Partial Purification of Soluble Protein from Mouse Skin to Which Carcinogenic Hydrocarbons Are Specifically Bound*

Judith G. Tasseron,† Heino Diringer,‡ Nechama Frohwirth,§ S. S. Mirvish,|| and Charles Heidelberger#

ABSTRACT: Partial purification has been carried out of the protein fraction from mouse skin to which carcinogenic hydrocarbons are covalently bound in direct relationship to their carcinogenic activity. This has been termed the *h* fraction.

Purification has been carried out on the 100,000*g* supernatant fraction of an extract of mouse skin taken 48 hr after topical application of a labeled polycyclic hydrocarbon. The centrifuged extract was passed through DEAE-cellulose and after concentration, a column of Sephadex G-100. A symmetrical radioactive peak was obtained, corresponding to a molecular weight of 40,000. This was then subjected to isoelectric focusing on a column, and a peak obtained at pH 8.05 contained the *h* protein from the carcinogenic 1,2,5,6-dibenzanthracene and 3-methylcholanthrene; this peak was not found with the noncarcinogenic 1,2,3,4-dibenzanthracene. This peak contained one radioactive and one nonradioactive protein, both with molecular weights of about 40,000 and represents (in the case of 3-methylcholanthrene) a purification of 560-fold. Electrophoresis of this radioactive protein fraction on polyacrylamide gel in the presence of sodium dodecyl sulfate and 8 M urea, gave a single sharp peak with a molecular weight of 20,000. Thus, the *h* protein consists of two similar or identical subunits.

Following the initial demonstration by Miller (1951) of the binding of carcinogenic hydrocarbons to mouse epidermal proteins using fluorescent techniques, a study of this phenomenon has continued in this laboratory by means of tracer methodology. Wiest and Heidelberger (1953) established the covalent nature of this interaction in the case 1,2,5,6-DBA,[1] and a rough correlation between the binding to total mouse skin proteins of various polycyclic aromatic hydrocarbons and their carcinogenic activity was demonstrated by Heidelberger and Moldenhauer (1956). On preliminary fractionation of the proteins of mouse skin, the hydrocarbons did not appear to be bound to any specific fraction (Davenport *et al.*, 1961). However, Abell and Heidelberger (1962) carried out starch gel electrophoresis of the soluble proteins of mouse skin following topical application of tritiated hydrocarbons and found a radioactive band with covalently bound carcinogens. They described the striking correlation between the binding to this protein fraction and the carcinogenic process; hence, it was of obvious interest to attempt to purify, isolate, and characterize this protein from mouse skin.

The partial purification of the rat liver protein to which the azo dyes and acetylaminofluorene are bound has been accomplished by Sorof and his colleagues (*cf.* 1963), using vertical column electrophoresis. He has termed this the h_2 fraction. His more recent work has been reviewed (Sorof, 1969). Barry and Gutmann (1966) have also partially purified the rat liver protein to which acetylaminofluorene is bound, by chromatography on Sephadex G-25 and DEAE-cellulose. Apparently the most complete purification of this rat liver protein to date has been accomplished by Ketterer *et al.* (1967). However, the identity of this as the main protein to which the azo dyes are bound has been questioned by Sorof (1969). Since the protein in mouse skin to which the hydrocarbons are bound in quantitative relationship to their carcinogenic activities has some properties similar to the *h* protein of Sorof, we will henceforth refer to this as the mouse skin *h* protein.

Materials and Methods

Animals. Female Swiss albino mice weighing 25–30 g, obtained from the A. R. Schmidt Co., Madison, Wis., were used. They were fed Rockland pellets and water *ad libitum*.

Chemicals. Tritiated 1,2,5,6-DBA, 1,2,3,4-DBA, and 3-MCA were obtained from Amersham-Searle. They were diluted with the corresponding nonradioactive hydrocarbons obtained from Eastman Organic Chemicals to a suitable final specific activity, in most cases 100 mCi/mmol. The radiochemical purity of the hydrocarbons was periodically checked by thin-layer chromatography, and only homogeneous samples were used.

The following proteins were used for the molecular weight determinations: ovalbumin from the Nutritional Biochemicals Corp., horse heart cytochrome *c* and papain from the Sigma Chemical Corp., deoxyribonuclease I and elastase from Worthington Biochemical Corp., transferrin from Behring-

* From the McArdle Laboratory for Cancer Research, The Medical School, University of Wisconsin, Madison, Wisconsin 53706. *Received November 13, 1969.* This work was supported in part by Grant CA-7175 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

† Present address: Department of Biochemistry, University of Leiden, Leiden, The Netherlands.

‡ Holder of a Postdoctoral fellowship from the Damon Runyon Memorial Fund. Present address: Max-Planck Institut fur Virusforschung, Tubingen, Germany.

§ Present address: Department of Biochemistry, University of Tel-Aviv Medical School, Tel-Aviv, Israel.

|| Present address: Eppley Institute for Cancer Research, Omaha, Neb.

American Cancer Society Professor of Oncology.

[1] Abbreviations used are: DBA, dibenzanthracene; MCA, methylcholanthrene.

werke, bovine serum albumin from Pentex Inc., and bromgrass mosaic virus.

Measurements of Radioactivity. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Aqueous solutions were counted in Scintisol (Isolab, Inc.).

Radioactivity was measured in protein precipitates by dissolving them in 1 ml of Soluene (Packard Instrument Co.) to which 10 ml of toluene–2,5-diphenyloxazole (Liquifluor, New England Nuclear Corp.) was added.

Slices of stained polyacrylamide gels were incubated with 0.5 ml of Soluene in capped counting vials at 50° overnight. This procedure made the pieces swell considerably and eluted the protein from the gel. After addition of 10 ml of toluene–2,5-diphenyloxazole the slices were counted. This method gives a higher counting efficiency than digestion of the gel slices by the hydrogen peroxide method of Young and Fulhorst (1965).

Preparation of the Skin Protein Extract. ^{3}H-Labeled hydrocarbon (0.1 mg in 0.1 ml of benzene/mouse, 100 mCi/mmol) was dropped on the backs of 100 mice, which had been clipped the previous day. After 48 hr, when maximum binding occurs (Abell and Heidelberger, 1962) the mice were killed with ether. The skins of the backs were dissected and the adipose tissue and blood vessels were removed by scraping the inside of the skin with razor blades. The skins were frozen in liquid nitrogen immediately. The frozen skins were pulverized inside four thicknesses of cheesecloth with a wooden mallet and homogenized in 170 ml of 0.01 M Tris-HCl buffer (pH 8.0), containing 0.01 M NaCl, with a Polytron (Type PT 10 O.D. Kinematica GMBA, Luzern, Switzerland). The homogenate was centrifuged at 100,000*g* for 1 hr and the pink supernatant was filtered through glass wool to remove the layer of fat.

Purification of the Mouse Skin h Protein. All purification steps were performed at 4° in a cold room.

STEP 1. SEPARATION OF THE LOW AND HIGH MOLECULAR WEIGHT SUBSTANCES. The high-speed supernatant fraction (175 ml from 100 mice) was passed through a Sephadex G-25 column (6.5 × 40 cm) with the same 0.01 M Tris-HCl buffer (pH 8.0) containing 0.01 M NaCl used for the extraction. The high molecular weight fraction was well separated from the low molecular weight material and was collected in fractions 10–26, each tube containing 16 ml. Elution of the low molecular weight material started at about fraction 28. The radioactivity in this latter fraction trailed over a big volume, which was found with all hydrocarbons. The adsorption of low molecular weight aromatic compounds to Sephadex is well known (*cf.* Determann and Walter, 1968).

STEP 2. DEAE-CELLULOSE CHROMATOGRAPHY. The Sephadex G-25 high molecular weight fraction (about 250 ml) was immediately passed through a DEAE-cellulose column (Serva, 0.7 mequiv/g, 15 g/100 skins, column 3.5 × 7 cm) in the same buffer as used for Sephadex G-25 fractionation. The least acidic proteins passed through the column without retention. This fraction was concentrated to 20–30 ml according to the method of Blatt *et al.* (1965) by ultrafiltration through a Diaflo UM10 membrane (Amicon Corp.), which retains substances of molecular weight greater than 10,000. Further concentration to 2–3 ml was achieved by reverse dialysis with Ficoll powder (Pharmacia). During these concentration procedures the small amount of precipitate that formed was checked for radioactivity, which was invariably low, and it was discarded.

STEP 3. SEPHADEX G-100 GEL FILTRATION. The concentrated DEAE-cellulose breakthrough peak was carefully layered on top of a Sephadex G-100 column (2.5 × 140 cm). The column was developed with 0.01 M Tris-HCl buffer (pH 8.0), containing 0.01 M NaCl and 0.005 M β-mercaptoethanol at a flow rate of approximately 15 ml/hr. Fractions of 8 ml were collected. The radioactive peak was concentrated by dialysis against Ficoll. The concentrated fraction was stored frozen after addition of one-tenth volume glycerol or was used fresh for the isoelectric focusing procedure. Repeated freezing and thawing did not affect the proteins, as shown on Sephadex G-100 and by isoelectric focusing in polyacrylamide gels, which gave the same patterns as the fresh unfrozen solution.

STEP 4. ISOELECTRIC FOCUSING PROCEDURE. The method described by Vesterberg and Svensson (1966) was used. The isoelectric focusing was performed in an LKB8100 electrofocusing column. The pH gradient used was either from pH 6 to 9 or from pH 7 to 9. These gradients were obtained with carrier ampholytes (LKB Instruments, Inc.). The anode was placed at the top of the column, the cathode at the bottom. Between the electrode solutions a sucrose density gradient containing 1% carrier ampholytes was arranged to prevent convection during the separation. The gradient was prepared in 24 tubes containing 4.6 ml of a mixture of systematically varied amounts of a 50% sucrose solution containing 1.5% ampholytes and a less dense solution (without sucrose) containing 0.5% ampholytes. The first tube contained 4.6 ml of the dense solution, the second contained 4.4 ml of dense solution and 0.2 ml of less dense solution, and so on. The radioactive *h* protein fraction eluted from the Sephadex G-100 column replaced the less dense solutions in the 11–13 tubes, after dialysis overnight against 1% ampholytes. The contents of the individual tubes were well mixed before they were successively poured down the glass wall into the column: the most dense solution was added first.

The column was run for 3 days and was cooled with water circulated at 4°. The voltage applied at first was 300 V, which was increased to 500 V after 24 hr and 600 V after 48 hr. After the run, fractions of 36 drops were collected. The pH of the fractions was measured with a Radiometer pH meter, Model PHM26. The fractions were assayed for radioactivity and optical density at 260 and 280 mμ. The radioactive fractions in the pH 8.05 region were concentrated by dialysis against Ficoll and kept frozen after addition of a 10% volume of glycerol for further testing. Replicate runs were highly reproducible.

Disc electrophoresis was carried out in the equipment made by Canalco, Rockville, Md. Three different types of electrophoresis were used.

BASIC SYSTEM, 7% POLYACRYLAMIDE GEL. The polymerization solution contained volumes of solutions A–B–C–H_2O of 1:2:4:1. Solution A contained 36.3 g of Tris, 48 ml of 1 N HCl, 0.23 ml of *N,N,N,N'*-tetramethylethylenediamine, and H_2O to 100 ml. Solution B contained 28.0 g of acrylamide, 0.735 g of *N,N'*-methylenebisacrylamide, and H_2O to 100 ml. Solution C (always freshly made) contained 0.14 g of ammonium persulfate in 100 ml of H_2O. The gels were 5.5 mm wide and 6 cm high. The tray buffer solution contained 0.0248 M Tris and 0.192 M glycine per l. (pH 8.3). The run was per-

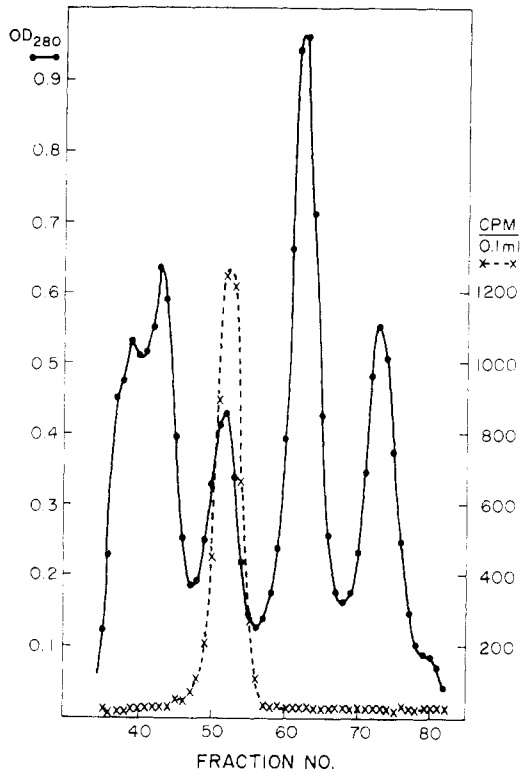

FIGURE 1: Sephadex G-100 gel filtration of the breakthrough peak from a DEAE-cellulose column. The fractions contained 7 ml. The buffer was 0.01 M Tris-HCl (pH 8.0) containing 0.01 M NaCl and 0.005 M β-mercaptoethanol. The mice had been treated with MCA.

formed at 4° and at 4 mA/gel. The gels were fixed and stained in an Amido Black solution in 7.5% acetic acid and destained by repeated washing in 7.5% acetic acid.

BASIC SYSTEM, DIFFERENT POLYACRYLAMIDE GEL CONCENTRATIONS. A variation of the first method was used for the estimation of molecular weight (Hedrick and Smith, 1968). Solution B contained 24 g of acrylamide and 0.8 g of *N N'*-methylenebisacrylamide. By proper dilutions gels were made containing 5, 7, 9, and 11% acrylamide. The molecular weight of proteins can be calculated from the slope of the graph where 100 log ($R_m \times 100$) is plotted against the gel concentration, where R_m is the mobility relative to bromophenol blue.

SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL SYSTEM. A modification of the system described by Shapiro *et al.* (1967) was used. The gels contained 0.1% sodium dodecyl sulfate, 0.1 M Tris (pH 7.3), 7% acrylamide, 0.4% *N,N'*-methylenebisacrylamide, and 8 M urea. The tray buffer contained 0.1 M Tris (pH 7.3), 8 M urea, 0.1% sodium dodecyl sulfate, and 0.1% 2-aminoethanethiol hydrochloride. This buffer was used in the bottom electrode bath. The same buffer without urea was used in the top electrode bath. The sample contained 50 μg of protein in 0.1 M Tris (pH 7.3), 8 M urea, 1% sodium dodecyl sulfate, and 0.1% 2-aminoethanethiol hydrochloride. The electrophoresis was carried out at room temperature at 7 mA/gel. The gels were fixed in 10% acetic acid, 50% methanol, and 40% H_2O for 2 hr, stained in 20% methanol, 0.5% Amido Black, and 2.2% acetic acid for 2 hr and destained by repeated washing with 35% ethanol and 7.5% acetic acid.

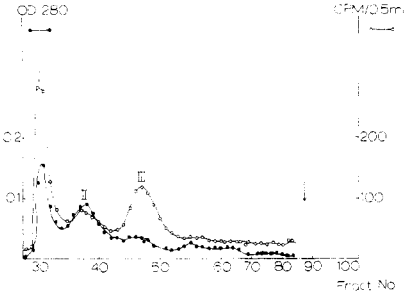

FIGURE 2: Sephadex G-100 gel filtration of the 100,000*g* supernatant fraction of the skin homogenate. The column was 2.5 × 140 cm, and 7.5 ml fractions were collected. The buffer was the same as for Figure 1, but without the β-mercaptoethanol. The mice had been treated with 1,2,5,6-dibenzanthracene.

Before measurements were made the gels were allowed to swell in 7.5% acetic acid. The system was standardized with proteins of known molecular weight.

Isoelectric Focusing in Polyacrylamide Gels. The method described by Wrigley (1968) was used. The gels consisted of A–H_2O (1:3, v/v). Solution A contained 0.8 ml of catalyst solution, 3.0 ml of acrylamide solution, and 0.3 ml of carrier ampholytes (40%). The catalyst solution contained 1 ml of *N,N,N',N'*-tetramethylethylenediamine and 14 mg of riboflavin, and was made to 100 ml with H_2O. The acrylamide solution contained 0.8 g of *N,N'*-methylenebisacrylamide and 30 g of acrylamide, and was made to 100 ml with H_2O. If the protein sample was mixed in the gels, 1 ml of gel mixture was used per tube (4 × 120 mm). If the sample was not mixed in the gel, 0.8 ml of gel mixture was used per tube. In the latter case the sample was put on top of the gel in 0.1 ml of 10% sucrose, on top of which 0.3 ml of 5% sucrose solution containing 1% carrier ampholytes was layered. The electrophoresis was carried out overnight in the cold room at 250 V; the last 1 or 2 hr were at 350 V. The gels were fixed and washed in 10% trichloroacetic acid, stained with coomassie brilliant blue in 10% trichloroacetic acid, and destained in 40% ethanol. Before measurements were made the gels were allowed to swell in 10% trichloroacetic acid.

For determination of the radioactivity the gels were cut into 1-mm slices with a gel slicer (Model 3015 Diversified Scientific Instruments, Inc.). The slices were then treated as described under measurements of radioactivity.

Results

Several extraction conditions for the mouse skin soluble proteins have been used during the years this work has been carried out. The most convenient method involves extraction with the same buffer (described in Materials and Methods) that is used for the subsequent column procedures. The homogenate thus extracted was centrifuged at 100,000*g* for 1 hr, and the supernatant fraction was passed through a Sephadex G-25 column. The high molecular weight fraction contained 25–30% of the OD_{280} units and radioactivity; the latter represents about 0.05% of the original amount of radioactivity applied to the skin as 1,2,5,6-dibenzanthracene-*t*. In the low molecular weight fraction, 90% of the label was accounted for as tritiated water, indicating that considerable

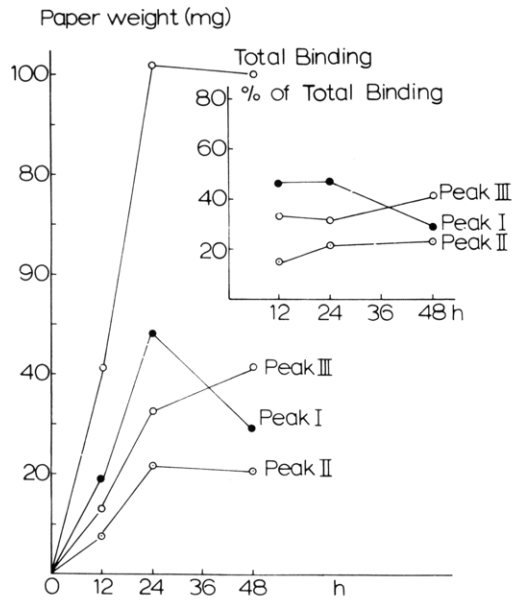

FIGURE 3: Time dependence of the binding of 1,2,5,6-dibenzanthracene-*t* to different high molecular weight fractions. At the indicated time after application, the 100,000*g* supernatant fraction was fractionated on Sephadex G-100 as shown in Figure 2, the radioactivity was determined in the individual fractions, and the results were graphed and expressed as the weight of the graph paper to give the integrated radioactivity.

exchange had occurred. However, this exchange could not account for the radioactivity in the *h* protein because of the large dilution, the nonuniform distribution among the various soluble proteins, and current studies in the laboratory on the structure of the protein-bound hydrocarbon derivatives.

The least acidic protein fraction was obtained by chromatography on DEAE-cellulose, by which Moore and Lee (1960) had separated the basic proteins of rat liver. They used an initial buffer of 0.005 M Tris–phosphate (pH 8.0) and subsequently a gradient to 0.5 M sodium phosphate, pH 6.5. We adopted a two-step elution first with 0.01 M Tris-HCl (pH 8.0) containing 0.01 M NaCl followed by 0.05 M sodium phosphate (pH 6.5) containing 1 M NaCl. The protein fraction (8–10%) that was not retained by the column, had a specific activity 2–2.5-fold higher than that from the Sephadex G-25. The protein fraction eluted with the second buffer had about a 5-fold lower specific activity than the first fraction.

When Sephadex G-100 gel filtration was carried out on the first fraction from the DEAE-cellulose column, a single symmetrical peak of radioactivity was obtained as shown in Figure 1 (MCA-treated mice) and as also reported by Umeda *et al.* (1968). With the 0.01 M Tris-HCl buffer (pH 8.0) containing 0.01 M NaCl, the recovery of radioactivity from the column was rather low (40–50%). The addition of 0.005 M β-mercaptoethanol increased the total recovery to 70–75%. A comparison of the elution volume of this radioactive peak from Sephadex G-100 with those of bovine serum albumin and horse heart cytochrome *c* gave, by the method of Andrews (1964), a molecular weight of about 40,000 for the *h* protein. When the second fraction eluted from the DEAE-cellulose column was run on a Sephadex G-100 column, nearly all the radioactivity was associated with molecules of a molecular weight greater than 40,000.

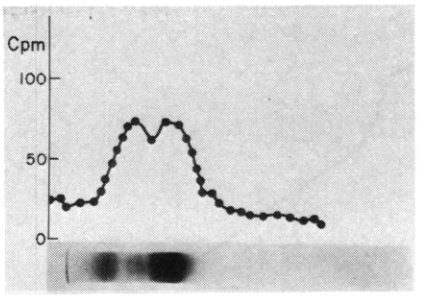

FIGURE 4: Polyacrylamide gel disc electrophoresis of the *h* protein fraction after Sephadex G-100 fractionation of the DEAE-cellulose eluate of proteins extracted from mice treated with 1,2,5,6-DBA. The graph shows the distribution of the radioactivity.

The Sephadex G-100 column was used directly to test the unfractionated skin supernatant fraction for the time course of the binding of 1,2,5,6-DBA. As shown in Figure 2, there were three peaks of high molecular weight with bound radioactivity, well separated from the small molecules. In agreement with the results of Abell and Heidelberger (1962), the maximum specific activity was found 24–48 hr after application of the hydrocarbons as shown in Figure 3. The mouse skin *h* protein fraction is found in the third peak of Figure 2, which shows an increasing amount of radioactivity until 48 hr.

The single sharp and symmetrical radioactive peak obtained on Sephadex G-100 fractionation of the DEAE-cellulose eluate from mice treated with MCA (Figure 1) suggested that at this stage of purification we might have had one protein to which the radioactive hydrocarbon was bound together with other nonradioactive proteins. However, polyacrylamide electrophoresis never resulted in a sharp radioactive peak (Figure 4) and the fact that chromatography of the skin *h* protein after Sephadex G-100 fractionation on CM-cellulose showed a radioactive pattern definitely not consistent with one radioactive protein (Figure 5), demonstrated that we had not yet achieved purity.

Further proof for the presence of more than one labeled protein in the radioactive *h* protein fraction after the Sephadex G-100 column came from a comparison, using these methods, of the binding of MCA, 1,2,5,6-DBA, and its non- (or very weakly) carcinogenic isomer, 1,2,3,4-DBA to the *h* protein fraction.

The results summarized in Table I show that MCA was bound to a greater extent than the two dibenzanthracenes at

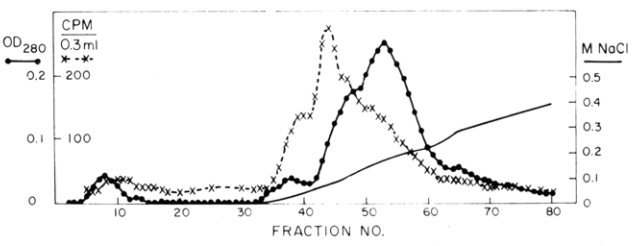

FIGURE 5: Carboxymethylcellulose chromatography of the same protein sample described for Figure 4. The column was 1.8 × 3.4 cm, and the buffer was 0.01 M Na_2HPO_4 plus 0.01 M acetic acid (pH 5.5) to which an NaCl gradient was added. Fractions of 1 ml were collected.

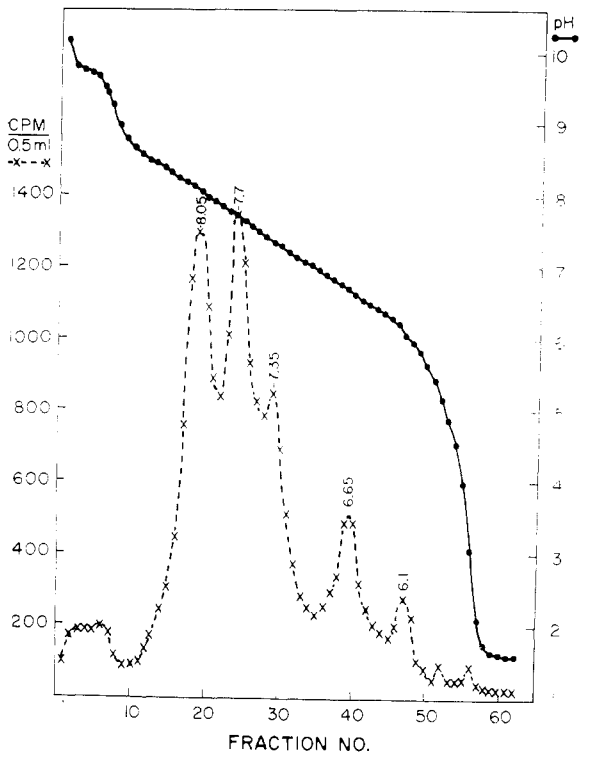

FIGURE 6: Isoelectric focusing in a column of the *h* protein obtained after the usual purification procedure following Sephadex G-100 fractionation. A pH gradient of 6–9 was used, and after 3 days of isoelectric focusing, fractions of 36 drops were collected. The radioactivity and the pH are graphed. The proteins were extracted from mice that had been treated with 1,2,5,6-dibenzanthracene-*t*.

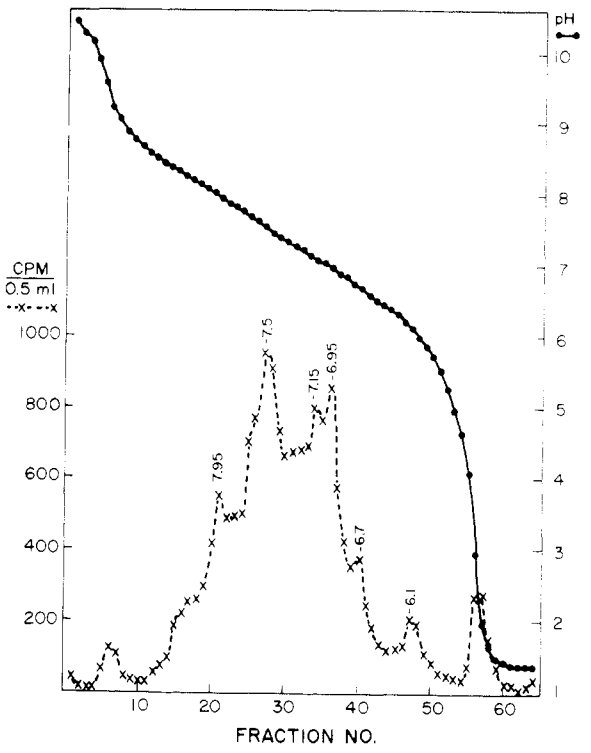

FIGURE 7: Same as Figure 6, except that the proteins were obtained from mice treated with 1,2,3,4-dibenzanthracene-*t*.

all stages of purification except the initial Sephadex G-25 column. Although there was some radioactivity from 1,2,3,4-DBA in the least acidic fraction (DEAE-cellulose breakthrough peak), starch gel electrophoresis of this fraction derived from the skins treated with both hydrocarbons confirmed the earlier results (Abell and Heidelberger, 1962) of a labeled band in the basic protein region that was much more radioactive in the case of the carcinogenic 1,2,5,6-DBA. However, when the partially purified proteins were compared on Sephadex G-100 columns, both were eluted in the same region. The only difference was an additional small peak (occurring in the region of fraction 45 in Figure 1), just before the main peak, that contained about 15% of the label from 1,2,3,4-DBA.

TABLE I: Binding of Hydrocarbons to the Protein Fractions at Different Stages of Purification.

Hydrocarbon	$\mu\mu$mol of Hydrocarbon/OD_{280}		
	MCA[a]	1,2,5,6-DBA[b]	1,2,3,4-DBA[c]
Sephadex G-25 high molecular weight fraction	21	25	20
DEAE-cellulose breakthrough peak	68	46	34
Sephadex G-100 radioactive fraction	494	233	140

[a] Average of 6 experiments, 1200 mice. [b] Average of 12 experiments, 1100 mice. [c] Average of 3 experiments, 300 mice.

Further separation and purification of the radioactive proteins in the Sephadex G-100 fraction was carried out by the isoelectric focusing method developed by Vesterberg and Svensson (1966), which has excellent resolving capability. The results obtained by this technic with a pH gradient from 6 to 9 on the proteins derived from 1,2,5,6-DBA, 1,2,3,4-DBA, and MCA are shown in Figures 6–8. Several replicate experiments were carried out with the proteins derived from application of each hydrocarbon with excellent reproducibility. These isoelectric focusing fractionations show clearly that the single symmetrical radioactive peak eluted from the Sephadex G-100 column contains more than one labeled protein. Furthermore, they show that with both carcinogens there was a sharp radioactive peak at pH 8.05 that was not found with 1,2,3,4-DBA. Therefore, by definition, this peak is the *h* protein from mouse skin.

It was rather difficult to obtain an accurate picture of the position of the nonradioactive proteins in the isoelectric focusing procedure because our samples contained very small amounts of proteins (6–8 OD_{280} units from 100 mice) and the ampholytes also absorb to some extent at 280 mμ, although considerably less than at 260 mμ. This fact also prevented us from obtaining accurate specific activities in all cases. However, in the experiment described in Table II,

TABLE II: Purification of *h* Protein Fraction Labeled with MCA.

	% Radioactivity in *h* Protein Fraction	Increase in Sp Act. (-fold)	Total Purificn[a] (-fold)
Sephadex G-25 high molecular weight fraction			0
DEAE-cellulose breakthrough peak	25	3	12
Sephadex G-100 fraction	100	7	84
Isoelectric focusing fraction pH 8.05[b]	30	2	560

[a] Since the *h* protein is only one of many radioactive proteins in the original extract, its increase in specific activity is not enough to indicate purification. In addition, it is necessary to determine on a given column or procedure, how much of the total radioactivity is present as *h* protein and divide the purity calculated from the specific activity by that value. In other words, the purification of the radioactive *h* protein involves its separation and removal from other radioactive proteins. [b] Since the protein concentration could not be determined in the presence of ampholytes by OD_{280} measurements, the tubes in this experiment were individually dialyzed extensively against water, and the protein concentration was then determined by OD_{280} values.

individual tubes were dialyzed and their protein contents were determined by OD_{280} measurements. This revealed that 30% of the radioactivity was present in the *h* protein fraction at pH 8.05 obtained from mice treated with MCA, and corresponds to a purification of 560-fold. In this pH 8.05 fraction, the actual specific activity was 240×10^3 dpm/OD_{280}, corresponding to 1090 $\mu\mu$mol of MCA/OD_{280}. Assuming a molecular weight of 40,000 for the protein, and that the bound hydrocarbon's specific activity was the same as that applied to the skin, 4–5% of the protein molecules in this fraction would have hydrocarbons attached. This fraction contained about 4% of the radioactivity in the original protein extract.

In order to gain further information on the protein distribution in the eluate from Sephadex G-100, isoelectric focusing was carried out in polyacrylamide gels by the method of Wrigley (1968). The results of a typical experiment are shown in Figure 9, in which two peaks of radioactivity were found at essentially the same pH as in the isoelectric focusing column (Figure 8). The first peak at pH 8.05 coincides with a faintly stained band that is very close to a heavily stained protein band. The second radioactive peak at pH 7.5 was in the region of a very faintly stained band, and no radioactivity was associated with a second heavily stained protein band (Figure 9). The heavily stained band adjacent to the pH 8.05 peak was also found in the fraction isolated from the corresponding peak of the isoelectric focusing column, as revealed both by conventional electrophoresis and isoelectric focusing in polyacrylamide gels, probably because of some band spreading during the collection of fractions from the column.

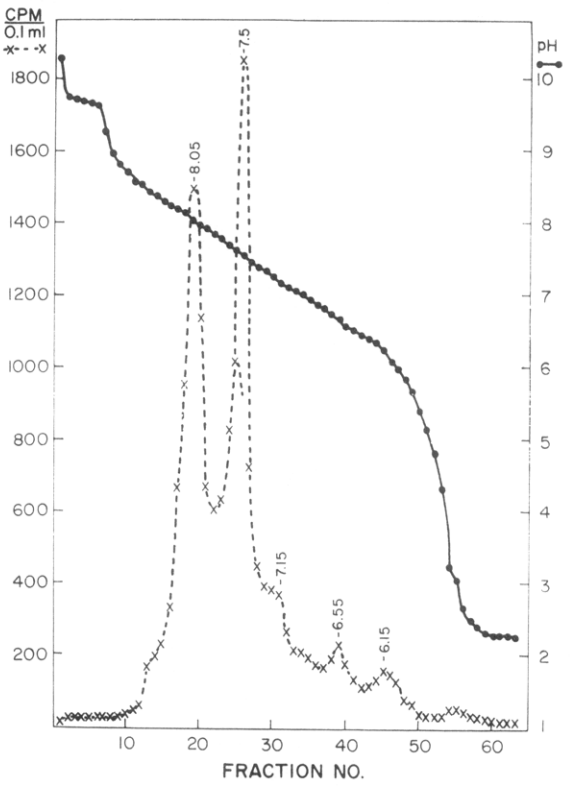

FIGURE 8: Same as Figure 6, except that the mice were treated with 3-methylcholanthrene-*t*.

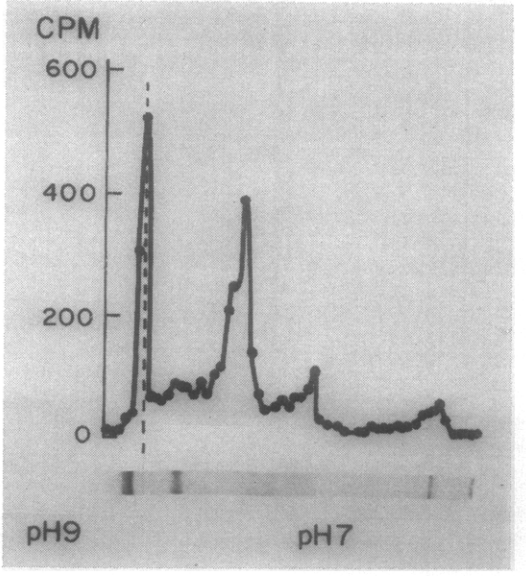

FIGURE 9: Isoelectric focusing in a polyacrylamide gel of the same protein fraction as described in Figure 8. Carrier ampholytes in the pH range of 7–9 were used. The sample was layered on the gel after polymerization.

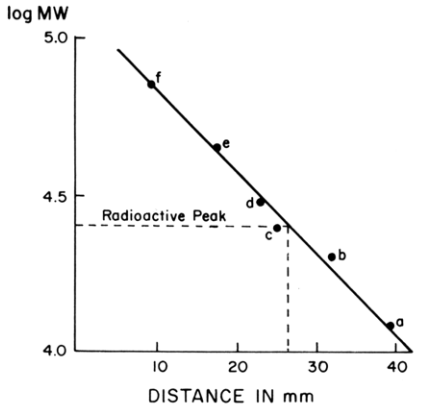

FIGURE 10: Standard curve for the sodium dodecyl sulfate, urea, and polyacrylamide gel system. The proteins used were (a) cytochrome *c*; (b) bromgrass mosaic virus; (*c*) elastase, (d) DNase I, (e) ovalbumin, and (f) transferrin.

At this stage, it appeared worthwhile to determine again the molecular weight of the *h* protein, which was found to be 40,000 on the original Sephadex G-100 column. After isoelectric focusing column purification of the protein derived from mice treated with MCA, the pH 8.05 peak contained so little protein that molecular weight determination on Sephadex G-100 was impossible. Therefore, we turned to polyacrylamide gel electrophoresis. When the molecular weight was determined by electrophoresis in gels of varying concentrations by the method of Hedrick and Smith (1968) a value of 40,000 was again found. Here, the gels contained one stained band and one sharp peak of radioactivity that coincided with the upper part of the stained band. We also resorted to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea, according to the method devised by Shapiro *et al.* (1967). This method was calibrated with six purified proteins of known molecular weight (Figure 10), and a better standard curve was obtained than we had found with the method employing different gel concentrations. Electrophoresis in the sodium dodecyl sulfate–urea system of the *h* protein eluted from the pH 8.05 peak in the preparative electrofocusing column gave, as shown in Figure 11, a single very sharp radioactive peak at a molecular weight of 20,000 coinciding with one of two bands. The nonradioactive band corresponded to a molecular weight of 46,000. This result suggests that the *h* protein consists of two subunits of mol wt 20,000, together with a contaminating nonradioactive protein with a molecular weight of 46,000. Similar experiments with the *h* protein purified in the same way from the skin of mice treated with 1,2,5,6-DBA also demonstrated that it consists of two subunits of 20,000 molecular weight.

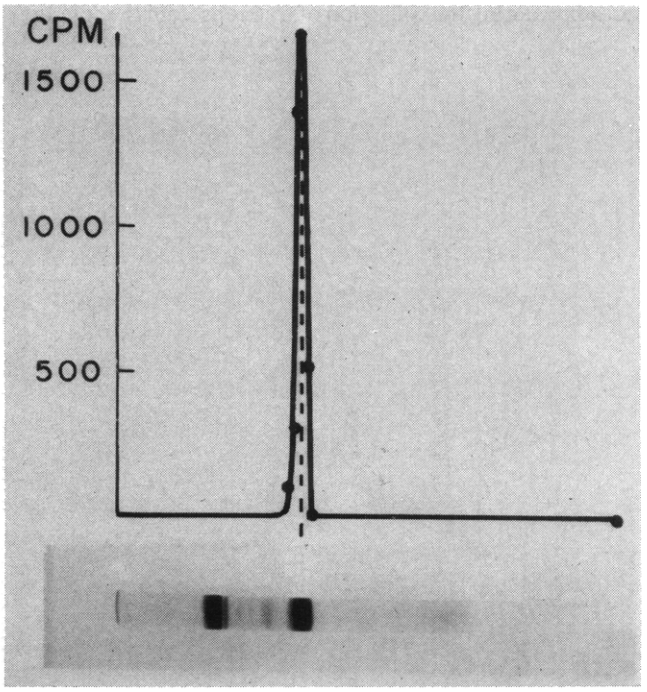

FIGURE 11: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the *h* protein fraction obtained from the pH 8.05 peak of the isoelectric focusing column. The mice had been treated with MCA.

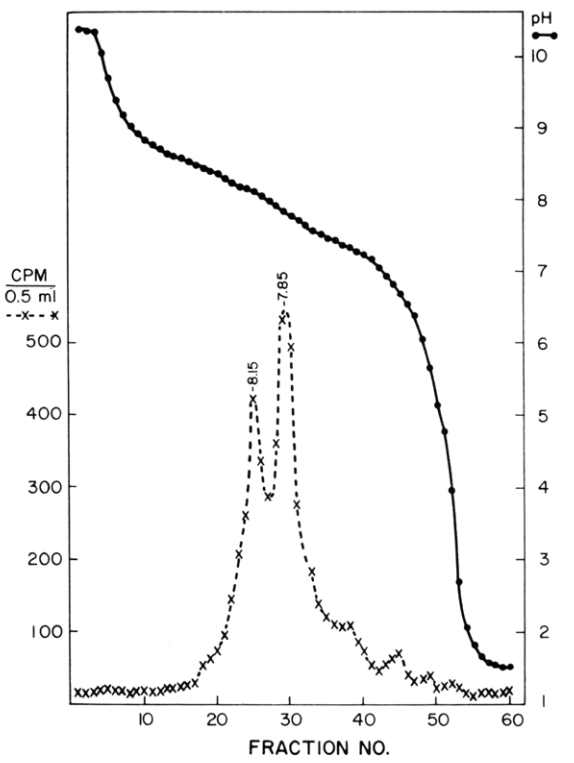

FIGURE 12: Isoelectric focusing on a column in a gradient of pH 7–9 of the same protein described in Figure 11.

Since the *h* protein obtained in the pH 8.05 peak from the electrofocusing column contained at least one nonradioactive protein contaminant of the single radioactive protein, attempts were made to purify it further by rerunning the peak on a second isoelectric focusing column, this time in a pH gradient of 7–9. Surprisingly, as shown in Figure 12, two peaks were obtained at pH 8.15 and 7.85. It seemed unlikely that this separation resulted from better resolution in this pH gradient. This was verified by the finding that when the *h* protein after Sephadex G-100 was run on an isoelectric focusing column in a pH gradient of 7–9 the distribution of radioactivity was

completely comparable with that found (Figures 6–8) at a pH gradient of 6–9. Furthermore, when the peak at pH 7.5 from the isoelectric focusing column was rerun, only a single labeled peak at the same pH was obtained. The two proteins obtained by reisoelectric focusing of the pH 8.05 peak were electrophoresed in gels of varying acrylamide concentrations, and the molecular weights of both were found to be about 40,000. This shows that the second isoelectric focusing procedure did not split the protein into the subunits that were produced by sodium dodecyl sulfate and urea. However, the splitting of the pH 8.05 peak into two proteins was also found in isoelectric focusing experiments in gels when the sample was present during the polymerization. Thus, the nature of this effect is unknown.

Discussion

In the present work we have accomplished the partial purification of the protein fraction from mouse skin to which carcinogenic hydrocarbons are covalently bound in a quantitative relationship to their carcinogenic activities; we have named this the mouse skin *h* protein, after the terminology of Sorof *et al.* (*cf.* 1967, 1969), who has been concerned with an apparently somewhat similar protein in rat liver to which hepatocarcinogenic amines are bound. Our purification procedure has involved successive fractionations on Sephadex G-25, DEAE-cellulose, Sephadex G-100, and isoelectric focusing. The final stage of this partial purification gives us a single radioactive protein with an isoelectric point of pH 8.05, contaminated with at least one other nonradioactive protein.

The degree of purification cannot be calculated in the same way in which enzyme purifications are, since in enzyme purifications there is usually only one given enzyme in an extract, whereas the *h* protein is only one of many radioactive proteins in the skin extract. Since radioactivity is our only measure of *h* protein, the degree of purification must be calculated so as to account for the removal from a given fraction of radioactive proteins other than the *h* protein, as explained in footnote *a* of Table II. In a typical experiment following application of MCA, the *h* protein in the pH 8.05 fraction from an isoelectric focusing column has been purified 560-fold (Table II) and represents a recovery of 4% of the radioactivity in the skin extract. This does not mean a 4% yield, since there are many other radioactive proteins in the extract.

We have demonstrated that the mouse skin *h* protein has a molecular weight of 40,000 and that it is made up of two subunits of 20,000 molecular weight. There appears to be some difference of opinion about the molecular weight of the corresponding protein obtained from rat liver. Sorof (1969) has reported a molecular weight of 60,000–80,000, determined by Sephadex G-200 chromatography, and Ketterer *et al.* (1967) found a molecular weight of 45,000 by Sephadex G-100 chromatography and sedimentation analysis. It is conceivable that the latter protein is a subunit of the former. If that is so, then the liver protein has twice the molecular weight of the mouse skin protein that we have been concerned with.

An interesting finding has been made by Freed and Sorof (1966, 1967) that an electrophoretic fraction of rat liver protein containing bound aminoazo dyes had the property of reversibly inhibiting the growth of cells. More recently Sorof *et al.* (1967) have shown that this growth inhibition is caused by arginase. We examined the mouse skin *h* protein fraction obtained after Sephadex G-100 fractionation, and found it to be devoid of arginase and growth inhibitory activities (Umeda *et al.*, 1968). Thus, the question of the involvement of arginase in chemical carcinogenesis remains open.

Why have we gone to the trouble to attempt to isolate and purify this problem? What is its role, if any, in chemical carcinogenesis? As described earlier (Abell and Heidelberger, 1962), there is an impressive correlation between the binding of hydrocarbons to the mouse skin *h* protein and the process of carcinogenesis. In fact, we believe that this correlation is better with respect to carcinogenesis than is the correlation of the covalent binding to mouse skin DNA (Goshman and Heidelberger, 1967). However, such correlations certainly do not constitute proof. Nevertheless, they justify the present work. Since it now seems to be clear that in almost all cases that have been properly studied chemical carcinogens are covalently bound to DNA, RNA, and protein, it is very difficult to determine conclusively which (if any) of these interactions is of causal significance in carcinogenesis. To our knowledge, no such determination has been made with any chemical carcinogen. Pitot and Heidelberger (1963) have offered a theory as to how the binding of a carcinogenic hydrocarbon to the *h* protein could give rise to a perpetuated change.

The recent development in our laboratory of a quantitative system for obtaining carcinogenesis *in vitro* of cells derived from C3H mouse prostate with carcinogenic hydrocarbons (Chen and Heidelberger, 1969a–c) provides the opportunity to test the possible role of the mouse skin *h* protein in the carcinogenic process.

References

Abell, C. W., and Heidelberger, C. (1962), *Cancer Res. 22*, 931.

Andrews, P. (1964), *Biochem. J. 91*, 222.

Barry, E. J., and Gutmann, H. R. (1966), *J. Biol. Chem. 241*, 4600.

Blatt, W. F., Feinberg, M. P., and Hopfenberg, H. B. (1965), *Science 150*, 224.

Chen, T. T., and Heidelberger, C. (1969a), *J. Natl. Cancer Inst. 42*, 903.

Chen, T. T., and Heidelberger, C. (1969b), *J. Natl. Cancer Inst. 42*, 915.

Chen, T. T., and Heidelberger, C. (1969c), *Intern. J. Cancer 4*, 166.

Davenport, G. R., Abell, C. W., and Heidelberger, C. (1961), *Cancer Res. 21*, 599.

Determann, H., and Walter, I. (1968), *Nature 219*, 604.

Freed, J. J., and Sorof, S. (1966), *Biochem. Biophys. Res. Commun. 22*, 1.

Freed, J. J., and Sorof, S. (1967), *Wistar. Inst. Symp. Monograph 7*, 15.

Goshman, L. M., and Heidelberger, C. (1967), *Cancer Res. 27*, 1678.

Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.

Heidelberger, C., and Moldenhauer, M. G. (1956), *Cancer Res. 16*, 442.

Ketterer, B., Ross-Mansell, P., and Whitehead, J. K. (1967), *Biochem. J. 103*, 316.
Miller, E. C. (1951), *Cancer Res. 11*, 100.
Moore, B. W., and Lee, R. H. (1960), *J. Biol. Chem. 235*, 1359.
Pitot, H. C., and Heidelberger, C. (1963), *Cancer Res. 23*, 1694.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Sorof, S. (1969), *Jerusalem Symp. Quantum Chem. Biochem. 1*, 208.
Sorof, S., Young, E. M., Luongo, L., Kish, V. M., and Freed, J. J. (1967), *Wistar Inst. Symp. Monograph 7*, 25.
Sorof, S., Young, E. M., McCue, M. M., and Fetterman, P. L. (1963), *Cancer Res. 23*, 864.
Umeda, M., Diringer, H., and Heidelberger, C. (1968), *Israel J. Med. Sci. 4*, 1216.
Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20*, 820.
Wiest, W. G., and Heidelberger, C. (1953), *Cancer Res. 13*, 250.
Wrigley, C. (1968), *Sci. Tools 15*, 17.
Young, R. W., and Fulhorst, H. W. (1965), *Anal. Biochem. 11*, 389.