[CANCER RESEARCH 38, 4440-4444, December 1978]
0008-5472/78/0038-0000$02.00

Isolation and Partial Characterization of a ^{67}Ga-binding Glycoprotein from Morris 5123C Rat Hepatoma[1]

DeSales Lawless,[2] David H. Brown,[3] Karl F. Hübner, Shirley P. Colyer, James E. Carlton, and Raymond L. Hayes

Medical and Health Sciences Division, Oak Ridge Associated Universities,[4] Oak Ridge, Tennessee 37830

ABSTRACT

A glycoprotein, particularly high in tumors, has been extracted from Morris 5123C rat hepatomas and purified. The compound constitutes a major binding component for ^{67}Ga in this hepatoma. It has a molecular weight of approximately 45,000. Its molecular weight was determined by sodium dodecyl sulfate:polyacrylamide gel electrophoresis and by Sephadex G-200 superfine gel filtration. The steps involved in its extraction and purification include ultrafiltration, gel filtration through Sephadex G-200 superfine, ion-exchange chromatography on diethylaminoethyl Sephadex A-50, and hydroxylapatite chromatography. The homogeneity of the compound was established by gel electrophoresis. The NH_2-terminal residue, the percentage of nitrogen, the nonamino carbohydrate content, and the amino acid composition are reported.

INTRODUCTION

Since 1969 the radionuclide ^{67}Ga has been used to detect certain cancers by scanning techniques (10, 16). In normal animals the liver, spleen, bone, and bone marrow show high concentrations of ^{67}Ga 1 day after i.v. injection. However, when ^{67}Ga is injected into animals bearing transplanted tumors, the concentration is highest in the tumor tissue (16).

Hayes and Carlton (15) reported that 2 major macromolecular components bind ^{67}Ga in liver and malignant tissues. The lower-molecular-weight component (4 to 5 × 10^4) was found to be particularly high in tumors in contrast with that in the liver. We report here a procedure for extracting, purifying, and partially characterizing this lower-molecular-weight species.

MATERIALS AND METHODS

Animals

Male Buffalo rats, 3 to 4 months old, weighing 300 to 350 g, were used in this work. Laboratory chow (Ralston-Purina Co., St. Louis, Mo.) and water were available to the animals *ad libitum*. Morris 5123C hepatomas were transplanted i.m. by trocar into the thighs of these rats.

Materials

Carrier-free ^{67}Ga citrate was obtained from New England Nuclear, Boston, Mass. Sephadex G-200 superfine, Sephacryl S-200 superfine, DEAE-Sephadex A-50, and concanavalin A-Sepharose 4B were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Hydroxylapatite (Bio-Gel HTP) and the protein assay reagent were purchased from Bio-Rad Laboratories, Richmond, Calif. The dansylation chemicals were obtained from Pierce Chemical Co., Rockford, Ill., and the polyamide sheets were from Gallard-Schlesinger Co., Carle Place, N. Y. Bovine serum albumin and 1-*O*-methyl α-D-glycopyranoside were purchased from Sigma Chemical Co., St. Louis, Mo.

Ultrafiltration materials were purchased from Amicon Corp., Lexington, Mass. Polyacrylamide gel electrophoresis was performed with an LKB 2117 Mulitphor apparatus (LKB Instruments, Inc., Rockville, Md.).

Purification of the ^{67}Ga-binding Compound

Homogenization. Morris 5123C tumor implants were allowed to grow for 6 to 8 weeks and the animals were then given i.v. injections of ^{67}Ga citrate (0.1 to 1.0 mCi ^{67}Ga per kg, 1 mg citrate per kg). After 24 hr the animals were sacrificed and the tumor tissue was excised, washed briefly with cold 0.25 M sucrose, minced with scissors in ice-cold 0.25 M sucrose (1:10, w/v), and then homogenized in a Thomas Teflon homogenizer (cooled in an ice bath) with 6 strokes of the pestle (operated at 1000 rpm).

Extraction. The homogenate was centrifuged in a SW-27 rotor at 6 × 10^6 g_{min} at 4° with a Beckman L-2 ultracentrifuge. The supernatant (S-I) was then decanted from the pellet (P-I) as completely as possible and discarded. The pellet was rehomogenized in distilled water, allowed to stand for 1 hr in the cold, and then centrifuged under the same conditions. The new supernatant (S-II) was retained for isolation of the ^{67}Ga-binding compound, and the pellet (P-II) was discarded. The S-II aqueous solution was concentrated approximately 10-fold with an Amicon ultrafiltration cell and a PM 30 Diaflo membrane which retains globular proteins with molecular weights greater than 30,000. The final volume was 13 ml with a protein concentration of 13.1 mg/ml.

Sephadex G-200 and Sephacryl S-200 Gel Filtration. Sephadex G-200 superfine gel filtration was performed as described previously (15). Two- to 3-ml samples of the S-II concentrate were used in individual runs. ^{67}Ga radioactivity appeared in 2 major peaks corresponding to molecular weights of 1.0 to 1.2 × 10^5 and 4 to 5 × 10^4 (see Chart 1). The latter fraction (Seph-I) was investigated in this study

[1] This work was supported by NIH, Department of Health, Education and Welfare, Grant CA-11858.
[2] Present address: Fordham University, New York, N. Y.
[3] To whom requests for reprints should be addressed.
[4] Operated for the United States Department of Energy under Contract EY-76-C-05-0033.
Received June 9, 1978; accepted September 6, 1978.

since it appeared to be characteristic of tumor tissue in general (15).

For a recovery experiment Sephacryl S-200 was substituted for Sephadex G-200. This gel filtration was performed in a manner similar to that used with Sephadex G-200, except that the flow rate was 30 ml/hr.

DEAE Ion-Exchange Chromatography. A 2.6- x 40-cm DEAE-Sephadex A-50 column was equilibrated at 4° with 0.1 M Tris-HCl buffer, pH 7.0, at a gravity flow rate of 6 ml/hr. The Seph-I material (3 ml) was added at the top of the column and washed through with the 0.1 M Tris-HCl buffer until no ^{67}Ga radioactivity and no protein could be detected in the effluent. The column was then washed with 0.1 M Tris-HCl:0.05 M NaCl buffer, pH 7.0, again until no ^{67}Ga or protein could be detected. The column was finally eluted in a like manner with the same buffer system made 0.3 M with NaCl. Fractions from this elution were pooled, dialyzed to remove NaCl, concentrated by Amicon ultrafiltration to a 3-ml volume, and labeled D-I.

Hydroxylapatite Chromatography. A 1.6- x 10-cm column of hydroxylapatite (Bio-Gel HTP) was equilibrated at 4° with 0.01 M phosphate buffer, pH 7.0. The D-I from the DEAE-Sephadex A-50 separation, adjusted to a phosphate concentration of 0.01 M, pH 7.0, was added to the top of this column. The column was then washed with the above buffer until neither protein nor radioactivity was detected in the effluent. Following this the buffer was adjusted to pH 7.0 with 0.05 M phosphate, and the column was eluted in 1-ml fractions at a rate of 6 ml/hr. The radioactive fractions of successive runs were pooled, concentrated, dialyzed, and lyophilized, and the product was labeled H-I.

Assays

Protein Determinations. Protein determinations were made by the Bio-Rad protein assay (5) and the technique of Lowry *et al.* (21).

Dansyl Technique. The NH_2-terminal residue of the protein was identified by dansylating (5-dimethylaminonaphthalene-1-sulfonyl) 0.1 mg of the purified H-I material using the technique of Gray (13). The hydrolysate from this procedure was resolved on 2-dimensional polyamide chromatography sheets (5 x 5 cm), with the technique of Weiner *et al.* (26). The first solvent used was 1.5% formic acid and the second was benzene:acetic acid (9:1). A standard run was made under similar conditions and with a Beckman amino acid mixture.

Carbohydrate Analysis. Concanavalin A is specific for α-D-mannopyranosyl, α- and β-D-glycopyranosyl, and β-D-fructofuranosyl groups (14). To determine whether our compound contained such structures, we prepared a 1.5- x 12-cm column of concanavalin A-Sepharose 4B and equilibrated it with 0.1 M Tris-HCl buffer, pH 7.0. To the column we added 1 ml of S-II (13.1 mg/ml protein 760,800 cpm). The column was washed with buffer, and the effluent was recycled through the column 5 times. The column was then eluted with 10% α-methyl-D-glucose followed by 0.05 M sodium borate buffer at pH 7.0 (19). The binding of our compound to concanavalin A indicated to us the presence of a carbohydrate moiety (12). This was confirmed by periodic acid-Schiff reagent stain on polyacrylamide gels (24). The presence of nonamino carbohydrate was estimated by the phenol-sulfuric acid method (8) with galactose as a standard.

Amino Acid Analysis. The amino acid ratios in the protein were determined by the method of Spackman *et al.* (23) with a Beckman 120C autoanalyzer. Duplicate samples were hydrolyzed for 24 hr by the procedure of Hirs *et al.* (17). Proline and tryptophan were not written into the program of the computer readout since a different assay would have had to be used for proline determination, and alkaline rather than acid hydrolysis would have been required for tryptophan determination.

Nitrogen Composition. The percentage of nitrogen content was estimated in 3 separate runs by the micro-Kjeldahl technique (27). Samples of 0.8 mg were used.

Molecular Weight Determination. Gel filtration was performed as previously described by Hayes and Carlton (15) with the technique of Andrews (1).

Analytical SDS[5]:polyacrylamide gel electrophoresis was carried out using a slight modification of the procedure of Weber and Osborn (25). Ten % slab gels and a LKB 2117 Multiphor apparatus were used instead of disc gels. Samples were either heated at 100° for 2 min in SDS buffer or put directly on the gels. Sample volumes were 10 μl at concentrations of 1 to 2 mg/ml. Electrophoresis was carried out at 20 mA for 10 min and then at 100 mA until the bromphenol blue marker dye reached the bottom of the gel. Gels were fixed in 10% trichloroacetic acid, stained for 1 to 2 hr in Coomassie Brilliant Blue R-250, destained, and preserved in a solution of distilled water:ethanol:acetic acid:glycerol (10:3:1:1).

RESULTS

Purification. Table 1 summarizes the results of a representative extraction of the tumor from a single male Buffalo rat. In other extractions as many as 36 animals were used. When more than one animal was used, ^{67}Ga citrate was injected into one animal and the tumor tissue from this animal was used to spike the tissue homogenate of all animals. Approximately 50% of the ^{67}Ga originally present in the tumor tissue was recovered in the S-II supernatant.

S-II was resolved into 2 major ^{67}Ga peaks by Sephadex G-200 superfine filtration (Chart 1). An aliquot of the 4 to 5 × 10^4 fraction was assayed for protein concentration and radioactivity, and the remainder was applied directly to a DEAE-Sephadex A-50 column after concentrating. Earlier experience with DEAE-Sephadex A-50 had indicated that the high-molecular-weight compound could be removed from the ion-exchange column when the buffer was made 0.05 M with NaCl and that, when the NaCl concentration was raised to 0.3 M, the lighter-molecular-weight fraction was removed. Thus, after washing the column with 0.1 M Tris-HCl buffer containing 0.05 M NaCl, we added a 0.3 M NaCl:Tris buffer and removed the lower-molecular-weight material (D-I). Its protein concentration and radioactivity were determined, and the compound was then desalted and concentrated before being applied to hydroxylapatite for final purification.

[5] The abbreviation used is: SDS, sodium dodecyl sulfate.

Table 1

Stepwise purification of the ^{67}Ga-binding glycoprotein with a molecular weight of 45,000

Sample	Specific activity (cpm/mg protein)	^{67}Ga recovery (%)	Purification factor
Homogenate	16,470	100	1
Supernatant II (S-II)	49,800	52	3.02
Sephacryl S-200 (Seph-I)[a]	666,700	15	40.5
DEAE-Sephadex A-50 (D-I)	1,075,800	8.6	65.3
Hydroxylapatite HTP (H-I)	10,687,500	5.17	649

[a] Sephacryl S-200 was substituted for Sephadex G-200 (as described in "Materials and Methods").

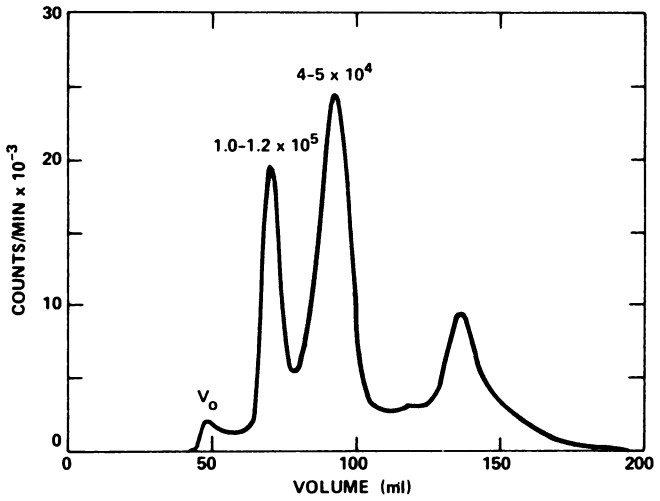

Chart 1. ^{67}Ga Sephadex G-200 superfine gel filtration profile of a distilled water extract of Morris 5123C rat hepatoma with the use of 0.1 M Tris buffer, pH 7.0. A flow rate of 6 ml/hr was used. The sample contained approximately 13 mg of protein. A 2.6- x 40-cm column was used.

Approximately 5.2% of the total activity in the starting homogenate was recovered in the purified hydroxylapatite fraction (H-I) with a purification factor of 649 (Table 1).

NH_2-terminal Residue. The results of dansylating the purified H-I protein and then submitting it to acid hydrolysis and chromatography on polyamide sheets in 2 dimensions indicated that a single species was present and that the NH_2-terminal residue was leucine. The spot visualized by UV was compared with the leucine in a Beckman standard amino acid mixture and also with dansylated leucine run separately against our preparation. A single spot was visible. The only other spot on the sheet was a trace of ϵ-lysine.

Amino Acid Content. The amino acid ratios obtained for H-I are reported in Table 2. The ratios are based on our estimate that the molecular weight of the compound is 45,000. Tryptophan was destroyed in acid hydrolysis and is not reported. Proline is likewise not reported since it did not react satisfactorily with ninhydrin under the conditions used. The ratios indicate that the protein is acidic since the number of acidic residues is higher than the number of basic residues.

Carbohydrate and Nitrogen Content. The phenol-sulfuric acid method for nonamino carbohydrate indicated that 3.6% of the compound by weight was nonamino sugar. We

Table 2

Amino acid composition of ^{67}Ga-binding glycoprotein with a molecular weight of 45,000

Amino acid	Found (nmol)	Nearest integer	Ratio	Ratio[a]
Lysine	24.0	24	6	42
Histidine	7.44	8	2	14
Arginine	11.5	12	3	21
Aspartylasparagine	22.7	23	6	42
Threonine	7.8	8	2	14
Serine	13.2	12	3	21
Glutanylglutamine	26.7	27	7	49
Proline				
Glycine	16.6	16	4	28
Alanine	27.1	27	7	49
Half-Cystine	6.6	8	2	14
Valine	4.63	4	1	7
Methionine	6.75	8	2	14
Isoleucine	3.16	4	1	7
Leucine	20.9	20	5	35
Tyrosine	7.9	8	2	14
Phenylalanine	10.9	12	3	21
Tryptophan				

[a] Based on molecular weight of approximately 45,000.

did not attempt to identify the individual sugars or test for the presence of amino sugars because of the limited quantity of purified compound. The micro-Kjeldahl determination for nitrogen showed 7.8% to be present; accordingly, our present estimate is that the substance contains 51% protein.

Molecular Weight Determinations. Our estimate of the molecular weight of the isolated H-I fraction is between 45,000 and 55,000 based on gel filtration studies with Sephadex G-200 superfine (Chart 2). SDS-PAGE produced a single band but indicated that the molecular weight of the purified compound was 83,000 (Chart 3). (The reason for the difference obtained between the 2 procedures is discussed later.)

Spectral Properties. The purified compound exhibited a typical protein UV spectrum in 0.1 M Tris-HCl buffer, pH 7.0. It was characterized by an absorption maximum at 278 nm. There was also a detectable absorbance at 400 nm, which was also apparent from the slight yellow color of the product. We estimated the 280:260 nm ratio to be 1.31, indicating a nucleic acid composition of under 1%. The molar absorbance is estimated from these studies to be 7.3.

Binding Properties. An aliquot of our S-II material showed a marked affinity for concanavalin A on Sepharose 4B. A sample containing 13.1 mg of protein was added to the column, and 0.161 mg was eluted with the radioactive material; 760,000 cpm were added and 288,000 cpm were washed out with the buffer. The protein adhering to the column along with its associated ^{67}Ga was eluted by 10% α-methyl-D-glucose followed by 0.05 N sodium borate buffer, pH 7.0 (68% recovery).

DISCUSSION

In 1969 Edwards and Hayes (9) reported an affinity of the radionuclide ^{67}Ga for certain soft tissue tumors in humans as the basis for the use of ^{67}Ga in the detection of cancer.

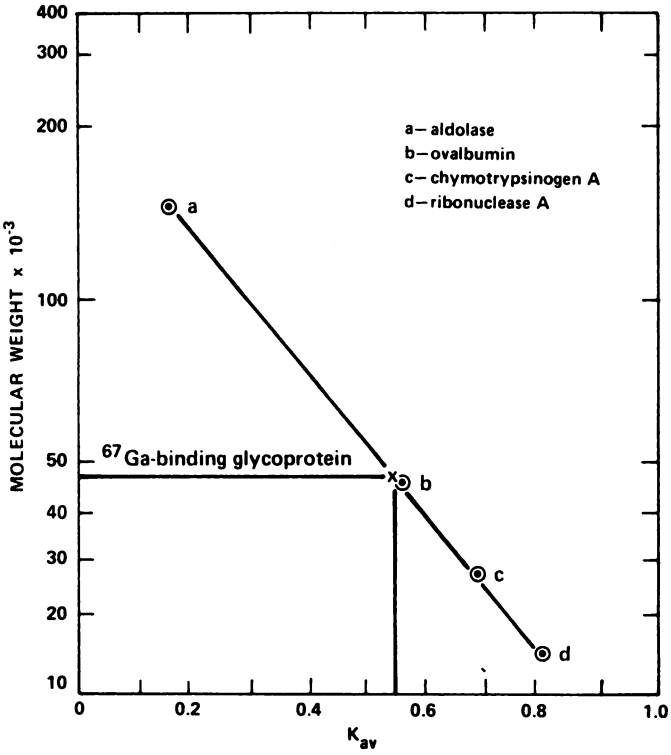

Chart 2. Estimation of the molecular weight of the hepatoma ^{67}Ga-binding glycoprotein by gel filtration. The glycoprotein sample was approximately 13 mg, and the standards contained 10 to 20 mg of protein. The gel filtration technique was the same as that used in Chart 1.

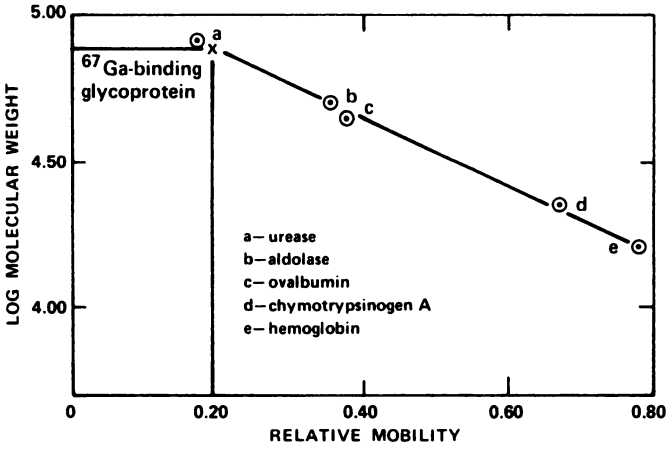

Chart 3. Estimation of the molecular weight of hepatoma ^{67}Ga-binding glycoprotein by SDS:polyacrylamide gel electrophoresis. The protein standards and ^{67}Ga-binding glycoproteins were suspended in 1% SDS and heated in a H_2O bath at 100° for 2 min. The solutions were chilled, and 10 μl of 2-mercaptoethanol and 10 μl of bromphenol blue (0.25%) were added to each 250-μl sample; 10 μl of each sample were then run for 2.3 hr at 7° and a field strength of 15 V/cm.

Since then animal studies have indicated that ^{67}Ga is associated with a lysosomal-like organelle in normal livers (7), but the majority of the ^{67}Ga was later found to be sequestered mostly in a smaller organelle in hepatomas (6). ^{67}Ga association has been further traced to 2 macromolecules within both these particles. A ^{67}Ga-binding macromolecule with a molecular weight of 4 to 5 × 10^4 has been found to be more abundant in hepatoma and other animal tumors than in normal liver (15). We have isolated and partially characterized this particular protein component that, although present in minute quantities, accounts for a large portion of the ^{67}Ga binding that occurs in the Morris 5123C hepatoma.

In the present study the ambiguity that arises in the molecular weight determinations by gel filtration and SDS:polyacrylamide gel electrophoresis may be the result of an anomalous behavior of the isolated glycoprotein in SDS gels (Chart 3). Although electrophoresis of most water-soluble proteins in SDS gels yields a molecular weight similar to that obtained by other methods, there are occasional artifactual results obtained by use of this analytical method (4, 11, 18, 20, 22). We have found that, if the purified ^{67}Ga-binding glycoprotein is subjected to electrophoresis conventionally or in the presence of gels containing SDS, but not pretreated with heat and denaturing amounts of SDS, the protein tends to band with standards having molecular weight values of 45,000 to 55,000. However, when we treated our isolated material with heat in 2% SDS [as is called for in the Weber-Osborn procedure (25)], we obtained a molecular weight of 83,000. At this point we can only speculate that heating resulted in dimerization of the molecule.

On the other hand it could be that an anomalous molecular weight value was obtained by gel filtration, since, as Andrews (2) has shown, glycoproteins often show anomalous behavior when molecular weights are determined by gel filtration. Most of the glycoproteins tested by Andrews showed an apparent increase in molecular weight of 10 to 150%, and in most instances the higher the carbohydrate concentration in the molecule the greater was the discrepancy from the accepted value. If the molecular weight value of 83,000 determined for our ^{67}Ga-binding glycoprotein by the SDS:polyacrylamide gel electrophoresis technique was correct, the gel filtration value would have had to be of necessity greater than the value obtained by electrophoresis. We find, however, that it is much lower. Therefore, we believe that the gel filtration value obtained for our ^{67}Ga-binding glycoprotein is the best estimate of its true molecular weight.

This report is intended as an initial communication on the purification and partial characterization of a ^{67}Ga-binding protein found in a transplanted animal hepatoma. This material appears to be present in tumor tissues in general, although it has been demonstrated previously that it occurs only in minute amounts, even in tumor tissues, *i.e.*, the mass action of the administration of a dose of as little as 25 μg of stable gallium per kg of body weight completely obliterates the ^{67}Ga binding of this material in tumor tissue (15). Initial experiments with antibody to the purified glycoprotein indicate that it probably does not originate from tumor tissue (D. H. Brown, K. F. Hübner, and R. L. Hayes, unpublished results); its function in tumor and normal tissue is not known.

REFERENCES

1. Andrews, P. Estimation of the Molecular Weights of Proteins by Sephadex Gel Filtration. Biochem. J., *91:* 222-233, 1964.
2. Andrews, P. The Gel-Filtration Behaviour of Proteins Related to Their Molecular Weights Over a Wide Range. Biochem. J., *96:* 595-606, 1965.
3. Ashwell, G. New Colorimetric Method of Sugar Analysis. Methods Enzymol., *8:* 93-95, 1966.

4. Atkinson, P. H., and Summers, D. F. Purification and Properties of HeLa Cell Plasma Membranes. J. Biol. Chem., *246:* 5162–5175, 1971.
5. Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem., *72:* 248–254, 1976.
6. Brown, D. H., Byrd, B. L., Carlton, J. E., Swartzendruber, D. C., and Hayes, R. L. A Quantitative Study of the Subcellular Localization of ^{67}Ga. Cancer Res., *36:* 956–963, 1976.
7. Brown, D. H., Swartzendruber, D. C., Carlton, J. E., Byrd, B. L., and Hayes, R. L. The Isolation and Characterization of Gallium-binding Granules from Soft Tissue Tumors. Cancer Res., *33:* 2063–2067, 1973.
8. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem., *28:* 350–356, 1972.
9. Edwards, C. L., and Hayes, R. L. Tumor Scanning with ^{67}Ga Citrate. J. Nucl. Med., *10:* 103–105, 1969.
10. Edwards, C. L., and Hayes, R. L. Scanning Malignant Neoplasms with Gallium 67. J. Am. Med. Assoc., *212:* 1182–1190, 1970.
11. Frank, R. N., and Rodbard, D. Precision of Sodium Dodecyl Sulfate-Polyacrylamide-Gel Electrophoresis for the Molecular Weight Estimation of a Membrane Glycoprotein: Studies on Bovine Rhodopsin. Arch. Biochem. Biophys. *171:* 1–13, 1975.
12. Goldstein, I. J., Hollerman, C. E., and Merrick, J. M. Protein-Carbohydrate Interaction. I. The Interaction of Polysaccharides with Concanavalin A. Biochim. Biophys. Acta, *97:* 68–76, 1965.
13. Gray, W. R. Dansyl Chloride Procedure. Methods Enzymol., *11:* 139–151, 1967.
14. Harmony, J. A. K., and Cordes, E. H. Interaction of Human Plasma Low Density Lipoprotein with Concanavalin A and with Ricin. J. Biol. Chem., *250:* 8614–8617, 1975.
15. Hayes, R. L., and Carlton, J. E. A Study of the Macromolecular Binding of ^{67}Ga in Normal and Malignant Animal Tissues. Cancer Res., *33:* 3265–3272, 1973.
16. Hayes, R. L., and Edwards, C. L. New Applications of Tumour-Localizing Radiopharmaceuticals. *In:* Medical Radioisotope Scintigraphy 1972, Vol. 2, pp. 531–552. Proceedings of a Symposium on Medical Radioisotope Scintigraphy held by the International Atomic Energy Agency in Monte Carlo, October 23 to 28, 1972. New York; Unipub, Inc., 1974.
17. Hirs, C. H. W., Stein, W. H., and Moore, S. The Amino Acid Composition of Ribonuclease. J. Biol. Chem., *211:* 941–950, 1954.
18. Katzman, R. L. The Inadequacy of Sodium Dodecyl Sulfate as a Dissociative Agent for Brain Proteins and Glycoproteins. Biochim. Biophys. Acta, *266:* 269–272, 1972.
19. Kennedy, J. F., and Rosevear, A. Assessment of the Fractionation of Carbohydrates on Concanavalin A-Sepharose 4B by Affinity Chromatography. J. Chem. Soc. Perkin Trans. I, 2041–2046, 1973.
20. Koga, S., Bolis, L., and Scanu, A. M. Isolation and Characterization of Subunit Polypeptides from Apoproteins of Rat Serum Lipoprotein. Biochim. Biophys. Acta, *236:* 416–430, 1971.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., *193:* 265–275, 1951.
22. McCumber, L. J., and Clem, L. W. Esterification of J Chain and Its Effect on Electrophoretic Mobility in Sodium Dodecyl Sulfate Polyacrylamide Gels. Biochim. Biophys. Acta, *446:* 536–541, 1976.
23. Spackman, D. H., Stein, W. H., and Moore, S. Automatic Recording Apparatus for Use in the Chromatography of Amino Acids. Anal. Chem., *30:* 1190–1205, 1958.
24. Wardi, A. H., and Michos, G. A. Alcian Blue Staining of Glycoproteins in Acrylamide Disc Electrophoresis. Anal. Biochem., *49:* 607–609, 1972.
25. Weber, K., and Osborn, M. The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. J. Biol. Chem., *244:* 4406–4412, 1969.
26. Weiner, A. M., Platt, T., and Weber, K. Amino-terminal Sequence Analysis of Proteins Purified on a Nanomole Scale by Gel Electrophoresis. J. Biol. Chem., *247:* 3242–3251, 1972.
27. Willard, H. H., Merritt, L. L., and Dean, J. A. (eds.). Instrumental Methods of Analysis, Ed. 5, pp. 791–795. New York: Van Nostrand Reinhold, 1974.