The Isolation of Carcinogen-Binding Protein from Livers of Rats given 4-Dimethylaminoazobenzene

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1. Three azo-dye-binding proteins were identified in the soluble cell supernatant fraction from livers of rats that had received 4-dimethylaminoazobenzene by intraperitoneal injection. 2. One is basic and was highly purified. It has an isoelectric point of pH 8.4 in barbital-sodium chloride buffer, 10.1, an $S_{20,w}$ value of 3.5s and a molecular weight determined by Sephadex chromatography of 45000. 3. It does not have *N*-terminal amino acids with free α -amino groups. 4. Digestion with Pronase gives rise to a single azo-dye-bound peptide, which on hydrolysis is shown to contain glycine, alanine, serine, threonine, glutamic acid and aspartic acid. The amino acid that binds the azo-dye was not identified. 5. On starch-gel electrophoresis the basic protein separates into a double band, indicating microheterogeneity. 6. The other two proteins were partially purified and occur in a fraction together. They have isoelectric points near neutrality and a molecular weight as determined by Sephadex chromatography of 13800. 7. The absorption spectra in formic acid of both the basic and the low-molecular-weight proteins are similar. The azonium ion has an absorption maximum at 518m μ and another adsorbed chromogen is present with an absorption maximum at 395m μ .

In experiments performed *in vivo* certain carcinogens have been shown to bind to cellular protein in tissues susceptible to their carcinogenic action. For example several aminoazo-dyes (Miller & Miller, 1947; Miller, Miller, Sapp & Webber, 1949), two aminofluorene derivatives (Miller & Miller, 1952; Weissburger, Weissburger & Morris, 1953; Weissburger & Weissburger, 1958) and ethionine (Sorof, Young & Fetterman, 1962) have been observed to bind to liver protein in the rat, and various polycyclic hydrocarbons have been shown to bind to proteins in the mouse skin (Miller, 1950; Wiest & Heidelberger, 1953; Heidelberger & Moldenhauer, 1956). The carcinogens appear to be chemically bound to the protein. With the azo-dyes it has not been possible to obtain the dye in small-molecular form from dye-bound protein except by digestion with proteolytic enzymes or strong alkali. The isolation of azo-dye-bound peptides from proteolytic digests has been reported by several workers (Hughes, 1959; Terayama & Takeuchi, 1962). Scribner, Miller & Miller (1965) demonstrated that alkali treatment of liver homogenates from rats given

4-dimethylaminoazobenzene released 3-mercapto-methyl-4-monomethylaminoazobenzene, which suggests that the dye may bind to protein through the sulphur atom of methionine.

Subfractionation of liver homogenates after administration of azo-dye shows that the dye is bound principally in the microsome fraction and the soluble cell supernatant (Hultin, 1956; Gelboin, Miller & Miller, 1958; Fiala & Fiala, 1959). Azo-dye-bound protein in the microsome fraction is insoluble, but the soluble fraction has been submitted to zone electrophoresis, whereupon the azo-dye binding has been shown to be restricted to three protein fractions, namely a major basic protein fraction and two minor more-acidic protein fractions (Sorof, Young, McCue & Fetterman, 1963).

The present paper is concerned with these soluble proteins that appear to bind azo-dye specifically and in particular with the isolation and properties of the basic azo-dye-binding protein. Interest in this latter protein has received impetus from the observation by Freed & Sorof (1966) that an electrophoretic fraction that apparently contains the basic azo-dye-binding protein can reversibly inhibit growth in certain cell cultures, e.g. mouse L-fibroblasts and HeLa cells.

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METHODS

Preparative zone electrophoresis

Preparative electrophoresis was performed after the method of Müller-Eberhard (1960). The vinyl copolymer Pevikon C-870 (Stockholms Superfosfat Fabriks A.B., Stockholm, Sweden) was used as the support. It was acid-washed, made into a thick slurry with either 0.05 M-barbital buffer, pH 8.0, or 2 mM-phosphate-citrate buffer, pH 7.0, and poured into a tray 12 cm. $\times$ 42 cm. $\times$ 1 cm. After being dried to a suitable consistency by blotting with filter paper a slot was cut in the resulting slab. The protein solution under study was applied to the slot as a slurry made with more Pevikon and electrophoresis was carried out at a potential difference of 300 v for 48–72 hr. Sections of the slab were removed with a spatula and protein was eluted from each section with 0.15 M-NaCl.

Starch-gel electrophoresis

Vertical starch-gel electrophoresis was performed in the apparatus designed by Smithies (1959). The most commonly used buffer was based on the discontinuous system of Ashton & Braden (1961). The gel buffer contained 5.6 g. of tris, 0.12 g. of LiOH, 1.4 g. of citric acid and 1.19 g. of boric acid/l. and the bridge buffer was composed of 1.2 g. of LiOH and 11.89 g. of boric acid/l. This system has a pH of 8.4. Other gel buffers used were 6 mM-phosphate-citrate buffer, pH 7.0, and 5 mM-acetate buffer, pH 4.5. The bridge solution for the latter gel buffers was 0.15 M-NaCl (Neelin, 1963).

Gels were made by heating 70–80 g. of starch ('starch hydrolysed'; Connaught Medical Research Laboratories, Toronto, Canada) with 500 ml. of buffer. Runs were performed overnight at a potential difference of 100–200 v.

After slicing, the gels were soaked first in 5% (w/v) trichloroacetic acid in 50% (v/v) ethanol to show the position of bound azo-dye, which in strong acid occurs largely as the red azonium ion (Cilento, Miller & Miller, 1956), and then in a nigrosine stain to show the position of the protein. The nigrosine stain consisted of 1.0 g. of nigrosine, 10 g. of tungstophosphoric acid and 10 g. of trichloroacetic acid in 1 l. of 50% (v/v) ethanol. Excess of the stain was removed by washing with 50% (v/v) ethanol.

Free electrophoresis

Moving-boundary electrophoresis was performed in the Electrophoresis Instrument model 35 manufactured by C. Zeiss, Jena, Germany. A 1% (w/v) solution of the protein in barbital-NaCl buffer, pH 8.4 and 10.1, was used. The run was performed for 4 hr. at 4° and a potential gradient of 6 v/cm.

High-voltage filter-paper electrophoresis

Basic azo-dye-binding protein preparations were subjected to high-voltage filter-paper electrophoresis in the apparatus of Ryle, Sanger, Smith & Kitai (1955), with Esso White Spirit as coolant. The filter paper was Whatman no. 3MM and the buffer pyridine-acetic acid-water (1:10:89, by vol.), pH 3.5. After electrophoresis at a potential gradient of 50 v/cm. for 30 min. the paper was

dried, treated with ninhydrin dipping reagent (Smith, 1957) and then stained for protein with bromophenol blue (Kunkel & Tiselius, 1951).

Sedimentation in the ultracentrifuge

Ultracentrifugal analysis was performed in the Spinco model E ultracentrifuge at a speed of 63650 rev./min. and a mean temperature of 18°. Protein was analysed as a 1% (w/v) solution in 0.15 M-NaCl–0.01 M-tris buffer, pH 7.0.

Estimation of molecular weight by gel filtration

The molecular weights of the azo-dye-binding proteins were determined in a series of experiments in which proteins of known molecular weight and the azo-dye-binding protein fraction were run simultaneously on the same column. For the basic azo-dye-binding protein 2 ml. of solution containing 10 mg. each of bovine γ -globulin, bovine serum albumin and ovalbumin was applied to a 2 cm. $\times$ 90 cm. column of Sephadex G-100 packed in 0.2 M-NaCl–0.01 M-tris buffer, pH 7.0. When, after the application of this standard protein solution, exactly 50 ml. of effluent had passed through the column, 10 mg. of basic azo-dye-binding protein in 2 ml. of solution was applied. The volumes of the effluent fractions were determined by weighing. For the small-molecular azo-dye-binding proteins bovine serum albumin, ovalbumin and horse-heart cytochrome c were used as standards. Molecular weights were calculated by the method of Whitaker (1963), who showed that the behaviour of most proteins on Sephadex is such that there is a linear correlation between the logarithm of the molecular weight of a protein and the ratio between its elution volume and the void volume of the column.

Absorption spectrum

The absorption spectra of small volumes of protein solution were determined by using the micro-cell attachment of the Unicam SP.800 recording spectrophotometer. To show the absorption due to the azonium ion, azo-dye-binding protein preparations were dissolved in 90% (w/v) formic acid. Before spectrophotometry, a loosely bound chromogen was largely removed from the basic azo-dye-binding protein by precipitating it in 50% (v/v) ethanol at pH 8.0 and washing the precipitate with 50% ethanol. The specific extinction coefficients were determined for all protein preparations by using dilute solutions of the protein in formic acid. The protein concentration of each preparation was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard. Bovine serum albumin and the basic azo-dye-binding protein gave almost identical standard curves.

Amino acid analysis

Azo-dye-binding protein was hydrolysed in triple-distilled constant-boiling HCl at 105° for either 24 or 48 hr. The hydrolysates were analysed in the EEL amino acid analyser (Spackman, Stein & Moore, 1958). Azo-dye-bound peptide was hydrolysed in constant-boiling HCl at

105° for 16hr. and the hydrolysate was analysed by two-dimensional chromatography on Whatman no. 1 filter paper with the solvents butan-1-ol-acetic acid-water (12:3:5, by vol.) and phenol-ammonia (Smith, 1957).

Determination of N-terminal amino acids by using 1-fluoro-2,4-dinitrobenzene

Reaction with fluorodinitrobenzene. Two methods were used. One was based on that of Phillips (1958). A 10mg. sample of the basic azo-dye-binding protein was dissolved in 1ml. of 6M-guanidine hydrochloride and 10mg. of NaHCO₃ was added. To this solution was added 0.1ml. of fluorodinitrobenzene and the resulting mixture was shaken overnight at room temperature. Unchanged fluorodinitrobenzene was removed by a single extraction with peroxide-free ether and the aqueous solution was diluted, acidified and dialysed. DNP-protein was precipitated out and was washed repeatedly with N-HCl, then acetone and finally water.

Alternatively 10mg. of protein was dissolved in 1ml. of M-acetate buffer, pH 6.0 (Elliott, 1952), then 1ml. of propan-2-ol and 0.1ml. of fluorodinitrobenzene were added and the reaction was carried out with shaking overnight (Weil & Seibles, 1961). Under these conditions DNP-(azo-dye-binding protein) was precipitated as it formed, but gave a much more finely divided precipitate than is obtained in the presence of ethanol and NaHCO₃ as used in the procedure of Fraenkel-Conrat & Porter (1952). The DNP-protein precipitate was washed as described above.

Hydrolysis. The dried DNP-protein was weighed, moistened with formic acid and hydrolysed at 105° under N₂ in a sealed tube for either 5hr. with 11N-HCl, when destruction of glycine is minimal, or for 16hr. with constant-boiling HCl (Levy, 1955).

Ether-soluble DNP-amino acids. The hydrolysate was diluted until it was 1N with respect to HCl, extracted four times with 5ml. of peroxide-free ether and the ether extracts were combined and washed once with N-HCl. The resulting ether-soluble material was then submitted to two-dimensional chromatography on Whatman no. 1 filter paper that had been soaked in 0.05M-potassium hydrogen phthalate buffer, pH 6.0, and dried. The solvent for the first dimension was the organic phase of a system composed of the above phthalate buffer equilibrated with pentan-1-ol (Pairent & Williamson, 1960). The solvent for the second dimension was either 1.5M- or 2.0M-phosphate buffer, pH 6.0 (Levy, 1955). Descending chromatography was used in both dimensions.

Water-soluble DNP-amino acids. The water-soluble components of the hydrolysate were submitted to continuous ether extraction in the apparatus of Mills (1952) according to the recommendation of Biserte, Holleman, Holleman-Dehove & Sautière (1959) to remove selectively any di-DNP-histidine. This ether extract and the remaining water-soluble material were both evaporated to dryness and analysed by two-dimensional chromatography on Whatman no. 1 filter paper. The first dimension was with either the organic phase of a 0.1% (w/v) ammonia and butan-1-ol system (Koch & Weidel, 1956) or the organic phase of a system composed of 3-methylbutan-1-ol and 0.05M-phthalate buffer, pH 6.0. In the latter case the paper was impregnated with the phthalate buffer (Blackburn & Lowther, 1951). In either case the second dimension was

with 1.5M-phosphate buffer, pH 6.0. Descending chromatography was used in both dimensions.

RESULTS

Isolation of basic azo-dye-binding protein

Adult male Wistar rats of an inbred strain were fed on the Rowett diet 86, which contains 20% of protein. Animals weighing between 180 and 250g. were selected, given 50mg. of 4-dimethylamino-azobenzene in 2ml. of corn oil by intraperitoneal injection and killed with chloroform after 16hr. Immediately after death the livers of six to ten rats were perfused with 20ml. of cold 0.25M-sucrose, removed, minced and homogenized with an equal volume of 0.25M-sucrose. All subcellular particles were removed by centrifuging for 120min. in the 8 × 50ml. angle rotor of the MSE Super-Speed 40 ultracentrifuge at 30000 rev./min.

The resulting soluble cell supernatant was made 0.15M with respect to sodium chloride, 2mM with respect to both calcium chloride and copper sulphate and adjusted to pH 4.5 with N-acetic acid. The heavy precipitate that developed during 30min. was discarded. The concentrations of calcium chloride and copper sulphate in the supernatant were each increased a further 1mM, the pH was adjusted to pH 7.4 with M-triethanolamine and the volume was reduced by dialysis against solid A.R. sucrose in the cold. Two or three batches of material concentrated in this way were combined and thoroughly dialysed against 1.25mM-calcium chloride-2.5mM-triethanolamine buffer, pH 8.0. Precipitated protein was removed by centrifuging and the supernatant was passed through a 3cm. × 30cm. (diam. × ht.) column of DEAE-cellulose that had been equilibrated against the same buffer. The unadsorbed material was made 0.05M with respect to sodium chloride, adjusted to pH 6.8 with N-acetic acid and passed through a 5cm. × 1.5cm. CM-Sephadex C-50 column equilibrated against 0.05M-sodium chloride-5mM-triethanolamine buffer, pH 6.8. The unadsorbed material was diluted to 0.03M with respect to sodium chloride, adjusted to pH 5.8 and passed through a 3cm. × 12cm. column of CM-Sephadex C-50 equilibrated against 0.026M-sodium chloride-0.01M-sodium acetate buffer, pH 5.8. Basic azo-dye-binding protein remained adsorbed to this column while many of the impurities including less basic azo-dye-binding protein passed through with the free volume. The basic azo-dye-binding protein was eluted with 0.2M-sodium chloride-0.01M-acetate buffer, pH 5.8. The eluted material was made 2.7mM with respect to EDTA, 5mM with respect to mercaptoethanol and adjusted to pH 8.6 with triethanolamine. The volume was reduced by ultrafiltration and the concentrated material was

applied to a 3cm. $\times$ 90cm. column of Sephadex G-100 packed in 0.2M-sodium chloride-0.08M-phosphate buffer, pH 7.4. The yellow basic azo-dye-binding protein that emerged at about 160ml. was concentrated by ultrafiltration, dialysed against 2.5mm-disodium hydrogen phosphate and passed through a 1cm. $\times$ 5cm. column of DEAE-Sephadex A-50 at pH 8.5. The unadsorbed protein represented the final product.

Fig. 1. Vertical starch-gel electrophoresis of azo-dye-binding proteins in LiOH-borate-tris-citrate buffer, pH 8.4. Experimental details are given in the text. (a) Small-molecular azo-dye-binding protein fraction freed from major contaminating proteins by Pevikon electrophoresis at pH 7.0. (b) h-protein fraction obtained from soluble cell supernatant by Pevikon electrophoresis at pH 8.0. (c) Purified basic azo-dye-binding protein. Pinmarks on the gel show the position of bound azo-dye.

Properties of the basic azo-dye-binding protein preparation

Starch-gel electrophoresis. A single nigrosine-staining band was obtained at pH 4.0 and a double band at pH 7.0 and pH 8.4 (Fig. 1). Regions on the gel staining with nigrosine coincided with regions on the gel giving a pink colour with aqueous tri-chloroacetic acid owing to the presence of bound azo-dye.

Free electrophoresis. Basic azo-dye-binding protein was found to be isoelectric in barbital-sodium chloride buffer, pH 8.4 and 10.1, at 4°. After 4hr. electrophoresis no evidence of heterogeneity could be detected in either the ascending or the descending limbs of the apparatus (Fig. 2).

Sedimentation in the ultracentrifuge. In 0.15M-sodium chloride-0.01M-tris buffer, pH 7.0, the azo-dye-binding protein sedimented as a single boundary with an $S_{20,w}$ value of 3.5s (Fig. 3).

Behaviour on Sephadex G-100. Basic azo-dye-binding protein emerged as a single symmetrical peak after passage through a column of Sephadex G-100. When compared with proteins of known molecular weight chromatographed simultaneously, it behaved as if it had a molecular weight of 45000, having an elution volume indistinguishable from that of ovalbumin.

Amino acid analysis. The amino acid analysis of 24hr. and 48hr. hydrolysates of the basic azo-dye-binding protein are shown in Table 1. The number of residues of each amino acid has been calculated on the basis of a molecular weight of 45000 for the protein. Tryptophan would have been destroyed during the hydrolysis and has not been analysed for separately. Compared with published amino acid analyses for a number of other proteins (Tristram & Smith, 1963) the basic azo-dye-binding protein is noteworthy in having a relatively high methionine content and a relatively low content of hydroxy-amino acids.

Fig. 2. Moving-boundary electrophoresis of purified basic azo-dye-binding protein at pH 8.4 in barbital-NaCl buffer, 10.1. The photograph of the initial boundary in the descending limb is superimposed on a photograph of the schlieren pattern obtained after 120min.

Fig. 3. Sedimentation of a 1% (w/v) solution of purified basic azo-dye-binding protein in 0.15M-NaCl-0.01M-tris buffer, pH7.0, at 63650 rev./min. in the Spinco model E ultracentrifuge. The schlieren pattern after 48min. is shown.

Fig. 4. Absorption spectra in 90% (w/v) formic acid., 17 μ M-4-Dimethylaminoazobenzene; —, 1% (w/v) purified basic azo-dye-binding protein; ----, 1% (w/v) purified basic azo-dye-binding protein precipitated in 50% (w/v) ethanol at pH8.0 and washed several times with 50% ethanol.

Table 1. Amino acid analyses of 24 and 48hr. hydrolysates of basic azo-dye-binding protein

The results are expressed as residues/mol. of protein of mol.wt. 45000. Each set of values is the mean of two analyses, which agreed within the expected 5% error.

Amino acid	Amino acid composition (residues/mol. of protein)	
	24hr. hydrolysate	48hr. hydrolysate
Gly	18.3	19.9
Ala	26.9	27.3
Val	16.9	18.1
Leu	46.1	49.3
Ile	18.3	19.2
Phe	17.4	18.0
Tyr	14.2	10.9
Trp	*	*
Pro	17.8	16.4
CyS	2.1	1.8
Met	15.0	14.5
Ser	14.8	13.5
Thr	12.3	11.1
Asp	36.0	36.8
Glu	41.2	39.9
His	5.2	5.3
Lys	34.4	33.5
Arg	20.6	22.5

* Tryptophan is destroyed during acid hydrolysis.

Binding of amino acids. If basic azo-dye-binding protein was precipitated at pH8.0 in 50% (v/v) ethanol a variety of amino acids was found in solution in the aqueous ethanol, indicating the release of adsorbed amino acids. Bound amino acids

could also be demonstrated by submitting preparations of the protein to high-voltage paper electrophoresis, when the amino acids moved away from the origin at their characteristic velocities and the protein remained adsorbed at the origin.

Dinitrophenylation. No water-soluble α -DNP-amino acids were found in hydrolysates of DNP-(basic azo-dye-binding protein), but small and variable amounts of ether-soluble α -DNP-amino acids were always present. The yield of the latter was little affected by the method of dinitrophenylation used; it remained small when the reaction was carried out in 6M-guanidine hydrochloride at pH8.0, even though under these conditions the DNP-protein remained in solution and was presumably in the unfolded state, with the result that minimal hindrance would have been offered to the reaction of fluorodinitrobenzene with free α -amino groups of *N*-terminal amino acid residues had they been present. The factor that affected the yield of ether-soluble α -DNP-amino acids markedly was the thoroughness with which the DNP-protein was washed before hydrolysis; the more thorough the washing the lower the yield. If, after dinitrophenylation, the supernatant above the DNP-protein precipitate was combined with various washings and chromatographed a number of α -DNP-amino acids were identified that presumably originated from the free amino acids that the protein had adsorbed on it.

Thus it appears that the basic azo-dye-binding protein does not have *N*-terminal amino acids free to react stoichiometrically with fluorodinitrobenzene. The small amounts of α -DNP-amino acids that are found probably originate from the free amino acids that are adsorbed on the protein.

Absorption spectrum. Fig. 4 shows the absorption spectra of solutions, in 90% formic acid, of 17 μ M-

4-dimethylaminoazobenzene, purified basic azo-dye-binding protein (1%, w/v) and the same protein (1%, w/v) that had been precipitated at pH 8.0 in 50% (v/v) ethanol and washed several times with 50% ethanol.

It is seen that the purified basic azo-dye-binding protein has two absorption maxima, one at 395m μ and the other at 518m μ . The former maximum is due to a substance that is presumably not chemically bound since treatment of the protein with ethanol caused a large decrease in the absorption. The absorption maximum at 518m μ is due to the azo-dye in its azonium ion form. Very little modification of the spectrum of the azonium ion has occurred as a result of the azo-dye's being bound to the protein.

The plot of E_{518} against protein concentration is not linear but falls away with increasing protein concentration. If E_{518} is determined at a protein concentration of 0.05% (w/v) the average basic azo-dye-binding preparation has $E_{518}^{1\%}$ 1.25 at 518m μ and ϵ_{518} 5.4×10^3 .

The yield of basic azo-dye-binding protein from 100g. of liver was about 25mg.; 25mg. of the protein contained the equivalent of approx. 0.1 μ -mole of azo-dye, assuming that the free and the bound azo-dye have the same extinction coefficient. If it is also assumed that each mole of azo-dye-binding protein is capable of binding only 1mole of azo-dye, then only 20% of the molecules in a typical basic azo-dye-binding protein preparation have azo-dye bound to them. The amount of apparent bound azo-dye in the soluble cell supernatant derived from 100g. of liver was determined by the method of Miller & Miller (1947) and found to be approx. 1 μ mole. Thus 10% of the apparent bound azo-dye initially present is recovered in the basic azo-dye-binding protein fraction.

Comparison between the purified basic azo-dye-binding protein fraction and the 'h-protein fraction'

A fraction similar to the 'h-protein fraction' described by Sorof *et al.* (1963) was prepared as follows. The soluble cell supernatant from the livers of ten rats that had received 4-dimethylaminoazobenzene was concentrated to 10ml. and subjected to zone electrophoresis on a Pevikon slab at pH 8.0. After 72hr. a yellow band due to the presence of azo-dye-binding protein was clearly separated on the cathode side. This band was taken to indicate the position of the h-protein fraction and was eluted. On starch-gel electrophoresis at pH 8.4 it gave rise to a number of bands, only one of which contained bound azo-dye (Fig. 1). This single azo-dye-binding protein band appeared to correspond to the faster-moving azo-dye-binding protein band found in the purified basic azo-dye-

binding protein preparations. The h-protein fraction had $E_{518}^{1\%}$ 0.7 at 518m μ and the equivalent of 0.15 μ mole of azo-dye was recovered from 100g. of liver.

Isolation of small-molecular azo-dye-binding proteins

Azo-dye-binding protein of lower molecular weight was also present in the soluble cell supernatant. Some of this protein passed through the DEAE-cellulose column together with the basic azo-dye-binding protein and was separated from the basic protein on the CM-Sephadex C-50 column equilibrated with 0.026M-sodium chloride-0.01M-sodium acetate buffer, pH 5.8; the small-molecular protein was not absorbed. More small-molecular azo-dye-binding protein was eluted from the DEAE-cellulose column by buffer made 0.05M with respect to sodium chloride. Both fractions were combined and reduced in volume to 2ml. by dialysis against a concentrated solution of polyethylene glycol and applied to a 2cm. $\times$ 90cm. column of Sephadex G-100. The small-molecular azo-dye-binding protein was eluted at approx. 210 ml. This preparation separated into four major protein bands on starch-gel electrophoresis at pH 8.4, two of which were azo-dye-binding proteins. Further purification was achieved by zone electrophoresis on Pevikon at pH 7.0 for 48hr. Under these conditions the major contaminating proteins were removed (Fig. 1).

Properties of the small-molecular azo-dye-binding proteins

At pH 7.0 on zone electrophoresis the two small-molecular azo-dye-binding proteins appeared to be close to their isoelectric points and to be undergoing slow separation from each other. Compared with proteins of known molecular weight chromatographed simultaneously on Sephadex G-100 these proteins behaved as if they had a molecular weight of 13800.

The absorption spectrum of the small-molecular azo-dye-binding protein preparation is very similar to that of the basic azo-dye-binding protein. As well as the azonium ion, the chromogen with an absorption maximum at 395m μ is also present and to the same degree relative to the azonium ion as in the basic azo-dye-binding protein. The small-molecular azo-dye-binding protein preparations have a lower specific extinction coefficient than the basic protein; a typical small-molecular azo-dye-binding protein, free of major contaminants, has $E_{518}^{1\%}$ 0.9 at 518m μ . The properties of the azo-dye-binding proteins are summarized in Table 2.

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Table 2. *Summary of the properties of the azo-dye-binding proteins*

Basic azo-dye-binding protein	
Isoelectric point	pH 8.4 (barbital-NaCl, 10:1)
Sedimentation coefficient ($S_{20,w}$)	3.5 s
Molecular weight (Sephadex)	45 000
N-Terminal amino acids	None
Azo-dye-bound peptide	(Gly, Ala, Ser, Thr, Glu, Asp)*
Small-molecular azo-dye-binding proteins	
Isoelectric points	Near neutrality
Molecular weight (Sephadex)	13 800

* The amino acid binding the dye is not known.

Preparation and analysis of azo-dye-bound peptide

Basic azo-dye-binding protein in solution in 0.01 M-ammonium hydrogen carbonate was digested with 5% its weight of Pronase B (Calbiochem, Los Angeles, Calif., U.S.A.) at 37° overnight. Azo-dye-containing material was extracted from the digest with butan-1-ol and subjected to high-voltage electrophoresis between cooled plates in the apparatus of the Locarte Co. Ltd. (London, S.W. 7) with the buffer pyridine-acetic acid-water (1:10:89, by vol.), pH 3.5. After electrophoresis the section of the paper containing azo-dye-bound material was cut out and sewn on to a second sheet of paper and submitted to two-dimensional chromatography with butan-1-ol-acetic acid-water (12:3:5, by vol.) in one dimension and 3-methylbutan-1-ol-pyridine-water (7:7:6, by vol.) in the second dimension (Baglioni, 1961). A single azo-dye-containing spot was obtained. This spot gave no detectable ninhydrin reaction, but after elution and hydrolysis it gave rise to the amino acids serine, threonine, glycine, alanine, aspartic acid and glutamic acid. It has not as yet been possible to identify the amino acid that binds the dye, although several faint ninhydrin-positive spots occur on chromatograms of hydrolysates of azo-dye-bound peptide; these may be breakdown products of the azo-dye-bound amino acid residue that had been produced during the acid hydrolysis.

DISCUSSION

Isolation of basic azo-dye-binding protein. The isolation procedure has been submitted to many variations, but the one described above gives the highest yield of protein with the highest specific optical extinction due to bound azo-dye.

Two-thirds of the apparent azo-dye-bound

protein is lost in the pH 4.5 precipitate in the presence of Ca^{2+} , Cu^{2+} and sodium chloride; however, the omission of this step does not increase the yield of azo-dye-binding protein and gives rise to a preparation that has a lower specific optical extinction due to bound azo-dye and that is obviously impure on starch-gel electrophoresis. The pH at which the pH 4.5 supernatant is concentrated and dialysed has an important bearing on the yield, and is optimum at about pH 7.0. The DEAE-cellulose column removes three-quarters of the protein passing through it, leaving only the more basic proteins. A critical step is that of chromatography on CM-Sephadex at pH 5.8 and approx. 0.03 M- Na^+ . The basic azo-dye-bound protein must be only lightly adsorbed if it is to be eluted without denaturation; grade C-50 rather than C-25 must be used, for with the latter very little adsorption takes place. Passage through the DEAE-cellulose column is important in reducing the load of protein to pass through the critical CM-Sephadex column, and a prior passage through the small CM-Sephadex column at pH 6.8 and 0.05 M-sodium chloride is useful in that it removes some protein that would otherwise be eluted with the basic azo-dye-binding protein from the column at pH 5.8. The Sephadex G-100 column not only separates contaminating proteins of differing molecular weights but also some aggregated basic azo-dye-binding protein. The purpose of the final step is to free the preparation of traces of less-basic proteins; this is done by passing it through a DEAE-Sephadex A-50 column at pH 8.5. It is noteworthy that the yield of bound azo-dye recovered in the basic azo-dye-binding protein fraction is not very different from that recovered in the h-protein preparation, which is prepared by a much simpler method.

Homogeneity of the basic azo-dye-binding protein. The basic azo-dye-binding protein behaves as a single protein in a number of tests. It gives a single component on prolonged free electrophoresis at its isoelectric point, a single symmetrical elution peak on chromatography on Sephadex G-100 and it sediments as a single boundary in the ultracentrifuge. Only trace amounts of α -DNP-amino acids can be found after hydrolysis of the dinitrophenylated protein, and these probably originate from the free amino acids that the protein has been shown to have adsorbed on it. It would appear that the basic azo-dye-binding protein has blocked N-terminal amino acid(s). Starch-gel electrophoresis at pH 7.0 and pH 8.4 gives rise to a double protein band; but, since both bands contain bound azo-dye, this is probably a case of microheterogeneity.

Although the basic azo-dye-binding protein appears to be homogeneous with respect to protein

it has a number of small molecules adsorbed to it that may be present when the protein is in its natural state in the cell, but that could equally well be contaminants picked up during the purification. These small molecules include amino acids and a chromogen with an absorption maximum at 395m μ .

Relationship between the azo-dye-binding protein fractions described in the present work and those described by Sorof et al. (1963). Sorof et al. (1963) submitted soluble liver proteins to column electrophoresis in barbital buffer at pH 8.6 and resolved them into eight classes, designated 'a' to 'h' according to their electrophoretic mobility. These authors observed three azo-dye-binding fractions in the soluble cell supernatant of livers from rats that had been given azo-dye; one was associated with the rapidly moving 'a' fraction, another moved more slowly in the slow 'g' region and the major fraction moved very slowly indeed in the slow 'h₂' fraction.

A comparison of the behaviour on starch-gel electrophoresis of the basic azo-dye-binding protein and the h-protein fraction (Fig. 1) suggests that the basic azo-dye-binding protein corresponds to the azo-dye-binding protein in the slow 'h₂' fraction of Sorof et al. (1963), and that the slower band of the basic azo-dye-binding protein that appears on starch-gel electrophoresis is an artifact of the purification procedure. The small-molecular azo-dye-binding proteins have isoelectric points near neutrality and therefore probably correspond to azo-dye-binding protein in the slow 'g' fraction. Nothing equivalent to the azo-dye-binding protein in the 'a' fraction has been observed in the present work. Sorof et al. (1963) suggest that it is a ribonucleoprotein, in which case it is probable that it would be found in the pH 4.5 precipitate.

A chromogen absorbing at 395m μ was found in the basic azo-dye-binding protein and in a fraction prepared in the same way from normal rats. It is also associated with the small-molecular azo-dye-binding fraction. Similarly the azo-dye-binding protein in the slow 'h₂' fraction of Sorof et al. (1963) possessed an absorption peak at 400m μ . This chromogen has yet to be identified.

Azo-dye-bound peptide. Pronase digestion of the basic azo-dye-binding protein gave only one azo-dye-containing fragment that proved to be a peptide containing the amino acids glycine, serine, threonine, alanine, glutamic acid and aspartic acid as well as the as yet unidentified amino acid that binds the azo-dye. Other workers have also isolated azo-dye-bound peptide material, but from digests of whole liver protein. The azo-dye-bound peptide fraction obtained by Hughes (1959) contained proline, leucine/isoleucine, valine and glycine as major components and glutamic acid, phenyl-

alanine, serine, alanine and aspartic acid as minor components. Terayama & Takeuchi (1962) isolated a fraction that on hydrolysis gave phenylalanine, serine, glycine, proline, valine, glutamic acid and aspartic acid. The discrepancies between these earlier results and the ones now published may be due to two factors. First, the peptide fractions described previously are less likely to have been obtained pure since the difficulties inherent in the isolation of a single peptide from a digest of whole liver protein, where peptides from a number of proteins have to be dealt with, are much greater than when a single protein is the starting point. Secondly, more than one azo-dye-binding protein exists and it is possible that the azo-dye may bind to different amino acid sequences in each protein, giving rise to several different azo-dye-bound peptides. Consequently, it is possible that different workers have been studying different peptides.

The isolation of 3-mercaptomethyl-4-monomethylaminoazobenzene from alkali-treated homogenates of livers from rats given 4-dimethylaminoazobenzene suggests that the azo-dye may bind to methionine residues, giving rise to a sulphonium compound that is unstable and that can undergo two reactions. First, in alkali it can split at the sulphur atom to give 3-mercaptomethyl-4-monomethylaminoazobenzene and a homoserine residue, and, secondly, it is capable of methyl group transfer, giving rise to azo-dye bound to protein through an alkali-stable thio ether bond (Scribner et al. 1965). Only traces of non-polar free dye could, however, be extracted from alkali-treated solutions of the basic azo-dye-binding protein. As a result, it seems possible that, if methionine is involved in the binding of the dye to the basic protein, methyl group transfer has occurred during the purification and the dye binding has become alkali-stable. It is noteworthy that the basic protein has a relatively high methionine content.

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