1. Ichelson, Rose, R., PROC. SOC. EXP. BIOL. AND MED., 1953, v95, 57.

2. Steiner, J., *J. Neuropathol. and Exp. Neurol.*, 1952, v11, 343.

3. Needham, G. M., Yoss, R. E., Daly, D., *Proc. Staff Meetings of Mayo Clinic*, 1958, v33, 395.

4. Mavor, H., Gallaher, F. W., Schumacher, G. A., *New England J. Med.*, 1958, v260, 860.

5. Harding, H. B., Pollock, L. J., Dobin, N., Ruge, D., *Quart. Bull., Northwestern Univ. Med. School*, 1958, v32, 144.

Received June 1, 1959. P.S.E.B.M., 1959, v102.

## Electrophoretic and Immunologic Studies of Rivanol-Fractionated Serum Proteins.*† (25198)

ABRAHAM SAIFER AND LEWIS E. LIPKIN (Introduced by Bruno W. Volk)
*Isaac Albert Research Inst., Jewish Chronic Disease Hospital, Brooklyn, N. Y.*

Rivanol (6,9 diamino-2 ethoxyacridine lactate) has been employed by Hořejsí and Smetana(1,2) for precipitation of various protein fractions from serum or plasma at room temperature and in slightly alkaline solution. In contrast to most other procedures, *e.g.*, alcohol, salt fractionation, etc., addition of rivanol to serum does not result in precipitation of gamma, and to a lesser extent, beta globulins. This unique property of rivanol has been utilized in simple methods for preparation of highly purified gamma globulin(2), for separation of beta-1-metal combining globulin(3) and preparation of ceruloplasmin from Cohn's fractions III and IV(4). The apparent contradiction of the original concept of rivanol as specific reagent for separation of gamma globulin from all other serum protein fractions (2) and its use by others in preparation of beta globulin components led to the present study of rivanol-serum protein interactions. Our studies demonstrate that the supernatant solution, following rivanol precipitation of more rapidly migrating electrophoretic serum protein fractions, contains some beta globulin in addition to gamma globulin, as shown by both paper and moving boundary electrophoresis. In addition, this rivanol-containing supernate may be injected intravenously into rabbits resulting in antihuman-globulin sera of relatively high titer.

*Materials and methods. Rivanol.* Preparation of rivanol in these studies was obtained from Special Chemical Dept. of Winthrop Labs. under trade name "Ethodin." (Lot #N325FA). A 0.4% solution of rivanol in distilled water was prepared. *Rivanol-serum preparations.* To one volume of normal, human serum were added 1,2,3,5 and 9 volumes of 0.4% rivanol solution. The mixtures were left overnight at 4° and the resulting supernates were separated from precipitates for each mixture by centrifugation at 2250 g (International Type II) for 10 minutes. Supernates were then analyzed for protein composition by paper electrophoresis as described below. Corresponding precipitates were dissolved in 2 ml of citrate buffer (pH 5.0, 0.2 M). Except for small amount of insoluble material, the remainder of precipitate was readily soluble in this buffer. The redissolved precipitates were then subjected to paper electrophoresis in the same manner as their corresponding supernates. *Paper electrophoresis procedure.* The free-hanging horizontal strip technic of Dettker and Anduren(5) was used for paper electrophoresis determinations. The technic was essentially the same as described by Block, *et al.*(6), using Whatman No. 1 paper, barbital buffer (pH 8.6, 0.05 ionic strength) 220V for 6 hours and staining with Amido-Black 10B. Multiple aliquots of 10 lambda in inverse proportion to protein content were applied to paper strips (Fig. 1).

* Aided by Grant from U.S.P.H.S.

† The authors wish to acknowledge the aid of Shirley Gerstenfeld in the paper electrophoretic patterns, of Francis Vecsler, in performing moving boundary electrophoresis experiments, and Lillian Salowitz in editing and typing of manuscript.

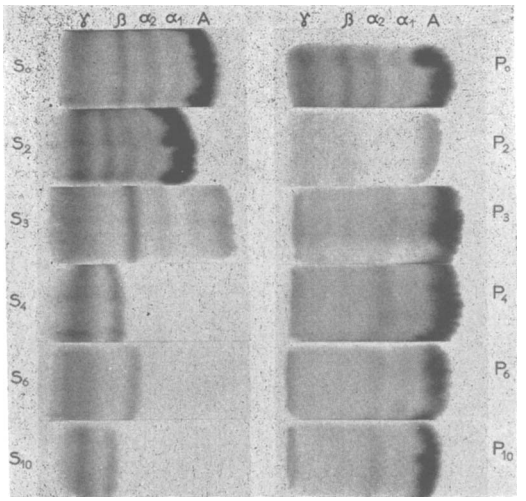


FIG. 1. Paper electrophoretic patterns obtained with barbital buffer (pH 8.6, .05 ionic strength), of the supernates and precipitates (dissolved in citrate buffer pH 5.0) of various rivanol-serum ratios. $S_0$, $P_0$ represents aliquots of original serum sample. $S_2$, $P_2$ $S_3$, $P_3$, etc. represent supernates and precipitates obtained by diluting serum 1:2, 1:3, etc. with .4% rivanol. Different aliquots of each sample were applied to the paper in inverse ratio to their protein content.

Samples of untreated serum were run simultaneously with both supernates and precipitates. Study of immunologic properties of rivanol-globulin complex employed 4 rabbits. Two of these were injected 4 times/week for 4 weeks, and 2 received subcutaneous doses once each week for same period. The dose was kept constant at 5 ml of supernatant solution derived from 1 vol. of serum mixed with 5 volumes of rivanol (1:6). In neither group were any untoward reactions observed, either local or generalized. Animals were bled weekly *via* ear vein, sacrificed at end of 4th week and autopsies performed. Serum obtained from weekly bleeding was titered against a 0.1% solution of purified human gamma globulin as the antigen.

*Results.* The electrophoretic runs (Fig. 1) demonstrate that as volume of 0.4% rivanol solution is increased in relation to a fixed volume of serum, the faster migrating electrophoretic fractions (albumin and alpha-globulins) are progressively removed from the supernate and appear in corresponding precipitates. In a 1:4 mixture the supernate contains only gamma and beta globulins. Further addition of 0.4% rivanol solution does not result in separation of any additional fractions as indicated in $S_6$, $P_6$, and $S_{10}$, $P_{10}$ patterns. When a 0.2% rivanol solution was used instead of 0.4%, a mixture of 1:6 or higher was required to obtain the supernate pattern corresponding to $S_4$ above. An analysis of the supernate equivalent to $S_6$ (Fig. 1) with the Tiselius moving-boundary electrophoresis (barbital buffer, pH 8.6, 0.1 ionic strength) showed presence of components in the following relative concentration (as % of the total protein): – gamma globulin (79%): beta globulins (13%) and alpha globulins (8%).

Autopsy of rabbits injected both intravenously and subcutaneously with the rivanol-globulin supernate revealed no gross abnormalities of any of the viscera. Microscopic examination of lung, liver, spleen, lymph node, kidney and adrenal showed no unusual features as compared to those seen in the usual process of immunization.

A progressive rise in precipitin titer to as high as 1:600 at end of 4 weeks, was obtained in sera of intravenously treated animals while subcutaneously injected animals showed a titer of 1:32. There was no cross precipitation with human albumin.

*Discussion.* Our study confirms the work of previous investigators(1,2,3,4) that addition of rivanol to serum or plasma at room temperature results in selective precipitation of protein fractions of greater mobility and retention in solution of those of lesser mobility. From our studies as well as previous studies of Hořejsí and Smetana(1,2) and of Boettcher, *et al.*(3), it appears that the major protein fractions remaining in the supernate at optimal rivanol (0.4%)-serum mixtures (>4:1) are gamma globulin and beta-1-metal combining globulin. The probable reason for lack of reactivity of many proteins, *e.g.*, gamma globulin, with organic cations and anions has been explained by Klotz(7,8) as due to internal binding between hydroxyl amino acids and carboxylic and cationic amino acid side chains supplemented by Van der Waals forces. In our opinion this does not rule out the possibility of soluble complex formation between these 2 protein fractions

and rivanol; this is being investigated.

The problem of possible denaturation and loss of biological activity of gamma globulin prepared by rivanol treatment has been extensively investigated by Hořejsí and Smetana (2) with respect to electrophoretic mobility, polarographic and potentiometric determination of SH groups, dye binding, viscosity and tryptic digestion. Of particular interest was their demonstration that rivanol prepared gamma globulin contained antibodies against polioencephalitis, several strains of influenza virus and infectious hepatitis of the same degree of protective capacity as did ethanolic preparations of gamma globulin. These results, which demonstrate unimpaired antibody activity of rivanol prepared gamma globulin, are supplemented by our finding of potent antigenic activity of such gamma globulin preparations. We have also shown that it is not necessary to remove rivanol prior to parenteral injection and that it is without deleterious effect on rabbits. Use of supernate without further purification, *e.g.*, charcoal absorption to remove rivanol, prevents loss of considerable proportion of original serum gamma globulin content.

While it is well known that most antibodies are gamma globulins, a number of antibodies have also been shown to have electrophoretic mobilities corresponding to beta-2-globulins (9). Therefore, the admixture of beta globulins with gamma globulins resulting from rivanol preparations may be advantageous, as for example in the immunohistochemical study of hypersensitivity states. It appears from the work of Boettcher, *et al.*(3), as well as our preliminary studies that the beta globulin component obtained with higher rivanol-serum ratios is the beta-1-metal combining globulin (transferrin or siderophilin). This component has recently been reported to possess antiviral activity(10).

These studies are being pursued along 2 parallel lines. Firstly, the antigenic properties and potency of various rivanol-plasma protein fractions are being investigated. Secondly, more detailed physico-chemical studies of these fractions are being carried out.

*Summary.* 1. Variation of ratio of 0.4% rivanol (6,9, diamino-2 ethoxyacridine lactate) solution to that of serum, provides a simple and rapid method for fractionation of serum proteins at room or refrigerator (4°) temperature. 2. Paper electrophoresis (barbital buffer, pH 8.6, 0.05 ionic strength) was used to determine protein fractions remaining in the supernate and those found in corresponding precipitates (soluble in pH 5.0 citrate buffer) at various serum-rivanol ratios. 3. The supernate of serum-rivanol mixtures of 1:4 or higher consisted almost entirely of gamma globulin (79%) and of the beta-1-metal combining globulin (13%) as determined by moving boundary electrophoresis. 4. Immunization of rabbits by direct intravenous injection of rivanol-globulin supernate caused no deleterious effects in animals and resulted in high titer antisera (1:600). 5. The possible advantages of using rivanol dilution as a simple method for preparation of concentrated plasma protein fractions containing substances with specific biological activity, *e.g.*, ceruloplasmin, gamma globulin, etc. is discussed.

1. Hořejsí, J., Smetana, R., *Collection Czechoslov. Chem. Commun.*, 1954, v19.

2. ———, *Acta Med. Scand.*, 1956, v155, 65.

3. Boettcher, E. W., Kistler, P., Nitschmann, H., *Nature*, 1959, v181, 490.

4. Steinbuch, M., Quentin, M., *ibid.*, 1959, v183, 323.

5. Dettker, A., Anduren, H., *Scand. J. Clin. and Lab. Invest.*, 1954, v6, 74.

6. Block, R. J., Durrum, E. L., Zweig, G., *Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, N. Y., 1955.

7. Klotz, I. M., Urquhart, J. M., *J. Am. Chem. Soc.*, 1949, v71, 1957.

8. Klotz, I. M., in Pauling, L., Itano, H. A., ed., *Molecular Structure and Biological Specificity*, Waverly Press, Baltimore, 1957, p91.

9. Oncley, J. L., in J. L. Tullis, ed. Blood Cells and Plasma Proteins, Academic Press, 1953, p180.

10. Martin, C. M., Jandl, J. H., *J. Clin. Invest.*, 1959, v38, 1024.

Received June 25, 1959. P.S.E.B.M., 1959, v102.