Effect of antioxidant vitamins A, C, E and their analogues on azo-dye binding protein in liver of rats treated with \( p \)-dimethylaminoazobenzene

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\( p \)-Dimethylaminoazobenzene (DAB) is an azo-dye and known to cause liver tumour in rats. Azo-dye binding protein is a specific cytosolic protein involved in the translocation of azo-dye carcinogen metabolites from liver cytoplasm into the nucleus. Administration of vitamin A (40,000 and 50,000 IU), L-ascorbic acid (500 and 1,000 mg) and vitamin E succinate (200–500 mg) reduced the amount of azo-dye binding protein in liver of rats treated with DAB. Supplementation of high doses of vitamin A acetate, vitamin A palmitate, sodium ascorbate, ascorbyl palmitate and vitamin E acetate had no effect on the quantity of azo-dye binding protein in liver. When the vitamin mixture was given, the level of azo-dye binding protein decreased in the liver at all the studied doses, which may be due to their synergistic effect.

**Keywords:** Antioxidant vitamins A, C and E, Azo-dye binding protein, \( p \)-Dimethylaminoazobenzene

The pioneering work of Miller and Miller\(^1\) established that aminoazo-dye, a known hepato carcinogen, binds to liver protein in rats; further *in vivo* studies on various animal models proved that the chosen carcinogens bound to cellular protein in tissues rendered them tissues susceptible to carcinogenic action. Azo-dye binding protein is a specific cytosolic protein involved in the translocation of azo-dye carcinogen metabolites from liver cytoplasm into the nucleus. The binding of azo-dye metabolites with nuclear macromolecule necessitate further prior processing that occur in the nucleus. The azo-dye binding protein is found in all the highly-differentiated hepatomas. It is not found in poorly differentiated hepatomas, anaplastic carcinomas and adenocarcinomas\(^2\). Thus the presence of azo-dye binding protein in tumour indicates the degree of cell differentiation. The hepatic binding protein is involved in the translocation of azo-dye carcinogen metabolites to the nucleus from liver cytoplasm. Interaction of azo-dye metabolites with nuclear macromolecules facilitates carcinogenesis that occurs in the nucleus\(^3\). Covalent binding of azo-dye metabolites to DNA occurs when the azo-dye metabolites are incubated with liver nuclei and not with isolated liver DNA. This is due to the specific soluble protein that controls the translocation form cytosol to the nucleus\(^4\).

The amount of protein-bound azo-dye is three times more in mice after 2-3 weeks of DAB feeding; in hamster, azo-dye binding to proteins increased gradually up to 6 weeks, and then remained constant\(^5\). Sub-fractionation of liver homogenates on administration of azo-dyes shows that the dye is bound principally with the microsomal fraction (insoluble) and the soluble supernatant\(^6-8\). The soluble fraction subjected to zone electrophoresis has shown three protein fractions, namely a major basic protein fraction and two minor mono-acidic protein fractions\(^9\). The present study is concerned with the soluble proteins that bind specifically to the azo-dye so as to evaluate the role of antioxidant vitamins A, C, E and their analogues in preventing the binding of azo-dyes in DAB-treated rats.

**Materials and Methods**

*Animals*—Male albino rats of Wistar strain (170 ± 20 g), reared and maintained in the animal house of...
the Department of Biochemistry (under the supervision of Institutional Animal Ethics Committee), J. J. College of Arts and Science, Pudukkottai, were used.

**DAB and vitamins**—The hepato carcinogen \( p \)-dimethylaminoazobenzene (\( p \)-DAB; CAS No. 60-11-7) was purchased from Sigma Chemical Co., St. Louis, USA. Vitamin A, C and E and their analogues vitamin A acetate, vitamin A palmitate, sodium ascorbate, ascorbyl palmitate, vitamin E acetate and vitamin E succinate with research grade purity were used for the present work.

**Individual vitamin treatment**—Rats (275) were divided into 3 groups. First group (n = 25) served as normal control. The second group (n = 25) received 0.06% of DAB for 4 months followed by basal diet alone for an additional 2 months and served as DAB control. The third group consisting of the remaining 225 rats was further subdivided into subgroups A, C and E (n = 75) based on the vitamins used. The subgroups A, C and E were further divided into subsets of A1, A2 and A3, C1, C2 and C3 and E1, E2 and E3 respectively consisting of 25 rats each. Finally, all the subsets were further divided into 5 sub-subsets (Sub-subsets I to V) of 5 rats each. All the rats were acclimatized to laboratory conditions for one week and fed ad libitum with a basal diet [containing 660 g glucose, 180 g milk casein, 40 g salt mixture, 10, 100 g corn oil, 20 g cod liver oil, 1.5 g choline chloride, 50 mg vitamin K3, 20 mg riboflavin, 20 mg thiamine, 20 mg pyridoxine, 60 mg calcium pantothenate, 50 mg nicotinamide, 1.8 mg folic acid, 0.6 mg biotin, 100 mg inositol, 50 mg \( p \)-aminobenzoate, 40 µg cyanocobalamin per kg diet]. Animals of second and third groups were sacrificed with ether after 6 months and used for the isolation of basic azo-dye binding protein. Immediately, excised livers of rats were perfused with 20 ml cold 0.25 \( M \) sucrose. All the sub cellular particles were removed by centrifuging at 30,000 rpm (65,390 \( g \)) for 120 min. The resulting soluble cell supernatant was used to isolate azo-dye binding protein as per Ketterer et al.16.

**Multiple vitamin treatment**—Multiple vitamin treatment study was performed with mixture13-15 of vitamin A, L-ascorbic acid and vitamin E succinate. For this study, 35 rats were divided into 7 groups of 5 rats each for normal, DAB, DAB + vitamin A (10,000 IU) + vitamin C (75 mg) + vitamin E (50 mg), DAB + vitamin A (20,000 IU) + vitamin C (150 mg) + vitamin E (100 mg), DAB + vitamin A (30,000 IU) + vitamin C (250 mg) + vitamin E (200 mg), DAB + vitamin A (40,000 IU) + vitamin C (500 mg) + vitamin E (400 mg), DAB + vitamin A (50,000 IU) + vitamin C (1000 mg) + vitamin E (500 mg). The mixture of vitamins was given to all the rats by gavage route once in a week for 6 months.

**Isolation of basic azo-dye binding protein**—Animals administered with 0.06% of DAB were sacrificed with ether after 6 months and used for the isolation of basic azo-dye binding protein. Immediately, excised livers of rats were perfused with 20 ml cold 0.25 \( M \) sucrose. All the sub cellular particles were removed by centrifuging at 30,000 rpm (65,390 \( g \)) for 120 min. The resulting soluble cell supernatant was used to isolate azo-dye binding protein as per Ketterer et al.16.

**Amino acid analysis**—Azo-dye binding protein was hydrolysed in constant boiling HCl at 105°C for 16 h and hydrolysate was analysed by chromatography on Whatman no.1 filter paper with the solvent butanol-acetic acid-water (12:3:5). Ninhydrin reagent (100 mg/ml of acetone) was used to spot the amino acids and the amino acids present in the sample were then identified by comparing the \( R_f \) values with that of the authentic amino acids, co-chromatographed.

**Determination of molecular weight by SDS-PAGE**—Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) was performed with 10% poly acrylamide gel following the procedure of Laemmli17. Solution of basic azo-dye binding protein in PBS (10mg/2 ml) was prepared and applied in SDS-PAGE. The molecular weight of azo-dye binding protein was determined using SDS-PAGE in which protein marker of known molecular weight and the azo-dye binding protein fraction were run simultaneously.
proteins present in the gel were stained for 4 h, using 0.2% Coomassie brilliant blue R-250 (Sigma) in 50% methanol and 7% acetic acid. The gel was later destained using destaining solution (7% acetic acid and 30% methanol) until the background was clear.

Estimation of bound azo-dye—The liver of rats administered 0.06% DAB were treated in a blender with 4 times its weight of distilled water until the tissue became a frothy pulp. The liver pulp was boiled for 5 min in acetate buffer and adjusted to pH 5 with acetic acid (5 ml for buffer /g of liver). After cooling, the coagulated protein was centrifuged and washed with buffer and then with 95% ethanol until no further colour could be extracted. Any remaining unbound azo-dye and lipid material was removed from the protein by extraction in a soxhlet apparatus with 95% alcohol for 48 h at 60°C. The protein mass was broken up and the ethanol was allowed to evaporate from the powder, which was then dried over P₂O₅ in a vacuum desicator²,¹₈.

Chemical assay—The liver protein powder was hydrolysed to release bound azo-dye and the released azo-dye was extracted with pentan-1-ol. The extracts were poured into 5 volumes of light petroleum and 10 ml of a freshly prepared mixture of concentrated-HCl and acetone (2:1, v/v) was added. The mixture was shaken and allowed to stand for 30 min. The lower layer containing the dye was transferred to a measuring cylinder and its extinction was measured at 520 nm in a spectrophotometer. The colour intensity became maximal after 30 min and remained constant for several hours. The extraction was corrected to a final volume of 10 ml and the bound azo-dye was expressed in arbitrary units of E₅₂₀/100 mg of protein¹⁸. Statistical analysis of the data was performed with Student’s ‘t’ test.

Results
The amino acid analysis of hydrolysates of the basic azo-dye binding protein shows the presence of following 17 amino acids: alanine (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), tyrosine (Tyr), threonine (Thr) and valine (Val). Amino acids such as Asn, Gln and Trp were not found in azo-dye binding protein (since tryptophan would have been destroyed during acid hydrolysis¹⁸, it is not been included in the list). The amino acid analysis of hydrolysates of basic azo-dye binding peptide after being digested with protease contains Ala, Asp, Glu, Gly, Ser, Thr, other amino acids were destroyed during digestion with protease. Isolated azo-dye binding protein had a molecular weight of 45 KDa as evident from the SDS-PAGE study (Fig. 1).

The amount of bound azo-dye in DAB-treated rats was found to be 0.41 arbitrary units/mg protein¹⁸. However, administration of vitamin A (40,000 and 50,000 IU), L-ascorbic acid (500 and 1,000 mg) and vitamin E succinate (200–500 mg) reduced the amount of azo-dye binding protein in liver (Fig. 2 to 4). Individual supplementation of high doses of vitamin A acetate, vitamin A palmitate, sodium ascorbate, ascorbyl palmitate and vitamin E acetate have no effect on quantity of azo-dye binding protein in liver (Fig. 5). When the vitamin mixture was given, the level of azo-dye binding protein decreased in the liver at all the studied doses (Fig. 6).

Discussion
Amino acid analysis of azo-dye binding peptide revealed the presence of Ala, Asp, Glu, Gly, Ser and Thr. The earlier studies on azo-dye bound peptide revealed that it contained Pro, Leu/Ile, Val and Gly as major components and Glu, Phe, Ser, Ala and Asp as minor components¹⁹. Terayama and Takeuchi²⁰ isolated a fraction on hydrolysis of azo-dye binding protein which was found to have Phe, Ser, Gly, Pro, Val, Glu and Asp. The discrepancies between the earlier results and the present study may be due to the fact that more than one azo-dye binding protein exists and the possibility of the azo-dye to bind with different types of amino acid sequences in each protein gives several different azo-dye bound peptides¹⁶.
The diet rich in riboflavin increased the induction period of liver tumours by azo-dyes. Similarly, rats fed with both riboflavin and cupric oxyacetate hexahydrate delayed the formation of liver tumour induced by azo-dyes. The results of the present study also indicate that supplementation of vitamin A, C and E offer protection against DAB by delaying the binding of the azo-dye. The binding of protein with azo-dye was lowered in vitamin treated rats. DAB can be inactivated in the liver by an enzyme system, azo-dye reductase, that reductively cleaves the molecule to non-carcinogenic amine moieties and that the coenzyme required is FAD. The protection given by vitamins is through the increased enzymatic destruction of the azo-dye. Dyes were bound with the proteins from the high-sulfur-protein area or high-tyrosine-protein area, depending on the level of dye dissolution. The passage of azo-dye binding protein through an immobilized protease column reduced the azo-dye metabolite translocation by 65%, concomitant with the degradation of proteins. Translocation from the cytoplasm into nucleus was not observed with protein-free metabolites and addition of protein promotes the metabolite translocation in rat liver. In the present study, supplementation of vitamin mixture inhibits the translocation of azo-dye binding proteins in liver of rats treated with DAB. In conclusion, antioxidant vitamin mixture prevents the binding of azo-dye in the liver by reducing the level of azo-dye binding protein.

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