catalytic interactions, the anomalous values appear to be restricted to the conditions of the catalytic reaction. It appears indeed (N. Citri and N. Zyk, in preparation) that the change is synchronous with the catalytic activity ("syncatalytic," Christen and Riordan, 1970) and that a distinction may now be possible between the stereospecific conformative response associated with the stage of binding, and the further, syncatalytic response which accompanies the catalytic activity of L-asparaginase.

References

Campbell, H. A., and Mashburn, L. T. (1969), *Biochemistry 8*, 3768.
Christen, P., and Riordan, J. F. (1970), *Biochemistry 9*, 3025.
Citri, N., and Zyk, N. (1965), *Biochim. Biophys. Acta 99*, 427.
Citri, N., and Zyk, N. (1967), *Biochem. Biophys. Res. Commun. 26*, 216.
Citri, N., and Zyk, N. (1972), *Biochemistry 11*, 2103.
Dixon, M. (1953), *Biochem. J. 55*, 170.
Dixon, M., and Webb, E. C. (1966), Enzymes, 2nd ed, Longmans, Green and Co., New York, N. Y., p 330.
Frank, B. H., Pekar, A. H., Veros, A. J., and Ho, P. P. K. (1970), *J. Biol. Chem. 245*, 3716.
Frieden, C. (1970), *J. Biol. Chem. 245*, 5788.
Glossmann, H., and Bode, W. (1971), *Hoppe-Seyler's Z. Physiol. Chem. 352*, 132.
Greenquist, A. C., and Wriston, J. C. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 3614.
Jackson, R. C., and Handschumacher, R. E. (1970), *Biochemistry 9*, 3585.
Kirschbaum, J., Wriston, J. C., and Ratych, O. T. (1969), *Biochim. Biophys. Acta 194*, 161.
Monod, J., Changeux, J.-P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.
Nakamura, N., Morikawa, Y., and Tanaka, M. (1971), *Agr. Biol. Chem. 35*, 743.
Theorell, H., and Tatemoto, K. (1971), *Arch. Biochem. Biophys. 143*, 354.
Wriston, J. C. (1971), *Enzymes 4*, 101.
Zyk, N., and Citri, N. (1967), *Biochim. Biophys. Acta 146*, 219.
Zyk, N., and Citri, N. (1968a), *Biochim. Biophys. Acta 151*, 306.
Zyk, N., and Citri, N. (1968b), *Biochim. Biophys. Acta 159*, 327.
Zyk, N., Citri, N., and Moyed, H. S. (1969), *Biochemistry 8*, 2787.
Zyk, N., Citri, N., and Moyed, H. S. (1970), *Biochemistry 9*, 677.

Determination of the h-Protein in Transformable and Transformed Cells in Culture†

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ABSTRACT: The h-protein, to which carcinogenic hydrocarbons are specifically bound, has been demonstrated in transformable cells in culture. The h-protein is present in cells of all the various species of rodents so far examined, but not in human fibroblasts. The isolation procedures for the h-protein consisted of three steps: Sephadex G-25 and DEAE-cellulose column chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis, by which the h-protein subunit was isolated as a sharp radioactive peak with a molecular weight of 22,000. The binding of hydrocarbons to the h-protein was firm, presumably covalent. When the binding to the h-protein of K-region derivatives of dibenz[*a*,*h*]anthracene was compared in mouse embryonic cells, the epoxide was bound about eight times more than the parent hydrocarbon, while with the *cis*-dihydrodiol and phenol negligible radioactivity was found in the h-protein region. This suggests that metabolism of hydrocarbon is involved in the binding to the h-protein. In transformed cells, a distinct band was present at a molecular weight of 22,000 (the h-protein region) in the same quantity as that of the normal or control cells, but no significant radioactivity was found in this band.

Studies of the interaction between carcinogenic hydrocarbons and macromolecules have been carried out extensively in mouse skin (Wiest and Heidelberger, 1953; Heidelberger and Moldenhauer, 1956; Abell and Heidelberger, 1962; Brookes and Lawley, 1964; Goshman and Heidelberger, 1967) and in systems of chemical carcinogenesis *in vitro* (Kuroki and Heidelberger, 1971). It now appears that the carcinogens are bound covalently to DNA, RNA, and proteins both *in vivo* and *in vitro*. However, the question remains open as to which of these interactions, if any, is most significant in carcinogenesis. A change produced in the genetic material could be causal in the heritable processes of transformation and mutation. However, as postulated by Pitot and Heidelberger (1963), it is also conceivable that the binding of a carcinogen to a specific protein (possibly a repressor) could give rise to a perpetuated change. This theory has been revived and amplified by Huebner and Todaro (1969). Early

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work in this laboratory had demonstrated the covalent binding of carcinogenic hydrocarbons to total mouse skin proteins, and a rough correlation was found between the extent of binding to the proteins and carcinogenic potency (Wiest and Heidelberger, 1953; Heidelberger and Moldenhauer, 1956). Abell and Heidelberger (1962) found that a better correlation exists between the carcinogenic potency of the compounds and their binding to a particular soluble protein fraction from mouse skin, which migrated toward the cathode on starch gel electrophoresis. Tasseron *et al.* (1970) partially purified this protein, and it has been named the h-protein of the mouse skin, following the terminology of Sorof (1969).

In the meantime, two systems of chemical carcinogenesis *in vitro* have been established. These include the transformation of hamster embryonic cells by carcinogenic hydrocarbons (Berwald and Sachs, 1965), 4-nitroquinoline 1-oxide (Kuroki and Sato, 1968), and dimethylnitrosamine (Huberman *et al.*, 1968), and the malignant transformation by carcinogenic hydrocarbons of cells derived from C3H mouse prostate (Chen and Heidelberger, 1969). We now consider these systems to be reliable models and to provide a new approach to investigate the cellular and molecular mechanism of chemical carcinogenesis. The present work was undertaken to study the h-protein in cultures of transformable and transformed cells.

Materials and Methods

Most experiments were carried out with the various cells involved in the systems of chemical carcinogenesis *in vitro*. Table I identifies the cells and their origins, and describes their ability to metabolize hydrocarbons to water-soluble material(s) (Huberman *et al.*, 1971) and to bind hydrocarbons to their DNA, RNA, and proteins (Kuroki and Heidelberger, 1971). All cells are fibroblastic.

Primary cultures of embryonic cells were prepared by enzymatic dispersion (0.1% trypsin) of minced whole embryos. Pregnant Syrian hamsters (12–14 days) were purchased from Con Olson Co., Verona, Wis. Pregnant C3H mice (12–14 days) were obtained from Charles River Laboratories, Boston, Mass., on allocation by the Cancer Chemotherapy National Service Center, and pregnant rats (15–17 days) were obtained from Holzman Co., Madison, Wis. Hamster embryonic cells and the cell line V79 from Chinese hamsters were cultured in Dulbecco's medium without phenol red plus 10% fetal calf serum; human fibroblastic cells were grown in F-12 medium plus 15% fetal calf serum. All other cells were cultured in Eagle's Basal Medium plus 10% fetal calf serum. For subculturing, cells were dispersed with 0.1% trypsin in Dulbecco's phosphate buffer solution. All media and sera were obtained from Gibco, Grand Island, N. Y. The cells were grown in plastic petri dishes (100 × 20 or 150 × 25 mm, Falcon Plastics, Los Angeles, Calif.). In some experiments, roller bottle cultures operating at a speed of 0.5 rpm were also employed.

Carcinogens. Tritiated MCA, DBA, and DB[ac]A[1] (generally labeled) were obtained from Amersham-Searle. The specific activities of MCA and DBA were 500 mCi/mmole, whereas that of the weakly carcinogenic DB[ac]A was 203 mCi/mmole.

K-region derivatives of DBA and their precursors were prepared from the above tritiated hydrocarbons, and were kindly supplied by Drs. P. Sims and P. L. Grover, Chester Beatty Research Institute, London, England. The K-region epoxide of DBA (dibenz[*a*,*h*]anthracene 5,6-oxide, specific activity 318 mCi/mmole) was prepared from the corresponding dialdehyde according to Newman and Blum (1964). The K-region phenol of DBA (5-hydroxydibenz[*a*,*h*]anthracene, specific activity 124 mCi/mmole) was synthesized from the corresponding dihydrodiol (specific activity 146 mCi/mmole) by the methods of Boyland and Sims (1965).

The purity of these compounds was examined by radioscans of thin layer chromatograms in benzene (100%), benzene–ethanol (19:1, v/v), and cyclohexane–dioxane (9:1, v/v). With all labeled carcinogens, the radiochemical purity was greater than 95%.

Preparation of Protein Extracts. Details of the preparation of the protein extract from mouse skin were described by Tasseron *et al.* (1970).

The labeled carcinogens were dissolved in Me_2SO and added to the medium to a final concentration of 0.5% Me_2SO. In the case of K-region derivatives, only freshly prepared solutions were used. The concentration of the compounds in the medium was 1 μg/ml (about 4×10^{-6} M), an optimal amount for transformation, (Berwald and Sachs, 1965; Chen and Heidelberger, 1969), and they were applied to exponentially growing cells. After incubation for 24 hr with tritiated hydrocarbons, the monolayer of cells was washed once with saline and harvested with 0.02% EDTA in saline. Each experiment required about 200×10^6 cells for the isolation of h-protein. The cell pellet was frozen at −80° until use.

The cells were swelled for 15 min with a cold hypotonic solution (0.01 M Tris-HCl (pH 8.0), 0.01 M NaCl, 0.005 M β-mercaptoethanol, and 0.005 M $MgCl_2$) at a concentration of $10–20 \times 10^6$ cells/ml. The cells were homogenized in a Douce homogenizer and were examined under a phase-contrast microscope to determine the presence of unbroken cells. The soluble cytoplasmic protein was obtained by two centrifugations at 800*g* for 8 min and then 100,000*g* for 1 hr.

Purification of the h-Protein. The standard buffer used for purification consisted of 0.01 M Tris-HCl (pH 8.0), 0.01 M NaCl, and 0.005 M β-mercaptoethanol. Steps 1–3 were carried out in a cold room, essentially as described in the previous paper, which described the necessity for the β-mercaptoethanol (Tasseron *et al.*, 1970).

STEP 1. DESALTING OF PROTEIN EXTRACTS. The high-speed supernatant fractions were desalted either by a Sephadex G-25 column (2.6 × 24 cm, 46 ml of void volume) or by ultrafiltration through a Diaflo UM-10 membrane (Amicon Co., Lexington, Mass.) with the standard buffer. The UM-10 membrane retains substances of molecular weight greater than 10,000. Recovery of protein in the high molecular weight fraction was approximately 70% in both systems.

STEP 2. DEAE-CELLULOSE CHROMATOGRAPHY. The high molecular weight fraction was then passed through a DEAE-cellulose column (Gallard-Schlesinger, 0.7 mequiv/g, 2.6 × 3.0 cm) in the standard buffer. The basic proteins passed through the column without retention.

STEP 3. SEPHADEX G-100 GEL FILTRATION. The DEAE-cellulose breakthrough fraction was concentrated to 2.5 ml by ultrafiltration through a Diaflo UM-10 membrane and layered on top of a Sephadex G-100 column (2.5 × 140 cm, 200 ml of void volume). The column was run with the standard buffer at a flow rate of approximately 24 ml/hr, and 8-ml fractions were collected. In the case of tissue culture cells,

[1] Abbreviations used are: DBA, dibenz[*a*,*h*]anthracene; DB[ac]A, dibenz[*a*,*c*]anthracene; MCA, 3-methylcholanthrene. Throughout this paper where the expression dodecyl sulfate is used, the sodium salt is implied.

TABLE I: The Cells Used in These Experiments.

Cells	Description	Metabolism of Hydrocarbons to Water-Soluble Form	Binding of Hydrocarbons to DNA, RNA, and Proteins	Origin
		A. Normal or Nontransformed Control Cells		
G23	Clone isolated from B_1 prostate cells, transformable by hydrocarbons	Medium (Huberman *et al.*, 1971)	Medium (Kuroki and Heidelberger, 1971)	This lab (Grover *et al.*, 1971)
Mouse embryonic cells	Secondary culture of C3H mouse embryo cells	High (Huberman *et al.*, 1971)	High (Kuroki and Heidelberger, 1971)	This lab
Hamster embryonic cells	Secondary culture from Syrian golden hamster	High (Huberman *et al.*, 1971)	High (Kuroki and Heidelberger, 1971)	This lab
Rat embryonic cells	Secondary culture of rat embryonic cells, Sprague-Dawley	High (Huberman *et al.*, 1971)	NE[a]	This lab
Chinese hamster cells	Cells obtained from lung of newborn Chinese hamsters, passage 8	High (Huberman *et al.*, 1971)	NE	Dr. T. Kuroki Tohoku Univ., Sendai, Japan
Human fibroblasts	Cells obtained from foreskin of newborn passage 8	Low (Diamond *et al.*, 1967)	Low (Diamond *et al.*, 1967)	Dr. R. DeMars Department of Genetics, U.W.
		B. Transformed Cells		
$4C_1$	MCA-transformed clone from prostate cells	Low (Huberman *et al.*, 1971)	Low (Kuroki and Heidelberger, 1971)	This lab (Mondal *et al.*, 1970)
T-10	MCA-transformed cells from prostate cells passaged serially through mice, then cultured	Low (Huberman *et al.*, 1971)	Low (Kuroki and Heidelberger, 1971)	This lab
G22	Spontaneously transformed clone derived from a clone of B_1 prostate cells	NE	NE	This lab (Embleton and Heidelberger 1972)
Sp	Spontaneously transformed Syrian hamster embryo cells	Low (Huberman *et al.*, 1971)	NE	Dr. K. K. Sanford, NCI (Sanford, 1968)
RB77	Rouse sarcoma virus (B77) transformed rat embryo cells	Low (Huberman *et al.*, 1971)	NE	Dr. H. M. Temin, this lab (Altaner and Temin, 1970)
V79	Spontaneous transformed male embryo Chinese hamster lung cells	Low (Huberman *et al.* 1971)	Low (Kuroki *et al.*, in preparation)	Dr. E. H. Y. Chu Oak Ridge (Chu, *et al.*, 1969)

[a] NE = not examined.

this step was not carried out because of the limited amount of protein.

STEP 4. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS. A modification of the gel electrophoresis system described by Maizel (1969) was used. The DEAE-cellulose breakthrough fraction or the radioactive peak from the Sephadex G-100 column (in the case of protein from the mouse skin) was concentrated to 0.5–1.0 ml by ultrafiltration. An aliquot of sample (20 or 25 μl) was taken for determination of radioactivity and amount of protein by the Lowry *et al.* (1951) procedure. Then the samples were denatured by mixing with one-fourth volume of a solution containing 5% dodecyl sulfate, 5% β-mercaptoethanol, 0.05 M sodium phosphate buffer (pH 7.2), 50% sucrose, and 0.02 mg/ml of Bromophenol Blue. The mixture was then heated in boiling water for 1 min. This denatured sample was stored frozen at $-20°$ after addition of one-tenth volume of glycerol, or was used fresh for gel electrophoresis. Repeated freezing and thawing

did not affect the proteins, as shown by gel electrophoresis, which gave the same pattern as a fresh unfrozen solution.

The gels contained 0.1% dodecyl sulfate 0.1 M sodium phosphate buffer (pH 7.2), 7.5% acrylamide, and 0.2% *N,N'*-methylenebisacrylamide and were polymerized with 1 mg/ml of ammonium persulfate and 0.5 μl/ml of *N,N,N',N'*-tetramethylethylenediamine. The tray buffer was 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% dodecyl sulfate. Usually, 20 to 200 μl of sample containing 100–200 μg of protein was carefully layered on top of the gels (0.5 × 8.5 cm). Electrophoresis was performed at room temperature at 90 V for 3 hr. The gels were fixed with 20% sulfosalicylic acid overnight, stained with 0.25% Coomassie Brilliant Blue R250 (Colab Laboratory) for 4 hr at room temperature (unless otherwise stated) and were destained by repeated washes with 7.5% acetic acid for 3 or 4 days. The stained gels were then scanned at 550 mμ with a Gilford linear scanner (Gilford Instrument Laboratory). The system was standardized with 20 μg/gel of bovine serum albumin fraction V (Sigma).

Determination of Molecular Weight. The molecular weight of proteins was determined in the dodecyl sulfate polyacrylamide gel system according to the method of Shapiro *et al.* (1967) and Weber and Osborn (1969). This method was calibrated with six purified proteins of known molecular weight: bovine serum albumin (fraction V, Sigma), ovalbumin (twice crystalized, Mann Research Laboratory), human γ-globulin (Mann Research Laboratory), pepsin (twice crystalized, Worthington), trypsin (twice crystalized, Worthington), and cytochrome *c* (type VI, Sigma). A linear standard curve was obtained.

Quantitation and Measurement of Radioactivity. Protein was quantitated by the Lowry procedure (Lowry *et al.*, 1951) with a standard of bovine serum albumin. Radioactivity was measured in Packard Tri-Carb liquid scintillation spectrometers. Aqueous solutions were counted in Scintisol (Isolab, Inc.) while radioactivity in protein precipitates was measured by dissolving them in 0.5 ml of Soluene (Packard Instrument Co.) to which 10 ml of toluene–2,5-diphenyloxazole was added. For determination of radioactivity in the gels, they were cut with a gel slicer into 2-mm slices after freezing in Dry Ice. The slices were then incubated with 0.5 ml of 30% hydrogen peroxide in capped counting vials at 66° overnight, following the method of Young and Fulhorst (1965). After addition of 0.5 ml of distilled water and 10 ml of Scintisol, the slices were counted. This method did not produce any serious quenching as tested by external and internal standards. The recovery of radioactivity was about 60%.

The relative amounts of protein after separation on the gel were determined on the basis of area (height × width) recorded by the gel scanner. Under our experimental conditions, the recorded area was proportional to the amount of protein added to the gels. Specific activities of binding to the h-protein, which was separated by gel electrophoresis, were expressed by μμmoles of carcinogen per area corresponding to that of 20 μg of bovine serum albumin (BSA unit).

Results

One of the difficulties in biochemical studies with tissue culture cells lies in the limited amount of material. For example, the skin of the back of one mouse (about 5 cm^2) yielded approximately 40 OD_{280} units of cytoplasmic soluble proteins, while the amount extractable from 100 × 10^6 pro-

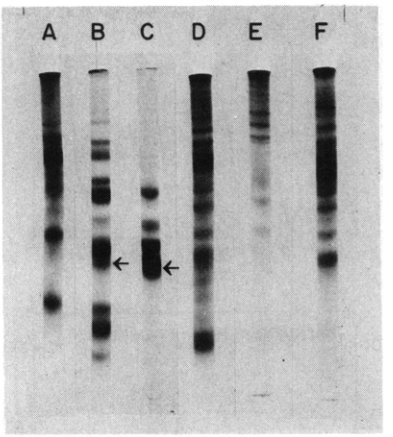

FIGURE 1: Dodecyl sulfate polyacrylamide gel electrophoresis of mouse skin proteins at each step of purification; arrows indicate the h-protein. (A) Total cytoplasmic soluble proteins; (B) basic proteins (breakthrough of DEAE cellulose column); (C) the h-protein fraction after Sephadex G-100 fractionation of the basic protein; (D) acidic proteins, which were eluted from the DEAE-cellulose column with buffer containing high salt concentration; (E and F) subfractions of acidic proteins, obtained after the Sephadex G-100 column, in which two peaks of OD_{280} associated with radioactivity were obtained.

state cells was only 10–12 OD_{280} units; thus, 300–400 × 10^6 cells correspond in quantity of soluble proteins to one mouse skin. We therefore attempted to scale down the technique of isolation of the h-protein used previously (Tasseron *et al.*, 1970), in which 100 mice were used in one experiment, to be suitable for such a small amount of protein.

The authentic h-protein was purified from fifty mouse skins treated with [^{3}H]MCA through three steps of purification: desalting with a Sephadex G-25 column, DEAE-cellulose column chromatography, and Sephadex G-100 column chromatography (Tasseron *et al.*, 1970). The fractions at each step of purification were then subjected to dodecyl sulfate polyacrylamide gel electrophoresis after concentration and dissociation by heating in dodecyl sulfate. Figures 1–3 show the gel electrophoresis patterns at each step of purification. The basic proteins from the DEAE-cellulose column consisted of 10 bands (Figure 1B and 2A) and further purification with a Sephadex G-100 column gave 4 well separated bands on the gel (Figure 1C and 2B). The patterns from the total soluble proteins (Figure 1A), acidic proteins, and their subfractions on a Sephadex G-100 column were more complicated than the basic fraction and consisted of more than 15 bands (Figures 1D–F, and 3). A single sharp radioactive peak was found both in gels of the basic proteins and their subfractions on the Sephadex G-100 column; their mobilities were identical (Figure 2). However, there was no radioactive peak in the samples from total proteins, acidic proteins, and their subfractions on Sephadex G-100 columns. Therefore, by definition, this labeled peak is the h-protein subunit from the mouse skin. It was calculated that the specific activity of the radioactive peak of the basic protein on the gel was at least 40 times higher than the bands of acidic proteins. (The specific activity of the radioactive peak of the basic protein on the Sephadex G-100 column was about 10 times higher than that of the acidic proteins; the former consisted of 4 bands but the latter consisted of more than 15 bands.)

The molecular weight of this radioactive band was determined by its mobility in the gel. It was found to have a molecular weight of 22,000 both in samples from the basic pro-

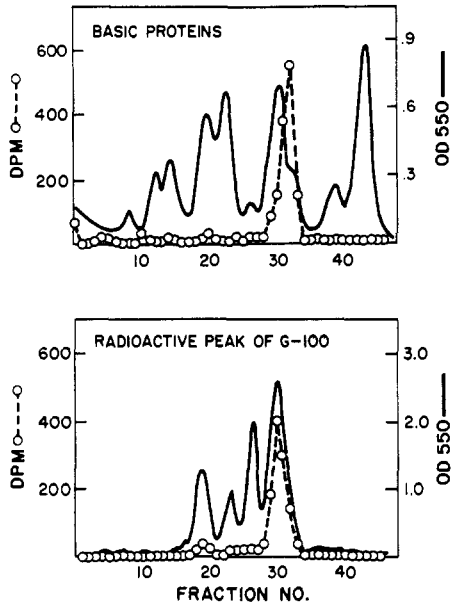

FIGURE 2: Profiles of dodecyl sulfate polyacrylamide gel electrophoresis of the basic protein (A) and following its further purification on a Sephadex G-100 column (B), obtained from mouse skin after application of [^{3}H]MCA. The gel in B was stained overnight.

teins and after subfraction on a Sephadex G-100 column. This molecular weight of 22,000 is identical with that obtained previously after further purification by an isoelectric focusing column and dissociation in dodecyl sulfate (Tasseron *et al.*, 1970). Nonradioactive bands of the subfraction on the Sephadex G-100 column corresponded to a molecular weight of 44,000, 34,000, and 27,000, respectively (Figures 2A and 2B).

Thus, the h-protein subunit was clearly demonstrated as a sharp radioactive peak on the gel after fractionation on only two columns: Sephadex G-25 and DEAE-cellulose.

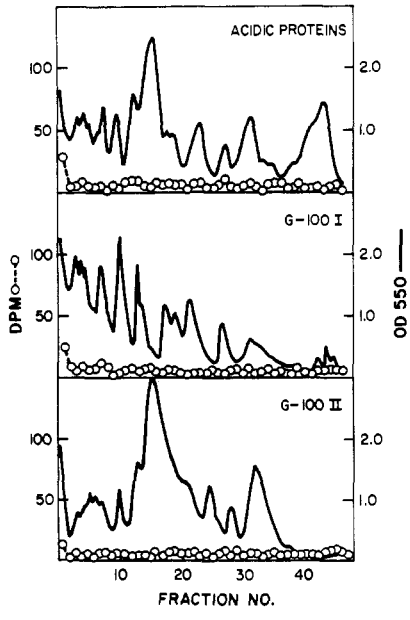

FIGURE 3: Profiles of dodecyl sulfate polyacrylamide gel electrophoresis of acidic proteins (A) and their subfractions on Sephadex G-100 columns (B and C), obtained from mouse skin after application of [^{3}H]MCA.

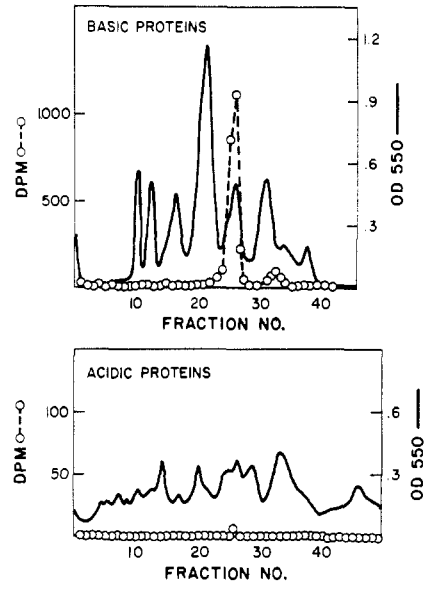

FIGURE 4: Profiles of dodecyl sulfate polyacrylamide gel electrophoresis of the basic (A) and the acidic (B) proteins of G-23 prostate cells, treated with [^{3}H]MCA for 24 hr.

Such excellent resolution in this gel system with a small quantity of material made it possible to apply this technique to the tissue culture cells.

Demonstration of the h-Protein in the Transformable or Normal Cells in Tissue Culture. With this simplified technique, we attempted to isolate the h-protein from various tissue culture cells that are known to be transformable by carcinogenic hydrocarbons. Exponentially growing cells were labeled with 1 μg/ml of [^{3}H]MCA for 24 hr and the soluble cytoplasmic proteins were extracted following separation of nuclei and high speed centrifugation. The initial soluble protein extract usually contained 30–40 OD_{280} units. After desalting with a Sephadex G-25 column or ultrafiltration through the UM-10 membrane, a DEAE-cellulose column was run with the two-step elution. Similarly to the case of the mouse skin, 6–7% of protein was not retained by the column and had a specific activity of about fivefold higher than that eluted with the second buffer containing a high-salt concentration. The breakthrough fraction of the DEAE-cellulose column usually contained 2–3 OD_{280} units or 2–3 mg of protein. The recovery of proteins from the DEAE-cellulose column was almost 100%. These basic and acidic proteins were then concentrated to about 0.5 ml by ultrafiltration through a UM-10 membrane. After denaturation and dissociation by heating with dodecyl sulfate, an aliquot of sample containing 100–200 μg of proteins was applied to the dodecyl sulfate polyacrylamide gel. Figure 4 shows the profiles of gel electrophoresis of the basic and acidic proteins from G-23 prostate cells. The basic fraction had 8 protein bands and a single sharp radioactive peak at a molecular weight of 22,000; no radioactive peak could be detected in the acidic proteins. Similarly, embryonic cells from Syrian hamsters and C3H mice gave a single radioactive peak at a molecular weight of 20,000 and 21,000 (Figure 5). These profiles on the gel were identical with those obtained from the mouse skin.

The h-protein was also found in secondary cultures of rat embryonic cells and an early passage (eight) of newborn Chinese hamster lung cells, which are considered to be "normal" in their morphology and chromosomal constitution; but it is not yet known whether or not these cells can be

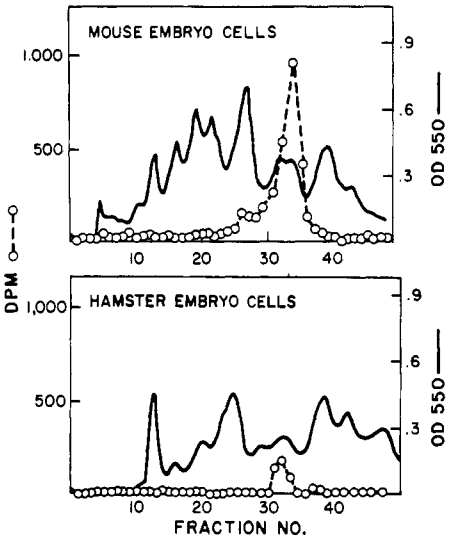

FIGURE 5: Profiles of dodecyl sulfate polyacrylamide gel electrophoresis of the basic proteins obtained from C3H mouse embryonic cells and hamster embryonic cells. The cells were treated with 1 μg/ml of [^{3}H]MCA for 24 hr.

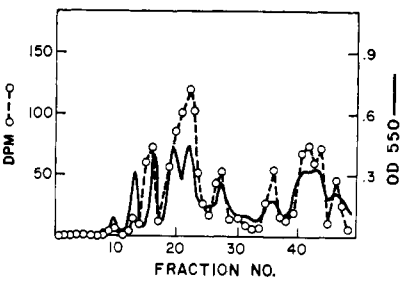

FIGURE 6: Incorporation of L-[^{14}C]leucine into the basic proteins obtained from C3H mouse embryonic cells. The cells were labeled with 0.5 μCi/ml of L-[^{14}C]leucine (sp act. 312 mCi/mmole) for 30 min, and the basic protein was isolated and analyzed by dodecyl sulfate polyacrylamide gel electrophoresis.

transformed. The quantity of h-protein was estimated from the weights of papers cut from the optical scan of the gels. As shown later in Table IV the quantity of the h-protein was almost constant among different species of rodents, and was approximately 12% of the basic protein, and 0.7% of cytoplasmic soluble proteins. As shown in Table II, there were differences in the specific activities of the h-proteins of the various cells. For example, mouse embryonic cells gave the highest specific activity. Hamster embryonic cells, however, had h-protein with about six times less specific activity than the mouse embryonic cells. Thus, it can be concluded that the binding of MCA to h-protein occurs in all of the various species of rodent cells so far examined, although there are considerable quantitative differences.

The incorporation of [^{14}C]leucine into the basic proteins was studied. As shown in Figure 6, the radioactivity was approximately proportional to the absorbance throughout the fractions, which suggests that the high affinity of the carcinogen for the h-protein is not due to its rapid turnover.

Characteristics of Binding to the h-Protein. The nature of the binding of carcinogens to the h-protein was investigated by repeated extraction with organic solvents. The h-protein subunit of mouse embryonic cells labeled with [^{3}H]DBA was isolated from the crushed gel by stirring with 0.1 M sodium phosphate buffer containing 0.1% dodecyl sulfate. An aliquot of the h-protein was washed with 80% ethanol, 100% ethanol, and once with ether. Of the radioactivity, 81% remained in the insoluble fraction after repeated washing with alcohol and ether, while washing with 5% cold trichloroacetic acid resulted in only a 9% release of radioactivity. This suggests that almost all of the carcinogen is bound firmly and probably covalently to the h-protein, since the hydrocarbons are readily extracted by alcohol and ether.

Comparison of the Binding of DBA and DB[ac]A to the h-Protein. An inconsistency in the correlation between carcinogenic potency of compounds and their binding to cellular constituents has been that the very weakly carcinogenic DB[ac]A was bound to all cellular constituents, particularly to total proteins, to a greater extent than its more carcinogenic isomer, DBA (Heidelberger and Moldenhauer, 1956; Goshman and Heidelberger, 1967; Kuroki and Heidelberger, 1971). However, the results in Table III show that the h-protein labeled with the carcinogenic DBA had a somewhat higher specific activity than that obtained from its weakly carcinogenic isomer DB[ac]A in embryonic cells from C3H mice and hamsters. This finding confirms our earlier results with h-protein of mouse skin (Abell and Heidelberger, 1962; Tasseron *et al.*, 1970).

Binding of Metabolites of the Hydrocarbon to the h-Protein. It was reported by Grover and Sims (1969) and Gelboin (1969) that carcinogenic hydrocarbons that are not themselves chemically reactive are metabolically converted by microsomal enzyme(s) to some form(s) that binds covalently to

TABLE II: The Specific Activity of the h-Protein of Transformable or Normal Control Cells, following Treatment with 1 μg/ml of [^{3}H]MCA for 24 hr.

Cells	Mol Wt	Sp Act.[a]
Mouse skin	22,000	6.8
C3H mouse embryo	21,000	4.4 ± 0.4
G23 prostate	22,000	1.7
Syrian hamster embryo	20,000	0.65 ± 0.10
Chinese hamster lung	22,000	1.9
Rat embryo	20,000	1.9

[a] pmoles/area corresponding to 20 μg of bovine serum albumin.

TABLE III: Comparison of Binding to the h-Protein of DBA and Its Weakly Carcinogenic Isomer DB[ac]A.

Cells	Carcinogen[a]	Mol Wt	Sp Act.[b]
Mouse embryo	DBA	22,000	1.7
	DB[ac]A	24,000	1.2
Hamster embryo	DBA	21,000	0.6
	DB[ac]A	21,000	<0.1

[a] Carcinogens were added to exponentially growing secondary cultures of embryonic cells for 24 hr at a concentration of 1 μg/ml. [b] pmoles of carcinogen/area corresponding to that of 20 μg of bovine serum albumin.

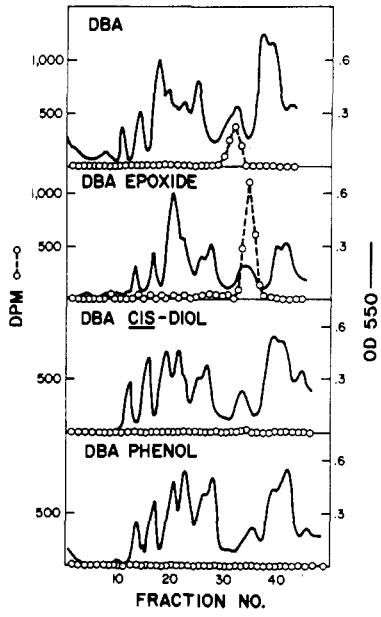

FIGURE 7: Profiles of dodecyl sulfate polyacrylamide gel electrophoresis of the basic proteins of C3H mouse embryonic cells, treated with equimolar amounts (1 μg/ml) of DBA and its K-region derivatives. (A) DBA, (B) DBA-epoxide, (C) DBA-*cis*-dihydrodiol, and (D) DBA-phenol.

DNA and histone. A recent investigation in this laboratory (Selkirk *et al.*, 1971) has confirmed experimentally the suggestion by Boyland and Sims (1964) that an epoxide is formed as an intermediate in the metabolism of DBA. We have also demonstrated that K-region epoxides are more active than their parent hydrocarbons, *cis*-dihydrodiols and phenols, in the production of malignant transformation both in hamster embryonic cells and in mouse prostate G-23 cells (Grover *et al.*, 1971). It was, therefore, of interest to investigate the binding of K-region derivatives of hydrocarbons to the h-protein.

TABLE IV: Quantity of the h-Protein in Control and Transformed Cells.

Species	Control Cells	%	Transformed Cells	%
C3H mouse	Embryo	11.0 ± 3.2[b]		
	G23	14.0 ± 1.0[f]	$4C_1$	11.4 ± 0.8[f]
			T-10	11.7 ± 0.5[f]
			G-22	12.8 ± 1.0[f]
Rat	Embryo	12.8 ± 1.4[f]	RB77	13.1
Hamster	Embryo	13.4 ± 1.7[c]	Sp	12.3 ± 0.9[f]
Chinese hamster	Lung	18.9	V79	16.9
Average		12.4 ± 3.1[d]		13.8 ± 2.3[e]

[a] Per cent of h-protein of the basic proteins calculated on the basis of the paper weight of the profiles of dodecyl sulfate polyacrylamide gel. [b] Average of 11 samples, mean plus and minus standard deviation. [c] Average of 5 samples, mean plus and minus standard deviation. [d] Average of 21 samples, mean plus and minus standard deviation. [e] Average of 11 samples, mean plus and minus standard deviation. [f] Average of 2 samples, mean plus and minus standard deviation.

TABLE V: Specific Activities of the h-Proteins of Transformed Cells Treated with 1 μg/ml of [^{3}H]MCA for 24 hr.

Cells	Transformed by	Mol Wt	Sp Act.[a]
$4C_1$	Prostate, MCA	20,000	0.2
T-10	Prostate, MCA	21,000	<0.1
G-22	Prostate, spontaneous	21,000	0.4
Sp	Hamster, spontaneous	21,000	<0.1
V79	Chinese hamster, spontaneous	21,000	<0.1
RB77	Rat, viral	21,000	0.3

[a] pmoles/area corresponding to 20 μg of bovine serum albumin.

Exponentially growing mouse embryo cells were treated with an equimolar concentration (1 μg/ml) of tritiated DBA, its K-region epoxide, *cis*-dihydrodiol and phenol for 24 hr, and the h-protein was isolated. As shown in Figure 7, the parent hydrocarbon and its K-region epoxide gave a single radioactive peak at a molecular weight of 22,000, while in the case of the *cis*-dihydrodiol and phenol, negligible radioactivity was found in the h-protein region. The extent of binding of epoxide was 13.5 pmoles/BSA unit, approximately eightfold higher than its parent hydrocarbon, 1.7 pmoles/BSA unit. This result suggests that metabolism of the hydrocarbon is required for binding to the h-protein.

Binding of [^{3}H]MCA to the Proteins of Transformed Cells. Chemically, virally and spontaneously transformed cells were treated with 1 μg/ml of [^{3}H]MCA for 24 hr and the procedure for isolation of the h-protein was carried out. In all cells, a distinct protein band was present in the gel at a molecular weight of 20,000 to 24,000, which appears to correspond to the h-protein of control cells. As summarized in Table IV, there was no significant difference between control and transformed cells in the quantity of the protein in this region. The protein in the h-region averaged 12–14% of the total basic proteins. However, radioactivity was considerably lower in this region from the transformed cells (Table V) than from the nonmalignant cells from which they were derived (Table II).

Two explanations for these observations in transformed cells can be considered. The protein in the h-region is the h-protein, but a lack of metabolism prevented the conversion of the hydrocarbons into the reactive form (probably epoxide). Such a low metabolism in transformed cells has previously been reported (Diamond *et al.*, 1967; Huberman *et al.*, 1971). Another possibility is that the protein in the h-region differs in its structure, function, or conformation from the h-protein in the control cells. In an attempt to test these possibilities, transformed cells (T-10) were treated with DBA and its K-region epoxide, and the h-like protein was isolated on the gels. Radioactivity from DBA epoxide in the h-protein region was 0.4 pmole/BSA unit, and negligible radioactivity was detected in the h-protein from the cells treated with the parent hydrocarbon. Identical specific activities were obtained with $4C_1$ (Mondal *et al.*, 1970) transformed prostate cells. These findings are not sufficiently clear-cut to enable a decision to be made between the two possibilities considered above.

Binding of [³H]MCA to the Proteins of Human Fibroblasts. Diploid human fibroblasts were treated with 1 μg/ml of [³H]MCA or [³H]DBA epoxide for 24 hr, and the procedure for isolation of the h-protein was carried out. As shown in Figure 8A, a small protein band at a molecular weight of 21,000 was observed, but contained no radioactivity derived from bound MCA. Similarly (Figure 8B), DBA-epoxide did not bind to the basic proteins. Hence, a low metabolic activity of human cells (Diamond *et al.*, 1971) is not sufficient to explain the lack of binding of polycyclic hydrocarbons to the h-proteins, if, in fact, human cells have h-proteins.

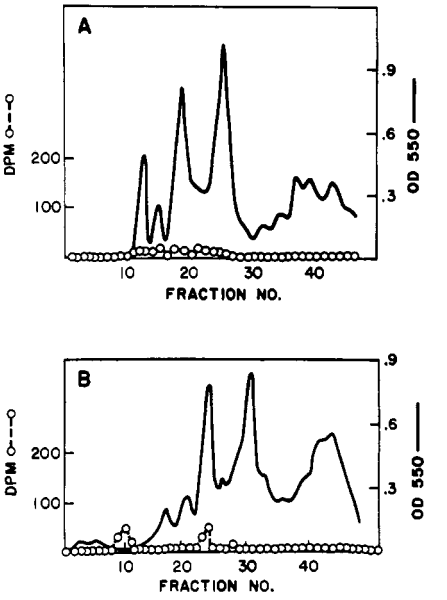

FIGURE 8: Profile of dodecyl sulfate polyacrylamide gel electrophoresis of the basic proteins from human fibroblastic cells which were treated (A) with 1.0 μg/ml of [³H]MCA and (B) with 1.0 μg/ml of [³H]DBA-epoxide for 24 hr.

Discussion

The concept is now firmly established that carcinogenic compounds, that are not themselves chemically reactive, must be converted metabolically into electrophilic substances prior to exerting their carcinogenic effect (Miller, 1970). We have recently presented evidence that carcinogenic hydrocarbons are metabolized to epoxides (Selkirk *et al.*, 1971), which are more active than the corresponding hydrocarbons in producing malignant transformation in two *in vitro* systems (Grover *et al.*, 1971). The observation that DBA-epoxide is bound to the h-protein to a considerably greater extent than DBA supports the view that hydrocarbons must be metabolically activated prior to binding to the h-protein and provides additional evidence that this binding is covalent. Epoxides are also bound to DNA, RNA, and total proteins of transformable cells to a greater extent than the parent hydrocarbons (Kuroki *et al.*, 1972). The fact that the chemically reactive epoxide binds only to the h-protein among the soluble basic proteins is intriguing and not easily explained. All evidence available at present indicates that the hydroxylation reaction (in which the epoxide is an intermediate) occurs exclusively in the microsomal fraction (Nebert and Gelboin, 1968). A possibility is that the h-protein may be an enzyme involved in glutathione conjugation of epoxides, since this activity is known to be soluble (Booth *et al.*, 1961; Boyland and Williams, 1965). The h-protein of mouse skin and rodent cells is similar in several properties, including a molecular weight of 42,000 and subunits of 21,000, to the azo-dye binding protein isolated from rat liver by Ketterer *et al.* (1967). Recently the same group demonstrated that under appropriate conditions of denaturation their protein released a glutathione conjugate (Ketterer *et al.*, 1971).

It is of considerable interest that the correlation of the carcinogenic activity of hydrocarbons and their derivatives with their binding to macromolecules appears to be better with the h-protein than with DNA, RNA, and total proteins (Heidelberger and Moldenhauer, 1956; Abell and Heidelberger, 1962; Goshman and Heidelberger, 1967; Tasseron *et al.*, 1970; Kuroki and Heidelberger, 1971). This suggests an involvement of the h-protein in the mechanism of hydrocarbon carcinogenesis, and appropriate biological experiments are currently being carried out in the system of carcinogenesis *in vitro* involving mouse prostate cells.

In search for function, Freed and Sorof (1966, 1967) found that a fraction containing the h-protein from rat liver inhibited the growth of cells in culture; this inhibition was shown to be caused by arginase, which had a different molecular weight from the h-protein (Sorof *et al.*, 1967). Umeda *et al.* (1968) demonstrated that mouse skin arginase activity was also easily separable from the mouse skin h-protein.

A possible function for the h-protein as a carrier of hydrocarbon from the cytoplasm to the nucleus was postulated by Abell and Heidelberger (1962). More recently a protein that specifically binds an anionic metabolite of corticosterone (Corticosteroid binder I) has been isolated by Morey and Litwack (1969) from rat liver. This protein has been found to be identical (Litwack and Morey, 1970) to one of the proteins from rat liver to which aminoazo dye carcinogens are bound (Ketterer *et al.*, 1967) and is also identical with the rat liver protein to which MCA is bound (Singer and Litwack, 1971). It has now been shown (Litwack *et al.*, 1971) that this protein is also identical to the organic anion-binding Y protein (Levi *et al.*, 1969; Arias, 1971) and it has been termed ligandin. In view of the great similarities in chromatographic behavior and molecular weight, it seems quite possible that our h-protein may also be the same as ligandin. Further work will be directed toward a determination of this identity and toward elucidation of the function of this protein and its role (if any) in the process of hydrocarbon carcinogenesis.

Acknowledgments

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References

Abell, C. W., and Heidelberger, C. (1962), *Cancer Res. 22*, 931.
Altaner, C., and Temin, H. M. (1970), *Virology 40*, 118.
Arias, I. M. (1971), *Chem. Biol. Interactions 3*, 237.
Berwald, Y., and Sachs, L. (1965), *J. Nat. Cancer Inst. 35*, 641.
Booth, J., Boyland, E., and Sims, P. (1961), *Biochem. J. 79*, 516.
Boyland, E., and Sims, P. (1964), *Biochem. J. 91*, 493.
Boyland, E., and Sims, P. (1965), *Biochem. J. 95*, 788.

Boyland, E., and Williams, K. (1965), *Biochem. J. 94*, 190.
Brookes, P., and Lawley, P. D. (1964), *Nature (London) 202*, 781.
Chen, T. T., and Heidelberger, C. (1969), *Int. J. Cancer 4*, 166.
Chu, E. M. Y., Brimer, P., Jacobson, K. B., and Merriam, E. (1969), *Genetics 62*, 359.
Diamond, L., Defendi, V., and Brookes, P. (1967), *Cancer Res. 27*, 890.
Embleton, M. J., and Heidelberger, C. (1972), *Int. J. Cancer* (in press).
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.
Gelboin, H. V. (1969), *Cancer Res. 29*, 1272.
Goshman, L. M., and Heidelberger, C. (1967), *Cancer Res. 27*, 1678.
Grover, P. L., and Sims, P. (1969), *Biochem. J. 110*, 159.
Grover, P. L., Sims, P., Huberman, E., Marquardt, H., Kuroki, T., and Heidelberger, C. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1098.
Heidelberger, C., and Moldenhauer, M. G. (1956), *Cancer Res. 16*, 442.
Huberman, E., Salzberg, S., and Sachs, L. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 77.
Huberman, E., Selkirk, J., and Heidelberger, C. (1971), *Cancer Res. 31*, 2161.
Huebner, R., and Todaro, G. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1087.
Ketterer, B., Beale, D., Litwack, G., and Hackney, J. F. (1971), *Chem. Biol. Interactions 3*, 285.
Ketterer, B., Ross-Mansell, P., and Whitehead, J. K. (1967), *Biochem. J. 103*, 316.
Kuroki, T., and Heidelberger, C. (1971), *Cancer Res. 31*, 2168.
Kuroki, T., Huberman, H., Marquardt, H., Selkirk, J. K., Heidelberger, C., Grover, P. L., and Sims, P. (1972), *Chem. Biol. Interactions* (in press).
Kuroki, T., and Sato, H. (1968), *J. Nat. Cancer Inst. 41*, 53.
Levi, A. J., Gatmaitan, Z., and Arias, I. M. (1969), *J. Clin. Invest. 48*, 2156.
Litwack, G., Ketterer, B., and Arias, I. M. (1971), *Nature (London) 234*, 466.
Litwack, G., and Morey, K. S. (1970), *Biochem. Biophys. Res. Commun. 38*, 1141.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Maizel, J. B., Jr. (1969), *in* Fundamental Techniques in Virology, Habel, K., and Salzman, N. P., Ed., New York, N. Y., Academic Press, p 334.
Miller, J. A. (1970), *Cancer Res. 30*, 559.
Mondal, S., Iype, P. T., Griesbach, L. M., and Heidelberger, C. (1970), *Cancer Res. 30*, 1591.
Morey, K. S., and Litwack, G. (1969), *Biochemistry 8*, 4813.
Nebert, D. W., and Gelboin, H. V. (1968), *J. Biol. Chem. 243*, 6242.
Newman, M. S., and Blum, S. (1964), *J. Amer. Chem. Soc. 86*, 5598.
Pitot, H. C., and Heidelberger, C. (1963), *Cancer Res. 23*, 1694.
Sanford, K. K. (1968), *Nat. Cancer Inst. Monograph 26*, 387.
Shapiro, A. L., Viñeula, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Selkirk, J., Huberman, E., and Heidelberger, C. (1971), *Biochem. Biophys. Res. Commun. 43*, 1010.
Singer, S., and Litwack, G. (1971), *Cancer Res. 31*, 1364.
Sorof, S. (1969), *Jerusalem Symp. Quant. Chem. Biochem. 1*, 28.
Sorof, S., Young, E. M., Luongo, L., Kish, V. M., and Freed, J. J. (1967), *Wistar Inst. Symp. Monograph 7*, 25.
Tasseron, J. G., Diringer, H., Frohwirth, N., Mirvish, S. S., and Heidelberger, C. (1970), *Biochemistry 9*, 1636.
Umeda, M., Diringer, H., and Heidelberger, C. (1968), *Israel J. Med. Sci. 4*, 1216.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wiest, W. G., and Heidelberger, C. (1953), *Cancer Res. 13*, 250.
Young, R. W., and Fulhorst, H. W. (1965), *Anal. Biochem. 11*, 389.