Fractionation of Azo-Peptides in Tryptic Digests of Rat Liver

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Since the pioneer demonstration in 1947 by Miller and Miller (8) of the incorporation of the carcinogen 4-dimethylaminoazobenzene (DAB) into protein of the target organ, rat liver, a growing body of evidence has accumulated which suggests that carcinogen binding is of causal significance in carcinogenesis (5, 9, 10). The importance of these findings has stimulated much further research into this binding of aminoazo dyes. These efforts have been directed toward establishing the binding site(s) in the carcinogenic molecule (11-13) and to characterizing the actual protein(s) involved (16, 17). However, at present little information is available concerning the reactive site(s) in the involved proteins. Kusama and Terayama (6) have fractionated the peptides resulting from digestion of rat liver with a protease obtained from a Streptomyces culture and have identified the following amino acids in enzymatically prepared polar dye—proline, glycine, phenylalanine, lysine (only in polar dye prepared from the particulate fraction of rat liver), leucine, and valine together with lesser amounts of aspartic acid, serine, alanine, and glutamic acid. These same authors have also synthesized polar dyelike compounds from aminoazo dyes, amino acids, and formaldehyde and on the basis of spectroscopic evidence consider a tyrosine-aminoazobenzene combination as the most probable model for the polar dye. In an effort to determine whether any specific amino acid sequence is associated with the dye binding site(s), the peptides resulting from tryptic digestion of the livers of rats given the dye have been fractionated, and some of the results are recorded here.

MATERIALS AND METHODS

Preparation of materials.—3'-Methyl-4-dimethylaminoazobenzene (3'-Me-DAB), m.p. 118° C., was synthesized by the method of Giese, Miller, and Baumann (4).

DAB-C¹⁴ labeled generally on the prime ring was prepared from aniline-C¹⁴ by diazotization and coupling with dimethylaniline. This was chromatographed on an alumina column, being eluted with benzene. After crystallization from ethanol the dye had a m.p. of 117° C. and an activity of 1 mc/5.0 mmoles.

Adult rats of Wistar strain, fed a stock diet of Barastoc (a local proprietary food), were injected intraperitoneally with either 1.0 ml/100 gm body weight of a 2 per cent solution of 3'-Me-DAB in olive oil or with 50 gc. DAB-C¹⁴ in olive oil. After 48 hours the rats were sacrificed and their livers homogenized in water in a Potter-Elvehjem homogenizer. After the addition of an aliquot of 1.0 mole acetate buffer, pH 4.0, the homogenates were boiled for 3 minutes, filtered, and the precipitate was washed with buffer and then with ethanol. The precipitated proteins were extracted in a Soxhlet apparatus for 48 hours with ethanol.

The dried liver powders were then digested at 37° C. with 3 per cent by weight of twice-crystallized trypsin² with 50 per cent MgSO₄ which had previously been dialyzed overnight against distilled water. The digest was prepared by suspending the extracted liver powders in water (50 ml/gm) and adding the trypsin and a little toluene as preservative. The pH was adjusted to 8.5-8.6 twice daily by the addition of ammonium hydroxide. When digestion was slow, fresh trypsin was added on the 4th or 5th day. After 10-14 days' digestion the digest was extracted with petroleum ether and then boiled for 3 minutes, filtered, and freeze-dried.

Preliminary investigations on the resulting peptide mixture involved paper chromatography, starch gel electrophoresis, and counter-current distribution.

In preliminary experiments samples of the peptide digest were subjected to starch gel electrophoresis by the method of Smithies (15) at various pH values over the range 2.0-8.6.

¹ Obtained from the Radiochemical Centre, Amersham, Bucks, England.
² Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.
As a result of preliminary studies, only one colored band which turned pink on acidification was found at each pH (see "Results"), and within the limits of the method the colored peptide appeared electrophoretically homogeneous. Starch gel electrophoresis was not used in subsequent preparative experiments owing to all available samples of starch giving a faint Biuret reaction.

Three- to 4-gm. samples of the peptide digest were subjected to trial in a counter-current distribution apparatus of Lathe and Ruthven (7) design by the solvent system n-butanol:acetic acid: water (4:1:5) at 23° ± 2° C. Fifty-one transfers were performed, and the original peptide mixture was introduced into two tubes (containing 30 ml. of each phase per tube). Each equilibration was effected by being shaken 100 times. K, which is defined as the ratio of colored peptide in the aqueous phase to that in the organic phase, is determined from counter-current distribution data by use of the formula $N = nK/K + 1$, where $n$ is the number of transfers and $N$ the tube in which maximal intensity is found.

Because of the presence of interfering fragments in the contents of many tubes, spectroscopic examination of the aqueous phase was found to be unsuitable for locating the colored peptide(s). Therefore, the following procedure was adopted. Sufficient glacial acetic acid (4.5 ml.) was added to the contents of each tube to give a single phase; 8 ml. of this phase and 1 ml. of 12 ± HCl were placed in long test tubes 0.5 cm. in diameter. These tubes were then examined in sunlight so that the light, after traversing the contents of the tube longitudinally, fell on a white background. In this way some tubes were found to show a pink color, while others were pale yellow.

The following method was used for recovery of the peptides from selected tubes. The contents of the appropriate tubes were pooled, and the volume was measured. Concentrated HCl was added to make the total volume 0.5 x. Five volumes of peroxide-free ether were added, and after being shaken well the mixture was allowed to settle. The aqueous phase was removed. The organic phase was re-extracted with 30 ml. of 1 N HCl, and the aqueous phases were combined. The pH of the combined aqueous phases was adjusted to 4.0 with concentrated KOH. Sufficient trichloroacetic acid (TCA), 100 per cent (w/v), was added to bring the TCA concentration to 5 per cent in the aqueous phase and then sufficient n-butanol (15 per cent volume) to give a small amount of extra phase. The colored material was extracted with shaking into the small volume of n-butanol. The aqueous phase was re-extracted with a small volume of n-butanol. The combined butanol extracts were washed once with $\frac{1}{3}$ volume of water. The colored material remained in the butanol. The dye was redistributed into $\frac{4}{10}$ volume 1 N HCl by adding 5 volumes of peroxide-free ether. The organic phase was re-extracted with $\frac{3}{10}$ volume 1 N HCl and the combined aqueous phases dried in aaeuo over KOH.

In subsequent preparative experiments the first step in the above concentration procedure was avoided by taking the colored peptide into the organic phase of the counter-current mixture with TCA just insufficient to give a single phase, discarding the aqueous phase, and then recovering the peptide from the organic phase with ether and HCl.

During the recovery of the colored peptide from the counter-current tube contents some material (presumably some of the larger peptides) precipitated at the interface. This material was collected and moistened with 12 N HCl; however, no pink color developed. All except the first two fractionations were performed with a Craig type of counter-current apparatus.

In view of the difficulties experienced above in detecting colored peptides in the contents of the counter-current tubes, it appeared that the use of C14-labeled dye would provide a sensitive method for locating peptides with bound carcinogen. The peptides obtained from tryptic digestion of the livers of rats given DAB-C14 were subjected to counter-current distribution fractionation by the same solvent system as before. The contents of each tube were recovered in the usual way, and the peptide concentrate was dried in vacuo over KOH on a-inch-diameter watch glasses. Counting was performed by a Geiger-Müller tube with a mica window 2.3 mg/sq cm.

A portion of the colored peptide concentrate (obtained from livers when unlabeled dye was used) was dinitrophenylated (14), and the DNP peptides were taken up in 2 drops of 5.7 N HCl in a capillary tube. The tube was sealed and hydrolysis performed overnight (16 hours) at 105° C. The hydrolysate was diluted to 1 N HCl and then extracted with four 5-ml. portions of peroxide-free ether. The organic and aqueous phases were taken to dryness and residues subjected to chromatography at 23° ± 3° C. on Whatman No. 1 paper using the developing solvent system n-pentanol: 2 N NH4OH (1:1). Pink spots of several DNP derivatives were found (see "Results").

Other portions of the colored peptide concentrate were subjected to starch gel electrophoresis at pH 8.6 (borate buffer) and to two-dimensional paper chromatography with n-butanol:acetic acid.
acid: water (4:1:5) and phenol: water: HCN as developing solvent systems. The colored peptide concentrate was dissolved in water (in which it was freely soluble) for introduction onto the paper (lithium hydroxide-washed Whatman No. 3 paper) (1). After being dried the chromatogram was exposed to HCl vapor and any change to a pink color noted. After standing in a hood for 1 hour it was stained with ninhydrin. Only an extremely faint spot appeared after ninhydrin staining. The area which gave a pink color with HCl did not correspond to that giving a faint ninhydrin reaction (see “Results”).

From other chromatograms of the colored peptide concentrate the area giving a pink color with HCl was cut out and eluted with a mixture of ethanol and 1 N HCl (1:1) and dried in vacuo over KOH. Both the eluted material and a sample of the colored peptide concentrate (from the counter-current distribution fractionation) were hydrolyzed in sealed tubes with 2 drops of 5.7 N HCl at 105°C for 16 hours. The hydrolysates were dried in vacuo and then chromatographed on washed Whatman No. 3 paper in n-butanol:acetic acid: water (4:1:5) and phenol: water: HCN in two dimensions. After being dried the papers were sprayed with ninhydrin.

In subsequent preparation experiments the crude peptide mixture was first subjected to a counter-current distribution fractionation, and the peptides were recovered from the appropriate tubes. These peptides were further purified by two-dimensional paper chromatography (n-butanol: acetic acid: water 4:1:5) and phenol: water: HCN. The area showing a pink color on exposure to HCl was then eluted and, after hydrolysis with 5.7 N HCl in sealed tubes at 105°C for 16 hours,

The phenol/water/HCN system used was prepared by adding 150 ml. water to 500 gm. of redistilled phenol; after the mixture was allowed to stand overnight, the phenol was saturated with HCN vapor.

RESULTS

In the counter-current distribution fractionation the tubes showing a pink color on acidification were always consecutive. With 51 transfers, tubes 3–10 contained the colored peptide when the aqueous phase was transferred, and tubes 40–48 when the Craig apparatus was used.

The use of C14-labeled carcinogen as an aid in detecting the colored peptide(s) was unsatisfactory. Equally high peaks of radioactivity occurred in tubes which gave no pink color with unlabeled dye as in those which showed a pink color on acidification. When the colored peptide concentrate obtained after counter-current distribution fractionation was subjected to electrophoresis in either borate or ammonium hydroxide buffer, the yellow material, which was now much more easily seen, again formed a single band. However, up to 30 per cent of the dye-peptide was lost in the process. Whether this is owing to absorption on the starch or to the separation of minor colored components into bands too faint to be seen has not yet been determined.

In determining the end groups of the peptides in the colored peptide concentrate obtained after counter-current distribution concentration, the following spots were found on the chromatograms besides the usual artefacts. The aqueous phase fraction showed a very faint spot of Rf value 0.20; the ethereal phase, three faint yellow spots of Rf values of 0.065, 0.28, and 0.56; and two extremely faint spots, Rf values of 0.15 and 0.225, which fluoresced white in ultraviolet light.

Two-dimensional chromatography of the colored peptide concentrate on Whatman No. 3 paper with the use of n-butanol: acetic acid: water (4:1:5) and phenol: water: HCN gave a single spot (Rf values of 0.61 and 0.87, respectively) which showed a pink color on exposure to HCl vapor. On subsequent staining with ninhydrin a pink spot appeared. This was roughly pyriform in shape (see Chart 1). The material eluted from the area which showed a pink color on exposure to HCl gave, after hydrolysis and chromatography, ten or eleven bright spots with ninhydrin. This material, which contained between 35 and 45 per cent of the bound dye present in the original liver, was ninhydrin-negative prior to hydrolysis. Elution of other ninhydrin-negative areas from these chromatograms and subsequent hydrolysis did not give any ninhydrin-positive material.

The following amino acids have been identified...
in the hydrolysate—major components (in order of concentration): proline, leucine isomers, valine, glycine; minor components: glutamic acid, phenylalanine, serine, alanine, and possibly aspartic acid. In addition, other ninhydrin-positive material of $R_f$ values of 0.00 in n-butanol:acetic acid:water, and 0.48–0.68 in phenol:water:HCN has been found in most hydrolysates. Elution of areas corresponding to this material from other chromatograms and further hydrolysis for 16 hours at 105°C in 5.7 N HCl did not yield any additional ninhydrin-positive spots. However, in view of the small amounts of material available, this result does not exclude this material's being small peptide(s). Dinitrophenylation of the colored peptide prior to hydrolysis did not yield any DNP amino acids after hydrolysis.

DISCUSSION

Attempts have been made to elicit information as to the specificity of binding sites of aminoazo dyes in rat liver proteins. Whole liver rather than any particular azo-protein concentrate was chosen as a substrate for trypic digestion. Tryptic digestion was employed because of its high specificity for bonds involving lysine and arginine, thus giving a lesser number of fragments than other methods of degradation.

Difficulty was encountered in detecting azo-peptides. Spectroscopy was found to be unsuitable because of high absorption in some tubes from interfering pigments and to the low concentration of the dye in all tubes. The use of C14-labeled dye was most disappointing. It appears that metabolites of the dye also become bound, and this limits the applicability of the technic. The binding of labeled aniline, p-phenylene diamine, and its methylated derivatives (3) indicates the limitations and need for caution in employing isotopically labeled carcinogens in this type of study.

Within the limits of the experimental methods used it appears that at least the major portion of the bound dye is associated with a particular peptide. Thus, starch gel electrophoresis at a variety of pH values, counter-current distribution fractionation, and paper chromatography did not resolve the material giving a pink color on acidification into more than one major component.

On the other hand, the loss of colored material during electrophoresis of colored peptide concentrate suggests that the colored material may have been resolved into other bands whose concentration was too low to be detected visually.

Gelboin, Miller, and Miller (2) have reported that the polar-bound dyes obtained after injection of a massive dose of carcinogen have chemical and spectral characteristics similar to those obtained after prolonged feeding, so that the results obtained in this study should be applicable to azo-peptides obtained after feeding experiments.

The most interesting feature of the colored material isolated from trypic digests is that, while it is itself ninhydrin-negative, it gives a plethora of ninhydrin-positive spots after hydrolysis. The material is evidently either a cyclic peptide or a peptide in which its terminal $\alpha$-amino group is masked, possibly by attachment of the dye.

The ninhydrin-positive material commonly found in hydrolysates of colored peptide which was not identifiable as any known naturally occurring amino acid is probably small peptide(s). Only minute quantities were available for further hydrolysis, so the failure to liberate fresh amino acids is probably not significant. Alternatively, this material may represent dye attached to an amino acid side chain.

It will be noted that the amino acid composition of the colored peptide shows a preponderance of those amino acids with an affinity for the organic phase in the n-butanol:acetic acid:water system used in the counter-current separation. In view of this it is quite possible that other azo-peptides, containing predominantly amino acids which favor the aqueous phase, were present in the digest but have not been recognized as such because of being masked by other pigments in the tubes in which they occur.

Apart from the apparent absence of lysine and possibly aspartic acid there is agreement (despite the different enzymes used in preparation) between the amino acids found in azo-peptide by Kusama and Terayama (6) and those found in these studies. However, both methods utilize steps involving extraction of azo-peptides with butanol, and this in itself would concentrate peptides with a preponderance of amino acids with lipophilic side chains.

While these results suggest that a specific protein site is involved in azo dye binding, they are by no means conclusively indicative of this. Such a conclusion must be considered in the light of the limitations of the technics involved and in particular of the possibility of overlooking other peaks of azo-peptides.

A possible criticism may be leveled at the consecutive use of the solvent system n-butanol:acetic acid:water (4:1:5) for both the counter-current distribution and subsequent paper chromatography of the peptides. However, absorption on the paper introduces an additional variable, and the system used was found satisfactory in that it gave some resolution of the material giving a
ninhydrin-positive reaction from that giving a pink color on acidification.

SUMMARY
1. Tryptic digests of the extracted proteins, prepared from the livers of rats given aminoazo dyes, have been fractionated by the technics of starch gel electrophoresis, counter-current distribution, and paper chromatography.

2. Difficulty was experienced in finding a suitable method for detecting dye-peptides. Both spectroscopy and the use of C14-labeled dye were unsuitable. When the relatively crude method of visual detection of a pink color was used after acidification, only one colored component could be demonstrated.

3. This material was ninhydrin-negative but gave a plethora of ninhydrin-positive spots after hydrolysis. It is probably a cyclic peptide, but the possibility that the carcinogen is attached to an α-amino group has not been excluded.

4. The following amino acids have been identified in hydrolysates of the peptide: proline, both leucine isomers, valine, glycine, glutamic acid, phenylalanine, serine, alanine, and possibly aspartic acid.

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REFERENCES
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