occur via independent mechanisms, and suggest that the 3′-Me-DAB effect is mediated by a metabolite. This metabolite appears to be electrophilic since *in vitro* addition of the nucleophiles GSH and L-cysteine decreased the inhibitory effect of 3′-Me-DAB on protein synthesis. There was no correlation between the inhibitory effect of a series of azo dyes (3′-Me-DAB, 2′-Me-DAB, 2-Me-DAB and DAB) on *in vitro* protein synthesis and their respective carcinogenic potencies.

In vivo administration to rats of a number of hepatocarcinogens leads to a marked impairment of liver protein synthesis [1–8]. General opinion suggests that in most cases this inhibitory effect is mediated by electrophilic metabolites of the carcinogens [9–11]. Few studies, however, have been reported to date directly verifying this proposal.

A recent preliminary report from this laboratory [12] showed that the addition of the aminoazo carcinogen 3′-Me-DAB to an *in vitro* cell-free protein synthesizing system, using PMS from rat liver, resulted in a marked inhibition of amino acid incorporation. The inhibitory effect on protein synthesis, however, was dependent on the concurrent presence of the cofactors NADP$^+$ and G-6-P in a preincubated medium. Since these two cofactors are also required for xenobiotic metabolism [13], the results suggested that a dye metabolite was involved in the inhibitory effect.

The two cofactors, however, almost doubled the level of [^{14}C]leucine incorporation into protein as compared with the incorporation level in the control system [12]. This stimulatory effect of the cofactors on protein synthesis was subsequently shown to be due to the apparent prevention by NADP$^+$ of the degradation of an unidentified factor involved in protein synthesis (data to be published). 3′-Me-DAB could possibly interact with, and impair the activity of, this factor.

A further possible explanation for the inhibitory action of the carcinogen is that involvement of NADP (as NADPH) in microsomal metabolism of the carcinogen might limit the availability of the dinucleotide for its apparent protective role in protein synthesis as previously mentioned.

Studies have been undertaken to determine which of these possible mechanisms would explain the inhibitory effect of 3′-Me-DAB on protein synthesis. Experiments were carried out using postmitochrondrial supernatant obtained from phenobarbitone pretreated rats, a treatment known to induce the microsomal drug metabolizing enzymes [14], or using Triton X-100 solubilized PMS, which destroys such enzymes [15–17]. The results suggest that the inhibitory effect of 3′-Me-DAB on *in vitro* protein synthesis is initiated by an electrophilic dye metabolite. No correlation could be made between the inhibitory effect on *in vitro* protein synthesis and the carcinogenic potency of a series of structurally related dye analogues.

MATERIALS AND METHODS

Reagents. 3′-Me-DAB (m.p. 119–121°) and 2′-Me-DAB (technical grade) were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. The 2′-Me-DAB (m.p. 66–67°) was purified by chromatography on alumina and recrystallization from methanol. 2-Me-DAB (m.p. 66–68°) and DAB (m.p. 117–118°) were gifts from Dr. Jill Blunck. L-cysteine and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and GSH from

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‡ The following abbreviations are used: 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene; 2′-Me-DAB, 2′-methyl-4-dimethylaminoazobenzene; 2-Me-DAB, 2-methyl-4-dimethylaminoazobenzene; DAB, 4-dimethylaminoazobenzene; PMS, postmitochondrial supernatant; G-6-P, glucose 6-phosphate; GSH, reduced glutathione; Hepes, [2-(*N*-2-hydroxyethylpiperazin-*N*′-yl)-ethanesulphonic acid].

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Calbiochem, San Diego, CA, U.S.A. All other reagents were either as previously described [12] or of analytical grade.

Animals. Random bred male Sprague–Dawley rats (200–250 g) were obtained from the colony maintained in this Institution. IRM pellets (supplied by the Victorian Wheat-growers Corp. Ltd., Nth. Melbourne) and water were available *ad lib.* to the rats until killing. Where indicated, rats were pretreated daily with sodium phenobarbitone (90 mg/kg in 0.9% NaCl) by i.p. injection for three consecutive days. The last injection was administered 24 hr prior to sacrifice. Contol rats were injected with normal saline.

Preparation of postmitochondrial supernatant. All rats were routinely sacrificed by a blow to the head and exsanguinated between 1100 and 1200 hr, thus minimizing diurnal variation. Livers were removed and weighed, and 6 g samples added to 12 ml of homogenizing buffer (5 mM $MgCl_2$, 50 mM KCl, 50 mM Hepes and 0.25 M sucrose, pH 7.3 at 20°). The samples were homogenized with eight passes of a Potter–Elvehjem homogenizer with a teflon pestle. These homogenates were centrifuged at 20,000 g_{max} for 8 min in a Sorvall RC2-B centrifuge and the resultant supernatant, the PMS, used within 15 min.

In experiments using Triton X-100, an 11% (w/v) solution of the detergent was prepared in homogenizing buffer. This was then diluted with a suitable volume of PMS to give a final Triton X-100 concentration of 1% (w/v). Control PMS was likewise diluted with homogenizing buffer. The homogenates were then kept ice-cold with occasional shaking for 2 hr, according to the procedure outlined by Koffer [18].

Amino acid incorporation studies. Two incubation mixtures were prepared. The first of these, referred to as Mixture I (preincubation medium), contained in a final volume of 1.0 ml: 5 mM $MgCl_2$, 0.1 M Hepes buffer (pH 7.8, 20°) and 0.2 ml of PMS. Other optional additions, where indicated in the Results section included the appropriate aminoazo dye (240 nmole) in 20 μl of ethanol with the corresponding controls receiving ethanol only, $NADP^+$ (1 or 2 μmole), G-6-P (5 μmole), GSH (1 μmole), L-cysteine (1 μmole) or dithiothreitol (1 μmole).

Mixture I was incubated for 15 min at 37° under air. Samples were then chilled and added to 1.0 ml of a second mixture (Mixture II) which contained: 5 mM $MgCl_2$, 140 mM KCl, 4 mM ATP, 20 mM phosphoenolpyruvate, pyruvate kinase (100 μg/ml), 8.92 μM L-[1-^{14}C]leucine (0.5 μCi), 0.1 M Hepes buffer (pH 7.8, 20°) and 0.1 ml of PMS. The 2.0 ml of combined mixture (Mixture III) was similar in composition to that described by Richardson *et al.* [19] as being optimal for [^{14}C]amino acid incorporation in a PMS system. GSH (1 μmole), L-cysteine (1 μmole) or dithiothreitol (1 μmole) were added to Mixture II if these compounds had been present in Mixture I.

The combined mixture (Mixture III) was then incubated for 10 min at 37° under air. Incorporation of [^{14}C]leucine into acid-insoluble protein was terminated by the addition of 2.0 ml of ice-cold 10% (w/v) trichloroacetic acid containing 0.1% (w/v) unlabelled leucine. The precipitated protein was washed, dried and dissolved in 1 M NaOH, and samples were then assayed for radioactivity in a liquid scintillation counter [12]. The incorporated activity was expressed on the basis of the RNA content of the final volume of PMS present in Mixture III (0.3 ml). RNA was determined by the Schmidt–Thannhauser method as described by Blobel and Potter [20].

Drug metabolizing enzyme studies. Xenobiotic metabolizing activity in Mixture I was investigated by assaying aminopyrine *N*-demethylase, aniline *p*-hydroxylase and 3′-Me-DAB reductase. The composition of Mixture I was as described above [5 mM $MgCl_2$, 20 μl ethanol, 0.1 M Hepes buffer (pH 7.8, 20°) and 0.2 ml PMS, final volume 1.0 ml], but contained the additions $NADP^+$ (1 μmole) and G-6-P (5 μmole) where indicated.

(*a*) *Aminopyrine N-demethylase.* Samples of Mixture I containing aminopyrine (2 μmole) and semicarbazide HCl (4 μmole) were incubated for 15 min at 37° under air. Formaldehyde production was measured by a modification of the Nash method as described by Cochin and Axelrod [21].

(*b*) *Aniline p-hydroxylase.* Samples of Mixture I containing aniline HCl (2 μmole) were incubated for 15 min at 37° under air. Production of *p*-aminophenol was measured by the method outlined by Mazel [22].

(*c*) *3′-Me-DAB reductase.* Samples of Mixture I containing 3′-Me-DAB (240 μmole) were incubated for 15 min at 37° under air. The amount of dye with an intact azo linkage remaining at the end of incubation was measured by the method described by Mueller and Miller [23].

The results for the three enzyme activities were expressed as nmole product formed/g wet wt liver/15 min.

RESULTS

In agreement with our previous report [12], the addition of the cofactors $NADP^+$ and G-6-P to Mixture I was found to virtually double the basal incorporation of [^{14}C]leucine into protein in Mixture III (from $16.94 \pm 0.53 \times 10^3$ cpm/mg RNA to $29.15 \pm 0.70 \times 10^3$ cpm/mg RNA, n = 46) (Fig. 1). This effect was attributable to $NADP^+$.

The addition of 3′-Me-DAB to Mixture I in the absence of the two cofactors, on the other hand, resulted in a slight inhibition of the basal level of [^{14}C]leucine incorporation (Fig. 1). If $NADP^+$ and G-6-P were present, however, the extent of this 3′-Me-DAB inhibitory effect was markedly increased. This effect was also shown to be attributable to the presence of $NADP^+$. G-6-P had no significant modifying action on either the $NADP^+$ stimulatory effect or the 3′-Me-DAB inhibitory effect on [^{14}C]leucine incorporation.

The xenobiotic metabolizing capacity of Mixture I was also dependent on supplementation of the system with exogenous $NADP^+$ (Fig. 2). Only in the instance of aniline *p*-hydroxylase activity did the inclusion of G-6-P cause a significant, though small, decrease in $NADP^+$-directed metabolism.

The addition of exogenous $NADP^+$ to Mixture I therefore appears to be the sole requirement for (a) the $NADP^+$ stimulatory effect on amino acid incorporation in Mixture III, (b) the 3′-Me-DAB inhibitory effect on [^{14}C]leucine incorporation in Mixture III and (c) xenobiotic metabolism in Mixture I.

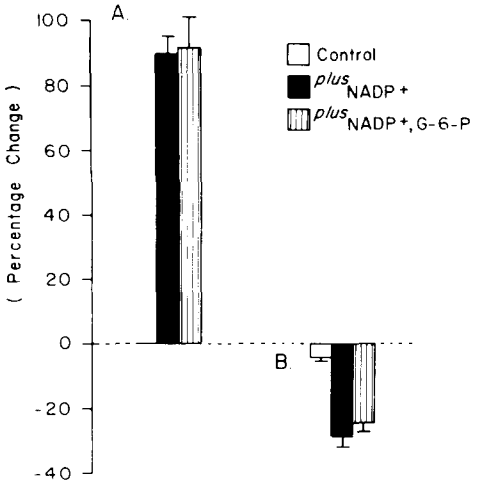

Fig. 1. The stimulatory effect of $NADP^+$ and the inhibitory effect of 3′-Me-DAB on protein synthesis in Mixture III using a cell-free liver system. A. Percentage stimulation from control. B. Percentage inhibition by 3′-Me-DAB. [^{14}C]Leucine incorporation into protein in Mixture III (i.e. Mixtures I and II) was measured as outlined in the Materials and Methods section. $NADP^+$ (1 μmole) and G-6-P (5 μmole) were added to Mixture I where indicated. 3′-Me-DAB (240 nmole) was added to Mixture I in the inhibitory studies (B). Results shown are the mean ± S.E.M. values obtained from 10 separate experiments, each carried out in duplicate.

The addition of 2 μmole $NADP^+$ to Mixture I instead of 1 μmole, as normally added, did not increase the magnitude of either the $NADP^+$ stimulatory effect or the 3′-Me-DAB inhibitory effect on protein synthesis in Mixture III (Table 1); the $NADP^+$ stimulatory effect was, in fact, slightly decreased. $NADP^+$ addition at these concentrations was therefore not a rate limiting factor in either effect. It is thus unlikely that the azo dye competes for $NADP^+$ with the factor necessary for stimulation of protein synthesis, a postulated mechanism for the inhibition of protein synthesis by the azo dye.

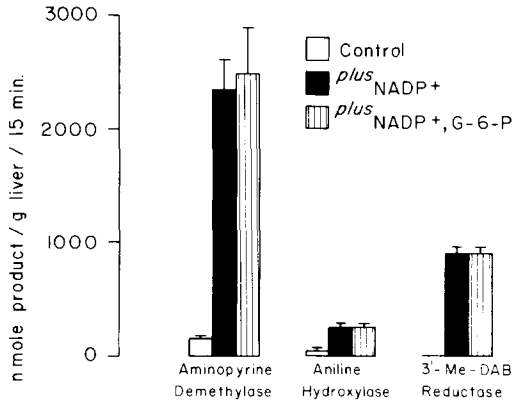

Fig. 2. The effect of $NADP^+$ and G-6-P on xenobiotic metabolism in Mixture I using a cell-free liver system. The metabolic activities of the 3 enzymes tested in Mixture I were assayed as outlined in the Materials and Methods section. $NADP^+$ (1 μmole) and G-6-P (5 μmole) were added where indicated. Results shown are the mean ± S.E.M. values obtained from a minimum of 3 separate experiments, each carried out in duplicate.

Table 1. The effect of doubling the concentration of $NADP^+$ on the $NADP^+$ stimulatory effect and the 3′-Me-DAB inhibitory effect on protein synthesis in Mixture III using a cell-free liver system*

$NADP^+$ (μmole)	Per cent stimulation by $NADP^+$	Per cent inhibition by 3′-Me-DAB
1.0	110.8 ± 9.1	23.3 ± 3.0
2.0	101.0 ± 8.5†	23.7 ± 1.4

* [^{14}C]Leucine incorporation into protein in Mixture III (i.e. Mixtures I and II) was measured as outlined in the Materials and Methods Section. $NADP^+$ (1 or 2 μmole) and G-6-P (5 μmole) were added to Mixture I in all cases. 3′-Me-DAB (240 nmole) was included only in the 3′-Me-DAB inhibitory studies. Results are the mean ± S.E.M. values obtained from 3 separate experiments, each carried out in duplicate.

† $P < 0.001$ from group containing 1 μmole $NADP^+$ (paired student's t-test).

Solubilization of the PMS with the nonionic detergent Triton X-100 resulted in a marked decrease in the basal level of [^{14}C]leucine incorporation into protein [from 18.33 ± 1.22 × 10^3 cpm/mg RNA to 5.22 ± 0.42 × 10^3cpm/mg RNA (n = 3)]. The 3′-Me-DAB inhibitory effect on [^{14}C]leucine incorporation in Mixture III was also markedly lessened in Triton X-100 treated PMS (Fig. 3), as were the activities of the three drug metabolizing enzymes assayed in Mixture I (Fig. 4). The $NADP^+$ stimulatory effect on [^{14}C]leucine incorporation in Mixture III, on the

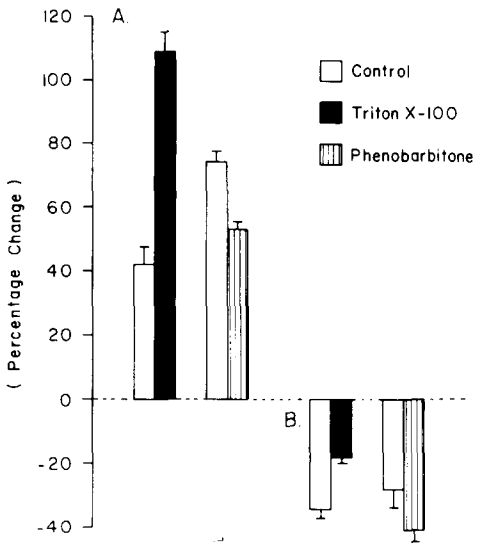

Fig. 3. The effect of Triton X-100 and phenobarbitone pretreatment on the $NADP^+$ stimulatory effect and the 3′-Me-DAB inhibitory effect on protein synthesis in Mixture III using a cell-free liver system. A. Percentage stimulation by $NADP^+$. B. Percentage inhibition by 3′-Me-DAB. Triton X-100 treated PMS and PMS obtained from phenobarbitone pretreated rats were prepared as outlined in the Materials and Methods section. [^{14}C]leucine incorporation into protein in Mixture III (i.e. Mixtures I and II) was measured according to the method also described in this section. $NADP^+$ (1 μmole) was added to Mixture I in all cases and 3′-Me-DAB (240 nmole) only in the inhibitory studies (B). The results shown are the mean ± S.E.M. values obtained from a minimum of 3 experiments each carried out in duplicate.

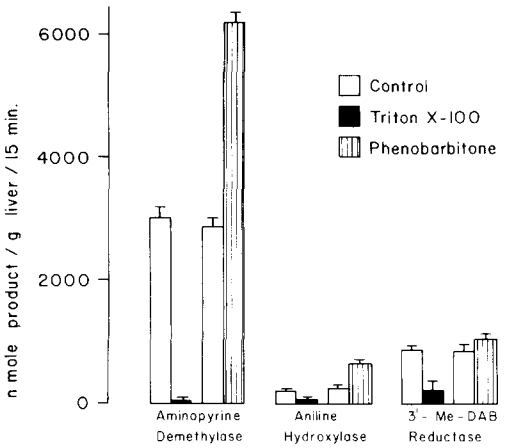

Fig. 4. The effect of Triton X-100 and phenobarbitone pretreatment on xenobiotic metabolism in Mixture I using a cell-free liver system. Triton X-100 treated PMS and PMS obtained from phenobarbitone pretreated rats were prepared as outlined in the Materials and Methods section. The metabolic activities of the three enzymes tested in Mixture I were assayed according to the methods also described in this section. $NADP^+$ (1 μmole) and G-6-P (5 μmole) were added in all cases. Results shown are the mean ± S.E.M. values obtained from a minimum of 3 separate experiments, each carried out in duplicate.

other hand, was more than doubled by Triton X-100 treatment. The effects of 3′-Me-DAB and $NADP^+$ on leucine incorporation therefore do not appear to be directly related.

Pretreatment of rats with the microsomal drug metabolizing enzyme inducer phenobarbitone resulted in an opposite pattern of results. The basal level of [^{14}C]leucine incorporation into protein was slightly, but significantly enhanced in the phenobarbitone pretreated rats [from $16.81 \pm 1.73 \times 10^3$ cpm/mg RNA to $21.67 \pm 1.56 \times 10^3$ cpm/mg RNA (n = 7)]. No significant difference in RNA content/g liver could be detected between the two groups. The 3′-Me-DAB inhibitory effect on [^{14}C]leucine incorporation in Mixture III (Fig. 3) and the aminopyrine demethylase and aniline hydroxylase activities in Mixture I (Fig. 4) were similarly enhanced by this pretreatment. The 3′-Me-DAB reductase activity was also increased, but not to a significant extent (Fig. 4). The $NADP^+$ stimulatory effect on [^{14}C]leucine incorporation in Mixture III, on the other hand, was significantly decreased in the PMS obtained from phenobarbitone pretreated rats (Fig. 3). Once again, it appears that the effects of 3′-Me-DAB and $NADP^+$ on leucine incorporation are not directly related. The results also support the involvement of a metabolite of 3′-Me-DAB in the carcinogen's inhibitory action on *in vitro* protein synthesis.

Table 2. The effect of added nucleophiles on the inhibitory effect of 3′-Me-DAB on protein synthesis in Mixture III using a cell-free liver system*

Addition	Per cent inhibition by 3′-Me-DAB
None	41.9 ± 0.7
1 mM GSH	31.5 ± 1.1†
1 mM L-cysteine	11.2 ± 1.9†
None	42.8 ± 1.7
1 mM Dithiothreitol	43.9 ± 0.9

* [^{14}C]Leucine incorporation into protein in Mixture III (i.e. Mixtures I and II) was measured as outlined in the Materials and Methods section. $NADP^+$ (1 μmole), G-6-P (5 μmole) and 3′-Me-DAB (240 nmole) were added to Mixture I in all cases. GSH (1 μmole), L-cysteine (1 μmole) or dithiothreitol (1 μmole) were added to both Mixtures I and II where indicated. Results shown are the mean ± S.E.M. values obtained from 3 separate experiments, each carried out in duplicate.

† $P < 0.001$ from control (paired student's *t*-test).

Table 3. The inhibitory effect of a series of azo dye analogues on *in vitro* protein synthesis

Dye	Per cent inhibition of protein synthesis*	Carcinogenic potency†
DAB	43.4 ± 0.3‡	6
2-Me-DAB	38.5 ± 1.0	0
3′-Me-DAB	37.4 ± 1.1	10–12
2′-Me-DAB	30.1 ± 2.2‡	2–3

* [^{14}C]Leucine incorporation into protein in Mixture III (i.e. Mixtures I and II) was measured as outlined in the Materials and Methods section. $NADP^+$ (1 μmole), G-6-P (5 μmole) and the appropriate dye (240 nmole) were added to Mixture I. Results shown are the mean ± S.E.M. values obtained from 3 separate experiments, each carried out in duplicate.

† From Miller, Miller and Finger (24).

‡ $P < 0.001$ from the inhibitory value for 3′-Me-DAB (paired student's *t*-test).

Addition to both Mixtures I and II of either of the two nucleophiles GSH or L-cysteine resulted in a marked reduction in the magnitude of the 3′-Me-DAB inhibitory effect (Table 2). The addition of the nucleophile dithiothreitol, however, had no significant effect. The evidence therefore suggests that the postulated active metabolite of 3′-Me-DAB may be electrophilic in nature.

A comparison was made between the inhibitory effect on *in vitro* protein synthesis and the carcinogenic potency of a series of structurally related dye analogues (Table 3). There was no correlation between the two properties.

DISCUSSION

Of the co-factors added to Mixture I, $NADP^+$ alone is necessary for three effects: (a) the $NADP^+$ stimulatory effect on protein synthesis in Mixture III, (b) the 3′-Me-DAB inhibitory effect on protein synthesis in Mixture III and (c) xenobiotic metabolism in Mixture I. However, no direct indication as to the precise nature of dinucleotide involvement in each of these three effects can be obtained from the results, for $NADP^+$ can undergo either reduction or conversion to products such as NAD^+ and nicotinamide mononucleotide in biological systems.

Xenobiotic metabolism is well known to require the reduced form of $NADP^+$ in the microsomal electron transport chain [13]. A significant amount of $NADP^+$ reduction might therefore be taking place in the system used in the present study. The reduction

step is catalyzed by G-6-P dehydrogenase which is present in cell sap. Inhibition of *in vitro* protein synthesis by 3′-Me-DAB also appears to require the reduced form of $NADP^+$. This conclusion stems indirectly from our demonstration that the inhibitory effect of the azo carcinogen on protein synthesis is probably mediated by a dye metabolite.

The $NADP^+$ stimulatory effect, on the other hand, may be mediated by the oxidized form of the dinucleotide since studies by Kalkhoff *et al.* [25] and Kroon [26] have suggested the involvement of oxidized $NADP^+$ in *in vitro* protein synthesis.

Possible mechanisms of inhibition of *in vitro* protein synthesis by the aminoazo carcinogen 3′-Me-DAB include the assumption that the quantity of $NADP^+$ present in the system was rate limiting for the $NADP^+$ stimulatory effect on protein synthesis and that the metabolism of the carcinogen merely competed for the available $NADP^+$. At the concentrations added to Mixture I, the quantity of $NADP^+$ in the system, however, was shown not to be a rate-limiting factor for either the $NADP^+$ or 3′-Me-DAB effects on protein synthesis.

A second possible mechanism of inhibition is the involvement of a dye metabolite. Triton X-100 treatment of PMS affords a means of destroying xenobiotic metabolism [15–17] without affecting protein synthesis [18, 27, 28]. If a dye metabolite is responsible for the inhibitory effect on protein synthesis observed in our system, Triton X-100 should abolish the effect. Unfortunately we found in contrast with earlier work [18, 27, 28], that the basal level of [^{14}C]leucine incorporation into protein in our cell-free system was greatly decreased in the Triton-treated PMS. This discrepancy may be due to differences in tissue preparation, since the previous workers [18, 28] had used Sephadex-filtered PMS. The inhibitory effect of 3′-Me-DAB on *in vitro* protein synthesis was markedly lessened by the detergent treatment. However, this effect could be due to either decreased metabolic activation of the dye or to a decrease in available target for the dye as a result of the solubilization process.

A further means of investigating the possible role of a 3′-Me-DAB metabolite in the mechanism of the inhibition of amino acid incorporation is by the use of PMS obtained from phenobarbitone pretreated rats. Phenobarbitone pretreatment is well known to induce the metabolism of xenobiotics in general [14, 17] and more specifically in the present context, the oxidative metabolism of the aminoazo dyes [29–31]. The azo reductase pathway for the dyes, however, is not induced by phenobarbitone pretreatment [29, 30, 32]. We confirmed some of these findings in the present study. This inductive effect is a result of *de novo* synthesis of drug metabolizing enzymes and is reflected by increased rates of *in vitro* amino acid incorporation as reported here and by others [33–35]. The extent of the 3′-Me-DAB inhibitory effect was enhanced by phenobarbarbitone pretreatment of rats. However, the stimulation could be due to either increased metabolic activation of the dye or an increase in target available to the dye as a result of *de novo* protein synthesis.

In summary, therefore, both the 3′-Me-DAB inhibitory effect and xenobiotic metabolism are dependent on supplementation of the system with $NADP^+$. Both are decreased by Triton X-100 solubilization of the PMS, and both are enhanced by phenobarbitone pretreatment of the rats. The evidence therefore suggests that the 3′-Me-DAB inhibitory effect is mediated by a metabolite of the carcinogen. Further evidence for this proposal is the finding that the *in vitro* addition of the nucleophiles GSH and L-cysteine results in a marked decrease in the extent of the 3′-Me-DAB inhibitory effect.

Both GSH and L-cysteine have been proposed to play a fundamental role in protecting body tissues from the detrimental effects of many carcinogens and toxins [36–38]. By virtue of their free sulphydryl groups, these nucleophiles can interact with the electrophilic metabolites of the carcinogens and toxins, and thus prevent the binding of such electrophiles to cellular proteins and nucleic acids [39–42]. Since 3′-Me-DAB itself is nucleophilic, the result obtained in the present study further support the concept that a dye metabolite is involved in the inhibitory effect on protein synthesis; this metabolite presumably being electrophilic. Dithiothreitol, on the other hand, had no effect on the 3′-Me-DAB inhibitory effect. This nucleophile has been previously shown to inhibit the binding of 2-acetylaminofluorene to transfer RNA [43]. Kadlubar *et al.* [44] have also shown it to be oxidized, as are cysteine and GSH, by *N*-hydroxy-4-monomethylaminoazobenzene, the proposed proximate carcinogen for the aminoazo dyes. The reason for the lack of effect of dithiothreitol in the present study is not known.

The precise nature of the electrophilic metabolite (ultimate carcinogen) responsible for the toxic and carcinogenic properties of the aminoazo dyes has not been established to date. The general consensus of opinion suggests this molecule to be a conjugate of the *N*-hydroxylated secondary amine [9–11]. Very recent studies by Kadlubar *et al.* [44, 45] have shown that such an eletrophilic azo dye metabolite can be generated *in vitro* by rat hepatic microsomes, the *N*-hydroxylation being catalyzed by the mixed-function amine oxidase of Ziegler and Mitchell [46], rather than by the cytochrome P-450 pathway. Furthermore, this metabolite binds to amino acids and nucleotides in a similar way to when the azo dyes are administered *in vivo*.

Although it is well established that either *in vivo* or *in vitro* administration of chemical hepatocarcinogens results in the inhibition of protein synthesis under certain circumstances [1–8], the role of this inhibitory effect in tumour induction is not understood. No correlation could be made in the present study between the inhibitory effect on *in vitro* protein synthesis and the carcinogenic potencies of a series of structually related dye analogues. Although the latter property varied considerably, all four dyes tested were virtually equipotent with respect to the inhibitory effect. This result has several implications. Firstly, it is possible that the inhibition of protein synthesis is an early step in tumour induction but is not the main factor in determining the carcinogenic potencies of the dyes. It is also possible that a detailed analysis of the proteins synthesized in the *in vitro* system may show qualitative differences in inhibition by the dyes, even though no differences in the inhibition

of total synthesis are observed. On the other hand, it is possible that the inhibitory effect on protein synthesis is merely a manifestation of the toxicity of the aminoazo dyes. Dietary feeding of the dyes, which leads to tumour formation, has not yet been shown to result in the inhibition of protein synthesis. From as early as one to ten days after the onset of dye feeding, protein synthetic rates are increased with respect to controls [47–49]. Only in acute *in vivo* dosage experiments, conditions which have not been shown to induce tumours, is protein synthesis inhibited [2, 3]. In these studies, however, the inhibitory effect is obtained when using high doses of the dyes: 200–250 mg/kg body wt, as opposed to the approximate 6-mg daily dose in dietary experiments. The inhibitory effect is also rapidly overcome by a secondary stimulatory action on protein synthesis, within 40 hr of dye administration [2]. Flaks and Teh [50] have concluded from their experiments that the high doses used in such acute dosage experiments produce structural effects not normally observed under conditions which lead to tumour induction. The inhibitory effect of the aminoazo dyes on protein synthesis observed in this and other studies, may eventually prove to be a toxic effect of the dyes rather than an essential factor in the process of tumour induction.

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