SUMMARY

The plant growth regulator 2,3,5-triiodobenzoic acid containing carbon-14 in the carboxyl position was synthesized and applied to field-grown soybeans. A biological half-life of 42.5 days was found for the radioactivity in the soybean plant. Radioactivity was translocated to sites of new growth within the plant. At harvest the seeds contained a residue equivalent to 167 ng. of TIBA* and/or its metabolites per Gm. Before an assessment of the health hazard associated with the use of this chemical on soybeans can be made, there must be further study as to the nature of the residue.

REFERENCES

(1) Galston, A. W., *Am. J. Botany*, **34**, 360(1947).
(2) Greer, H. A. L., and Anderson, I. C., *Crop. Sci.*, **5**, 229(1965).
(3) Wheeler, H. L., and Johns, C. O., *Am. Chem. J.*, **43**, 398(1910).
(4) Olivier, S. C. J., and Combe, W. P., *Rec. Trav. Chim.*, **69**, 22(1950); through *Chem. Abstr.*, **44**, 5330(1952).
(5) Munakata, K., and Nakai, A., *J. Agr. Food Chem.*, **7**, 176(1959).
(6) Ice, R. D., Breckinridge, C. E., Jr., and Christian, J. E., *J. Pharm. Sci.*, **55**, 497(1966).
(7) Jarboe, R., M.S. Thesis, Purdue University, Lafayette, Indiana, (1966).
(8) Klemme, C. J., and Hunter, J. H., *J. Org. Chem.*, **5**, 508(1940).
(9) Jarboe, R. H., Data, J. B., and Christian, J. E., *J. Pharm. Sci.*, **57**, 323(1968).
(10) Jaffay, H., and Alvarez, J., *Anal. Chem.*, **33**, 612 (1961).
(11) Schrodt, A., Gibbs, J., and Cavanaugh, R., "Quench Correction by Automatic External Standardization," Packard Instrument Co., Downers Grove, Ill., 1965.

Keyphrases

Carboxyl-^{14}C-2,3,5-triiodobenzoic acid—synthesis
Absorption, translocation, residue—soybean plants
TLC—analysis
Autoradiography—analysis
Liquid scintillation counting—^{14}C, soybean plants

Mechanism of Action of Phenolic Disinfectants VIII

Association of Phenolic Disinfectants With Proteins

By JON E. STARR and JOSEPH JUDIS

Association of phenol-^{14}C (P-C-14), *p-tert*-amylphenol-^{14}C (PTAP-C-14), and 2, 4-dichlorophenol-^{14}C (DCP-C-14) with human serum and bacterial (*Micrococcus lysodeikticus*) proteins was investigated by means of density gradient ultracentrifugation and Sephadex gel filtration. Definite association between the phenols, PTAP-C-14 and DCP-C-14, and human serum proteins could be demonstrated by sucrose density gradient ultracentrifugation and with Sephadex gel filtration. The major protein in human serum involved in this association appeared to be albumin. Sucrose density gradient ultracentrifugation provided data indicating association of bacterial proteins with the three phenolic compounds, but most clear-cut in the case of PTAP-C-14. Protein binding could explain interference of serum with germicidal effects of phenolic disinfectants and enzyme inhibition and structural damage may account for bactericidal action.

TWO MAJOR CONSIDERATIONS motivated the investigation of the possible association of proteins and phenolic disinfectants. The interference of organic matter with the action of many disinfectants including phenol derivatives is a well recognized, troublesome phenomenon (1) and the authors desired to gain direct evidence for the basis of this interference, namely, whether binding of disinfectant by protein occurs. Secondly, an understanding of the mechanism by which phenolic disinfectants are lethal to bacteria would be much more complete if it could be shown that certain microbial cell components are specifically attacked by these disinfectants. One could presume that if a chemical component of the microbial cell bound phenolic disinfectants, this component could be, hypothetically, the one damaged by these germicides. Proteins, of course, play a major structural and functional role in all living cells and the general toxicity of phenolic disinfectants could be best explained on the basis of affinity for a generally distributed cell component, such as proteins.

The proteins selected for the preliminary studies were human serum, human serum albumin,

Received April 28, 1967 from the College of Pharmacy, University of Toledo, Toledo, OH 43606
Accepted for publication January 10, 1968.
Abstracted in part from a thesis submitted by Jon E. Starr to the University of Toledo, Toledo, Ohio, in partial fulfillment of Master of Science degree requirements.

and an extract of *Micrococcus lysodeikticus* cells. The methods chosen for demonstrating possible binding of phenolic disinfectants by these proteins or more specifically, a protein-disinfectant complex, were density gradient centrifugation and Sephadex gel filtration. In previous work (2) three carbon-14 labeled phenol derivatives were used, phenol, 2, 4-dichlorophenol, and *p-tert*-amylphenol. The same compounds were utilized in the studies described here.

MATERIALS AND METHODS

Materials—The radioactive phenol derivatives used in the experiments were synthesized by New England Nuclear Corporation, Boston, Mass., and had the following specific activities: phenol-^{14}C (P-C-14), 50 μc. per 3.04 mg.; 2, 4-dichlorophenol-^{14}C (DCP-C-14), 50 μc. per 12.0 mg.; and *p-tert*-amylphenol-^{14}C (PTAP-C-14), 50 μc. per 30.4 mg. The label in all three compounds was uniformly distributed in the ring. Phenol-C-14 was used in aqueous stock solution while the other derivatives were dissolved in 0.1% sodium hydroxide and all stock solutions contained 5 μc./ml.

Human serum (tissue culture grade) was obtained from Difco Laboratories, Inc., Detroit, Mich. Albumin, human crystalline 4x, and crystalline D-sucrose, C.P., were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The albumin was reconstituted in distilled water to a concentration of 42 mg./ml. Sephadex G-200 and G-25 obtained from AB Pharmacia, Uppsala, Sweden, were used in the gel filtration experiments. All other materials used were reagent grade.

For the preparation of the bacterial extracts, *M. lysodeikticus* ATCC 4698 was grown on the medium previously used (2) and prepared in the following manner. Cells were washed twice with distilled water, and resuspended in distilled water to give a 1:3 w/v ratio. Glass beads measuring 25 μ in diameter were added in a 1:3 v/v ratio and the suspension subjected to sonic disintegration for 25 min. at 5° using a Branson sonifier, Branson Instruments Inc., Danbury, Conn., at the maximum power output. The material was then centrifuged at 12,000 × g for 10 min., and the supernatant, recentrifuged once. The supernatant contained 12.5 mg. of protein per ml., using the Lowry-Folin method (3) with crystalline human serum albumin as a standard.

Methods—Radio-assay was carried on in a Nuclear-Chicago Liquid Scintillation System and an aliquot of a sample was diluted when necessary with enough distilled water to produce a total volume of 2.0 ml., which was added to 13 ml. of a phosphor solution (2). Enough counts were accumulated in all experiments to give a nine-tenths error of less than 5%.

Density gradient separations were carried out as follows. In all experiments, 0.3 ml. of protein solution was added to 0.2 ml. of diluted radioactive phenol derivative. The stock solutions of the latter were diluted 8:50 (0.8 ml. of phenol derivative plus 4.2 ml. of 0.1% sodium hydroxide). In the experiments in which bacterial extract was used the ratio of the latter to the phenol derivative solutions was 9:1, using the stock solution of the phenol derivative undiluted. All mixtures were incubated for 10 min. at 25°. Gradients of 2–20% sucrose in 0.067 *M* tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), with a pH of 7.4, were used in the density gradient analysis involving human serum, serum albumin, and the radioactive phenols. Gradients of 1–20% sucrose in 0.067 *M* TRIS-HCl pH 7.4, were used in the studies with the bacterial extract. The gradients were produced in 5 ml. lusteroid centrifuge tubes using a gradient former constructed after the design of Britten and Roberts (4). In all of the analyses except those involving the bacterial extracts, 0.1 ml. of the sample was layered on the top of the sucrose gradient. In the experiments in which bacterial extract was used, 0.15 ml. of sample was taken for density gradient analysis. The tubes were centrifuged for 2–10 hr. at 165,000 × g at 3.5° in a Spinco model L-2 preparative ultracentrifuge using a SW50-L swinging bucket rotor. The contents of the tube were fractionated by piercing the bottom of the tube with a needle and eight drops were collected per fraction. Each fraction was diluted with 2.0 ml. of distilled water in all cases except those experiments involving the bacterial extract, in which case the fractions were diluted with 1.5 ml. of distilled water. One milliliter of each fraction was analyzed for protein using the Lowry-Folin method (3) and another aliquot of each diluted fraction was taken for measurement of radioactivity.

The procedure for gel filtration studies involving PTAP-C-14 was as follows. Sephadex G-200 was allowed to come to equilibrium in 1.0 *M* sodium chloride solution and a bed 30 cm. in height was prepared in a 2.5 × 45 cm. column obtained from AB Pharmacia, Uppsala, Sweden. The column was washed with 1.0 *M* sodium chloride for 3 days to stabilize the bed and a nylon gauze was placed on the surface of the bed to minimize its disruption when layering the samples (5). Reaction mixtures containing 2.0 ml. of human serum and 0.5 ml. of PTAP-C-14 (0.2 ml. of stock solution and 9.8 ml. of 0.1% sodium hydroxide), were incubated for 10 min. at 25°. Two milliliters of the mixture was layered on the bed and eluted with 1.0 *M* sodium chloride. Three-milliliter fractions of the eluant were collected on a fraction collector and 1 ml. of each fraction was diluted with 2.0 ml. of distilled water and the protein content determined by measuring light absorption at 279 mμ in a Bausch and Lomb Spectronic 600 spectrophotometer. The remaining 2.0 ml. of each fraction was used for measurement of radioactivity. In the experiments in which DCP-C-14 was used, Sephadex G-25 replaced G-200; 0.3 *M* sodium chloride was used as the eluant; and the reaction mixture consisted of 1.0 ml. of human serum, 1.0 ml. of 0.067 *M* phosphate buffer at pH 7.4, and 0.5 ml. DCP-C-14 (0.3 ml. stock solution and 2.7 ml. of 0.1% sodium hydroxide). Sephadex G-25 was used in place of Sephadex G-200 in the experiments with DCP-C-14 because Sephadex G-25 interacts less than G-200 with phenolic compounds (5) thus offering less competition to the proteins in the formation of complexes of the latter.

RESULTS AND DISCUSSION

Figures 1 through 3 represent the results of the density gradient studies of mixtures of human

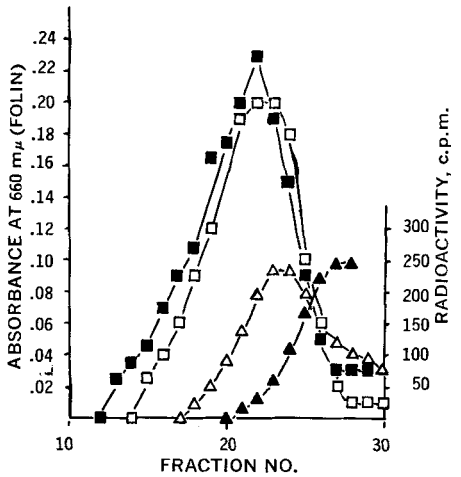

Fig. 1—Density gradient ultracentrifugation of human serum, PTAP-C-14, and a mixture of human serum and PTAP-C-14. The mixture was incubated for 10 min. at 25°. One-tenth ml. of the mixture was layered on a 2–20% sucrose gradient, centrifuged at 165,000 × g at 3.5° for 300 min., fractionated, and analyzed for protein and radioactivity. The initial mixture contained 0.3 ml. serum and 0.2 ml. of isotope (see Materials and Methods). *Key:* ■, *human serum alone;* □, *human serum mixed with PTAP-C-14;* ▲, *PTAP-C-14 alone;* △, *PTAP-C-14 mixed with human serum. In all figures,* □ ■ *refers to data obtained by analysis for protein and* △ ▲ *refers to radioactivity.*

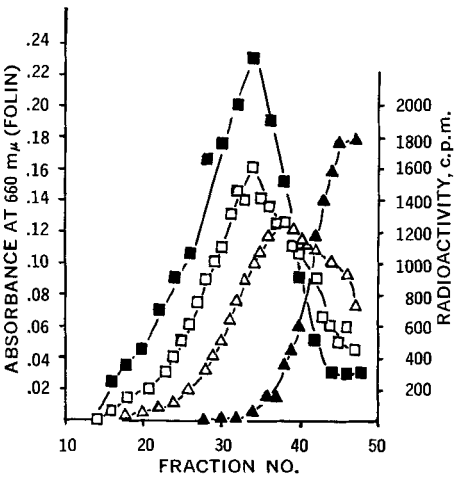

Fig. 2—Density gradient ultracentrifugation of human serum, DCP-C-14, and a mixture of human serum and DCP-C-14. Experimental conditions were the same as in Fig. 1 except the samples were centrifuged for 600 min. Key: ■, *human serum alone;* □, *human serum mixed with DCP-C-14;* ▲, *DCP-C-14 alone;* △, *DCP-C-14 mixed with human serum.*

serum and the radioactive derivatives of phenol. As one would expect, the phenol derivatives moved very little in the centrifugal field employed while the human serum proteins moved a considerable distance. The radioactive peaks obtained upon analysis of the mixtures of phenol derivatives with human serum are taken to represent bound phenol derivative. In most cases, the radioactive peak

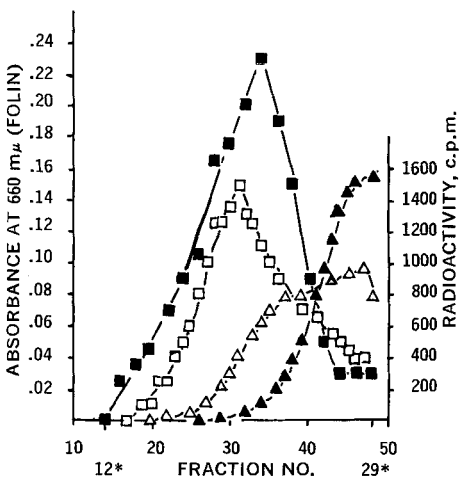

Fig. 3—Density gradient ultracentrifugation of human serum, P-C-14, and a mixture of human serum and P-C-14. Experimental conditions were the same as in Fig. 1. Key: ■, *human serum alone;* □, *human serum mixed with P-C-14;* ▲, *P-C-14 alone;* △, *P-C-14 mixed with human serum.*

from the mixtures did not correspond exactly with that of free protein and this slight difference in mobility may be due to the change of properties of the proteins when they are in combination with phenol derivatives. Chen (6) points out that serum albumin may have different physical properties when combined with fatty acids. The data in Fig. 3 would suggest that phenol does not form as tight a complex with serum proteins since the radioactive peak obtained from the phenol-human serum mixture is not as distinct nor was it located as close to the protein peak as it was in the cases of the other two phenol derivatives.

The data obtained from the experiments in which human serum albumin was substituted for human serum are given in Figs. 4 through 6. The results are similar to those obtained with human serum including the suggestion of relatively poor binding of phenol. While the radioactive peak obtained on analysis of phenol-albumin mixtures was in a significantly different position from that of free phenol (Fig. 6), the former certainly did not correspond well to the curve representing albumin.

Previous work from this laboratory (2) indicated that bacterial extracts did bind phenol derivatives in that the extracts interfered with binding of the phenol derivatives to whole cells. Density gradient analysis of mixtures of bacterial extract and radioactive phenols did show evidence for protein-phenol derivative complexes but most conclusively in the case of PTAP-C-14 (Fig. 7). The radioactive peak obtained in mixtures of DCP-C-14 and P-C-14 (Figs. 8 and 9) were significantly different in location from free phenol derivatives but not as distinctly as in the case of PTAP-C-14.

Evidence for protein-phenol derivative complexes was more unequivocal in the experiments utilizing gel filtration as the analytical method (Figs. 10 through 13). For example, PTAP-C-14 had a distinctly different rate of elution from the human serum proteins (Fig. 10) when subjected to gel filtration individually, but in a mixture, a radioactive peak appeared at a position (5) identical

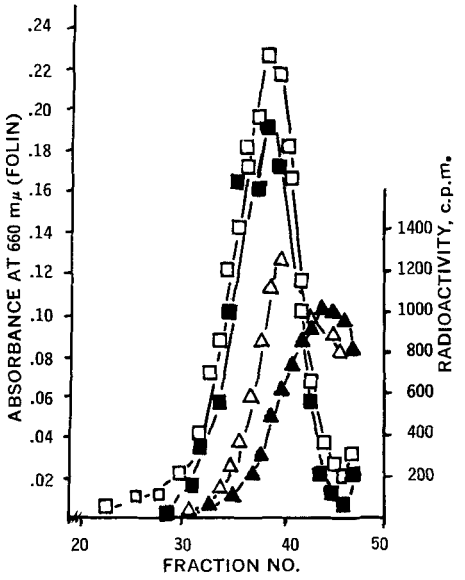

Fig. 4—Density gradient ultracentrifugation of human serum albumin, PTAP-C-14, and a mixture of serum albumin and PTAP-C-14. Experimental conditions were the same as in Fig. 1. Key: ■, albumin alone; □, albumin mixed with PTAP-C-14; ▲, PTAP-C-14 alone; △, PTAP-C-14 mixed with albumin.

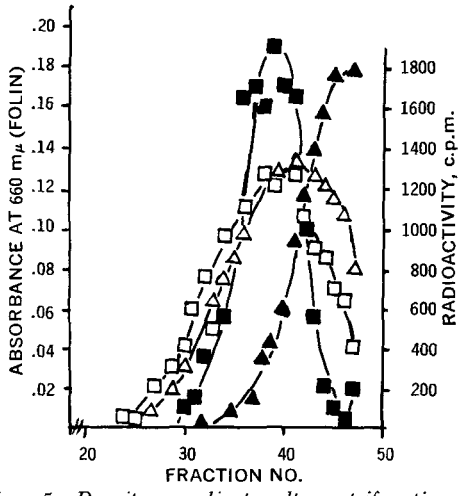

Fig. 5—Density gradient ultracentrifugation of human serum albumin, DCP-C-14, and a mixture of serum albumin and DCP-C-14. Experimental conditions as for Fig. 1. Key: ■, albumin alone; □, albumin mixed with DCP-C-14; ▲, DCP-C-14 alone; △, DCP-C-14 mixed with albumin.

with that of the human serum albumin peak (Fig. 11). Similar results were obtained with human serum and DCP-C-14 (Figs. 12 and 13) although in these experiments, the human serum proteins did not separate since Sephadex G-25 was used instead of G-200. Phenol-C-14 was not included in this series because preliminary experiments demonstrating definite association of the other two phenol derivatives with serum proteins indicated no binding of phenol-C-14.

In the opinion of the authors, the experimental

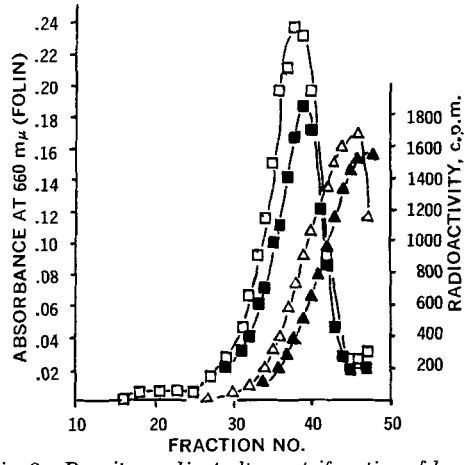

Fig. 6—Density gradient ultracentrifugation of human serum albumin, P-C-14, and a mixture of serum albumin and P-C-14. Experimental conditions as in Fig. 1. Key: ■, albumin alone; □, albumin mixed with P-C-14; ▲, P-C-14 alone; △, P-C-14 mixed with albumin.

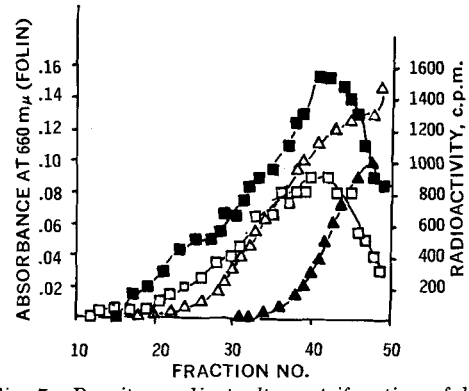

Fig. 7—Density gradient ultracentrifugation of bacterial extract, PTAP-C-14, and a mixture of bacterial extract and PTAP-C-14. Changes in experimental conditions from those in Fig. 1 were that 0.15 ml. of sample was layered on a 1–20% sucrose gradient, and centrifuged for 180 min. The initial mixture contained 0.9 ml. of bacterial extract and 0.1 ml. of phenol derivative (see Methods and Materials*). Key: ■, bacterial sonicate alone; □, bacterial sonicate mixed with PTAP-C-14; ▲, PTAP-C-14 alone; △, PTAP-C-14 mixed with bacterial sonicate.*

results present evidence for a protein-phenol disinfectant complex and would thus indicate that phenol derivatives are bound to serum proteins and presumably bacterial proteins, since the latter should constitute the major component of the bacterial extracts and the analytical method used would detect no other high molecular weight compounds other than proteins. These results are in accord with work previously reported from this laboratory (2) and that of Weinbach and Garbus (7–9) who reported the binding of several halophenols including DCP, to albumin. In all of the methods used in the studies reported here, the evidence for a protein-PTAP or DCP complex was much more clear-cut than for a protein-P complex in that the former were characterized by radioactive and protein curves corresponding rather

closely but in the case of the latter, correspondence was relatively poor. It is conceivable that the phenol derivatives did not associate with albumin as such but perhaps some other protein in the albumin fraction. Another explanation for the noncorrespondence of protein and phenol derivative curves could be that the phenol-protein complex had different mobility or rate of elution from those of the protein only. The same data might be considered in terms of relative affinities of the three phenol derivatives for proteins and one might expect that phenol is a poorer disinfectant because it is capable of less or looser binding to proteins. Although the specific activities of the three phenol derivatives were not identical, tightness of binding to proteins was considered to be related to identity of location of radioactivity and protein.

It was hoped that the experiments concerning bacterial extracts would result in clear-cut evidence for the existence of a protein-phenol derivative complex since previous work (2) described experiments indicating binding of phenol derivatives by whole cells. In mixtures of bacterial extracts and disinfectants PTAP-C-14 seemed to be located in a

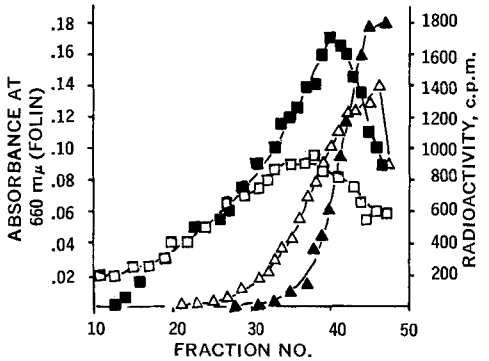

Fig. 8—Density gradient ultracentrifugation of bacterial extract, DCP-C-14, and a mixture of bacterial extract and DCP-C-14. Experimental conditions were the same as those in Fig. 7. Key: ■, bacterial sonicate alone; □, bacterial sonicate mixed with DCP-C-14; ▲, DCP-C-14 alone; △, DCP-C-14 mixed with bacterial sonicate.

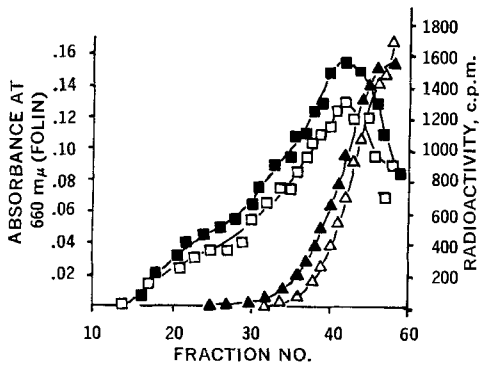

Fig. 9—Density gradient ultracentrifugation of bacterial extract, P-C-14, and a mixture of bacterial extract and P-C-14. The experimental conditions were the same as those used in Fig. 7. Key: ■, bacterial sonicate alone; □, bacterial sonicate mixed with P-C-14; ▲, P-C-14 alone; △, P-C-14 mixed with bacterial sonicate.

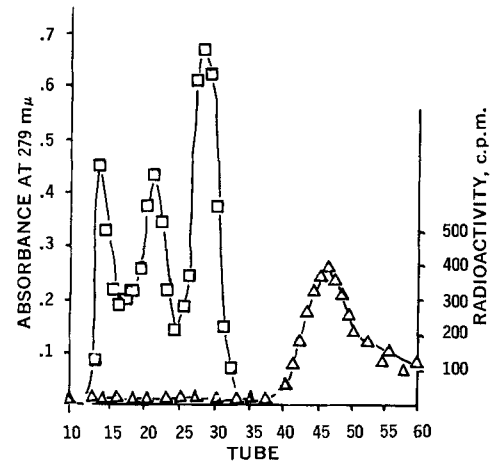

Fig. 10—Gel filtration of human serum and PTAP-C-14 separately. A 2.0-ml. sample was eluted from a Sephadex G-200 column with 1.0 M sodium chloride and 3.0 ml. fractions were collected and analyzed for protein and radioactivity. Key: □, human serum; △, PTAP-C-14.

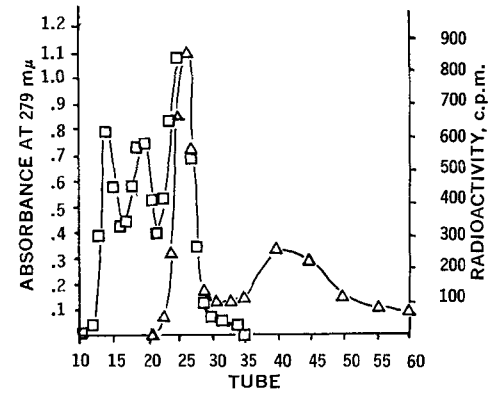

Fig. 11—Gel filtration of a mixture of human serum and PTAP-C-14. Experimental conditions were the same as in the case of Fig. 10. Key: □, human serum; △, PTAP-C-14.

centrifugal field similarly to protein in the bacterial extract (see Fig. 7), but the same was not true of DCP-C-14 or P-C-14 (Figs. 8 and 9). It is possible that in the process of preparing the bacterial extracts, major components responsible for binding of the phenol derivatives to whole cells were not obtained or were lost in the process of centrifugation of the sonic disintegrates. It is equally conceivable that the major component responsible for binding of phenol derivatives is present in such low concentrations in the extracts that it would not be detected by the method for protein analysis used. In any case, further work is presently in progress to obtain more conclusive evidence for binding of phenol derivatives by a component of these bacterial cells and characterization of this component. In addition, additional studies are being carried on to characterize the nature of binding of serum albumin to phenol derivatives, especially the number and nature of the binding sites involved. In conclusion, it is felt that the data herein presented suggest that phenols do bind to proteins including bacterial proteins and that this phenomenon could well

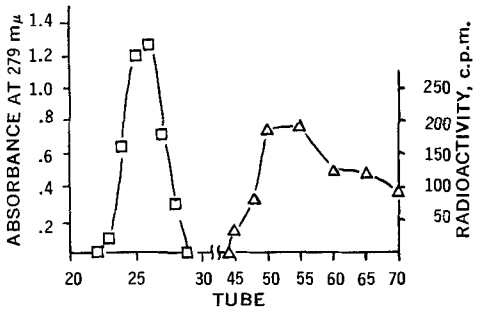

Fig. 12—Gel filtration of human serum and DCP-C-14 separately. Experimental conditions were the same as those used in the experiment represented by Fig. 10 except Sephadex G-25 was used and elution was done with 0.3 M sodium chloride. Key: □, human serum; △, DCP-C-14.

account for the antibacterial action of phenols. If binding is to the proteins in bacterial membranes, this association could well lead to disruption of the integrity of bacterial membranes, a phenomenon which has been demonstrated (10). Inactivation of enzymes by phenol is well documented (7, 11–15) and the latter could be a result of this binding process. It is interesting to note that there appears to be a correlation between the phenol coefficient of a given derivative and the extent of binding to whole bacterial cells. It is hoped that it may be possible to demonstrate a similar correlation in regard to binding to proteins in general.

REFERENCES

(1) Bennett, E. O., "Advances in Applied Microbiology," vol. 1, Academic Press Inc., New York, N. Y., 1959, p. 123.
(2) Judis, J., *J. Pharm. Sci.*, **55**, 803(1966).
(3) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265(1951).
(4) Britten, R. J., and Roberts, R. B., *Science*, **131**, 32(1960).
(5) Flodin, P., "Dextran Gels and Their Applications in Gel Filtration," A. B. Pharmacia, Uppsala, Sweden (1962).
(6) Chen, R. F., *J. Biol. Chem.*, **242**, 173(1967).
(7) Weinbach, E. C., and Garbus, J., *Science*, **145**, 824 (1964).
(8) Weinbach, E. C., and Garbus, J., *J. Biol. Chem.*, **240**, 1811(1965).
(9) *Ibid.*, **241**, 169 (1966).
(10) Joswick, H. L., Doctoral thesis, University of Michigan (1961).
(11) Weinbach, E. C., *Proc. Natl. Acad. Sci.*, **43**, 393(1957).

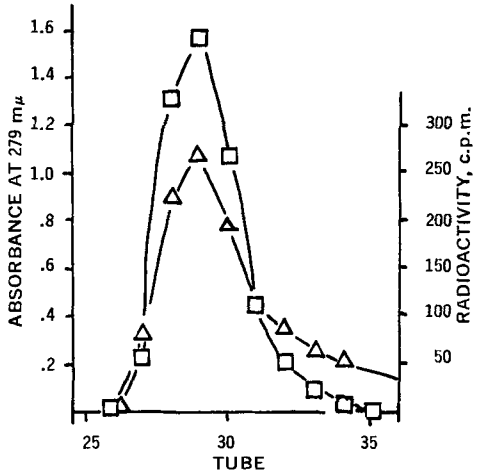

Fig. 13—Gel filtration of a mixture of human serum and DCP-C-14. The experimental conditions were the same as those of Fig. 12. Key: □, human serum mixed with DCP-C-14; △, DCP-C-14 mixed with human serum.

(12) Weinbach, E. C., and Bowen, W. J., *Biochim. Biophys. Acta*, **30**, 476(1958).
(13) Commager, H., and Judis, J., *J. Pharm. Sci.*, **54**, 1436 (1965).
(14) Judis, J., *ibid.*, **54**, 417(1965).
(15) *Ibid.*, **54**, 541(1965).

Keyphrases

Phenolic disinfectants—activity mechanism
Radioactive phenol derivates—test compounds
Protein–phenol disinfectant—association
Density gradient, sucrose—association confirmed
Gel filtration—association confirmed
Liquid scintillation counting—analysis
Spectrophotometry—analysis