The Preparation and Some Properties of Fibrinogen Precipitated from Human Plasma by Glycine.* (28553)

LOUIS A. KAZAL, SHELDON AMSEL, ORIN P. MILLER AND L. M. TOCANTINS†
Section on Plasma Fractionation, Charlotte Drake Cardeza Foundation, Jefferson Medical College, Philadelphia, Pa.

Blombäck and Blombäck(1) utilized the salting-in and salting-out property of glycine in presence of low concentrations of ethanol to prepare fibrinogen with 99% clottable protein from Cohn Cold Ethanol Fraction I. We have found that glycine in an aqueous system has a salting-in action on all proteins in plasma except on fibrinogen and a beta globulin (cold insoluble globulin), which are salted-out (precipitated). Under controlled conditions of temperature and glycine concentration, however, fibrinogen alone could be precipitated from plasma. These observations provided the basis for a simple and direct precipitation method for preparation of 98% clottable fibrinogen from plasma, without the use of organic solvents or of low temperature.

This report describes the procedure for and gives some of the properties of glycine-precipitated fibrinogen.‡

Methods. Analytical procedures. Total nitrogen (T.N.) was determined by the micro-Kjeldahl technic, protein from T.N. × 6.25 or by the biuret method(2), fibrinogen and fibrin by a spectrophotometric method(1,3)§ or the tyrosine method(4), and total solids by desiccation to constant weight at 110°C.

The Beckman DU spectrophotometer was used to measure absorbance, extinction coefficients(1,3) and opacity(5). The Durrum-Spinco technique (Model R System RIM-4, Beckman, Spinco Division) was used for paper electrophoresis. Moving boundary electrophoresis was performed after dialysis at pH 7.4 in phosphate-NaCl buffer, ionic strength 0.12 (0.05 μ PO_4 + 0.07 μ NaCl), at 20°C for 10,000 seconds.‖ The Spinco-Analytical Ultracentrifuge was employed for measurement of S20w at maximum velocity of 59,780 rpm for 96 minutes with 0.5% fibrinogen solution.¶

Fibrinogen was assayed for fibrin stabilizing factor in the presence of 0.1 M cysteine(6), prothrombin by the one-stage technique(7), plasmin(8), plasminogen(9), and antihemophilic globulin (AHG, Factor VIII) by the thromboplastin generation method(8). Thrombin times were measured by adding 0.1 ml thrombin (10 units) to 0.5 ml of fibrinogen solution.

Preparative technique. Glycine (calculated as mols per 1000 ml of solvent) was dissolved in plasma with gentle stirring in a beaker placed in a constant temperature bath. Precipitates were collected by centrifugation in the PR-2 International Centrifuge at 2900 rpm (1800 × *g*), or by high speed centrifugation with the Servall SS-1 angle centrifuge at 16,000 rpm or with the Spinco model L ultracentrifuge at 15,700 rpm with a No. 21 rotor. Large volumes (above 500 ml) were processed with the Sharples centrifuge, Type T-IP, with a clarifier bowl, at 38,000 rpm and a flow rate of 20 ml per minute. Solutions were clarified at high speed.

Experimental procedure. Preparation of fibrinogen with 2.2 moles (0.78 sat.) glycine at 20°C. Normal blood was collected carefully by venepuncture with 0.02 volume of 19% sodium citrate, and centrifuged twice (20 and

* Supported in part by U.S.P.H.S. grant, Nat. Inst. Health.

† Deceased, March 22, 1963.

‡ The technical assistance of Miss Jean Curry for some of the determinations of thrombin and lysis times is gratefully acknowledged.

§ We are indebted to Dr. Benjamin Sanders, Merck Sharp and Dohme Research Laboratories, West Point, Pa. for a sample of fibrinogen with 97% clottable protein, which was used to standardize the Blombäck assay.

‖ We are indebted to T. D. Gerlough, E. S. Squibb and Sons Research Laboratories, New Brunswick, N. J., for the moving boundary electrophoretic analysis.

¶ The ultracentrifugal analysis was made by Miss Alice Hogans and Dr. Benjamin Sanders, Merck Sharp and Dohme Research Laboratories, West Point, Pa., to whom we are greatly indebted.

TABLE I. Preparation of Human Fibrinogen by Glycine Precipitation at 20°C.

	Plasma	Adsorbed plasma	Glycine supernatant plasma	Solution 2×-ppt'd plasma	Lyophilized fibrinogen
Volume processed	1750	1530	1530*	500	—
Prothrombin time	22"	No clot	No clot	No clot	—
Protein, mg/ml	72.3	71.0	65.5	4.95	.89†
Fibrinogen, mg/ml	2.70	2.60	.17	4.84	.84†
% coagulability	3.0	2.97	—	97.8	94.3
Fibrinogen recovered, g	4.73	3.98	.26	2.42	—
% yield‡	—	84.5	5.5	51.3	—

* Based on adsorbed plasma volume.
† Dry wt basis, mg/6.59 mg solids.
‡ % of citrated, platelet-poor plasma.

60 min) at 2900 rpm to provide a platelet-poor plasma.

Prothrombin was adsorbed by the procedure of Surgenor and Noertker(10) as modified by Pennell(11) for citrated plasma at 20°C with 20 mM $MgSO_4 \cdot 7H_2O$ and 90 g $BaSO_4$ per liter for one hour. Two adsorptions were performed, followed by a third with a half-charge of $BaSO_4$ only if the prothrombin time was measurable. Traces of $BaSO_4$ in the supernatant were removed by high speed centrifugation.

In each liter of $BaSO_4$ adsorbed plasma at exactly 20°, 2.2 moles (165.15 g) of ammonia-free glycine were dissolved slowly with stirring (30 minutes), avoiding the formation of foam as much as possible. The glycine-precipitated fibrinogen was separated by high speed centrifugation for 20-30 minutes. It is essential in this step to centrifuge the precipitate into a well-packed paste in order to press out as much of the plasma proteins as possible, which otherwise would contaminate the fibrinogen. The gelatinous fibrinogen paste was dissolved with stirring for 30 to 60 minutes in a volume of 0.055 M sodium citrate at pH 7.4 equal to the volume of the first $BaSO_4$ supernatant, clarified and reprecipitated with 2.2 moles glycine as before. If a high speed centrifuge is not available, this step should be repeated at least once to insure removal of traces of plasma protein trapped in the centrifuged precipitate.

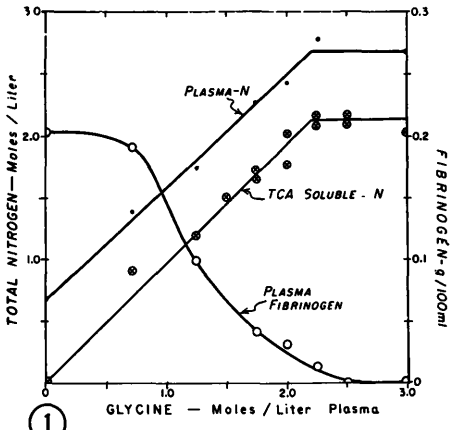
The last fibrinogen precipitate was dissolved in ¼ plasma volume of sodium citrate (0.055 M) at pH 7.4. The solution was usually clear; occasionally some particulate matter had to be removed by high speed centrifugation for 30 min. The supernatant solution was lyophilized in 10 ml aliquot portions for 48 hours, and the containers were sealed under nitrogen and stored at —15°C.

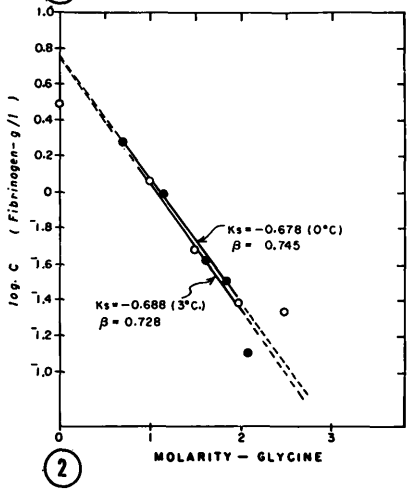
Results. Solubility of glycine in plasma. 1000 ml citrate plasma was saturated at 0°C by 2.2 moles of glycine (equivalent to a 1.88 M solution); at 20°C, by 2.83 moles (2.65 M). In distilled water, solubilities of 1.89 M at 0°C and 2.68 M at 20°C were obtained. Cohn *et al.*(12) reported the solubility of glycine at 20° to be 2.613 M. The solubility of glycine in plasma at 0°C is shown in Fig. 1.

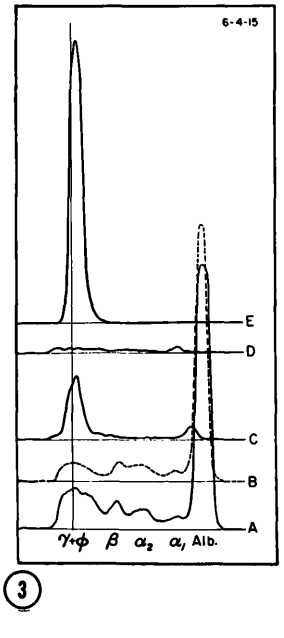
Precipitation of fibrinogen by glycine. At low temperatures (0 to 3°C) precipitation started around 0.7 mole and increased with increasing glycine concentration until it was almost complete at saturation (1.88 M in the example of Fig. 1). Since at this point only traces of fibrinogen remained in solution, it was difficult to determine fibrinogen concentration accurately and thus to determine the point of complete precipitation. Isolation experiments indicate complete precipitation occurred at saturation. The concentration of soluble fibrinogen was logarithmically proportional to glycine over most of the range of glycine concentration (Fig. 2).

Precipitates, obtained by saturating plasma with glycine at 0°C and washing well with saturated glycine solution to remove adhering plasma supernatant, contained only fibrinogen and beta globulin by paper electrophoretic analysis. The zones stained for carbohydrate but not lipoprotein.

Preparation of fibrinogen with 2.2 moles glycine (78% saturation) at 20°C. Table I describes the processing data obtained for a







preparation of fibrinogen with 98% clottable protein. Adsorption of prothrombin from plasma was necessary to eliminate spontaneous clotting of fibrinogen solutions. A temperature of 20°C and a glycine concentration of 0.78 saturation (2.07 M) was required for the precipitation of pure fibrinogen. Electrophoretic analysis of various fractions (Fig. 3) indicated clearly that an effective separation of fibrinogen from other plasma proteins was obtained by 2 such precipitations when high speed centrifugation was employed.

Precipitation of fibrinogen between 2.2 and 2.8 moles glycine at 20°C. About 5% of the fibrinogen remained unprecipitated in the plasma which was 78% saturated with glycine. This fraction was recovered by fully saturating the supernatant with glycine. The precipitate after washing with saturated

FIG. 1. Precipitation of fibrinogen from normal human plasma at 0°C by increasing concentrations of glycine. Glycine was added to 10 ml aliquots of plasma in amounts equivalent to moles of glycine in 1000 ml of solvent (plasma). The plasma was saturated above 2.2 moles glycine (1.88 M), as shown by the TCA-Soluble N curve; above this concentration the aliquots contained undissolved glycine. After 1 hr, the precipitated fibrinogen (and any undissolved glycine) was removed by centrifugation (1800 × *g*) and the supernatants analyzed for fibrinogen(4) (plasma fibrinogen), for total nitrogen (plasma-N), and for glycine (TCA-Soluble N), which was estimated from T.N. measurements on 5% trichloracetic acid supernatants, corrected for non-protein nitrogen of plasma. The TCA-N curve is a composite of data obtained with citrate and ACD plasmas at 0°C; the fibrinogen and plasma-N curve was obtained with ACD plasma.

FIG. 2. Solubility curves according to Cohn(18) for fibrinogen in human plasma collected with 19% sodium citrate (●) and in 18-day-old ACD bank blood plasma (○). Salting-out constants K_s and β were calculated from log $C = \beta - K_s m$. K_s is a measure of efficiency of protein precipitation. The value for glycine is lower than that reported for inorganic salts (*cf* 19,20) and is closest to NaCl ($K_s = 1.07$). Since glycine is considered according to electrostatic theory to be qualitatively similar to NaCl(20), glycine appears to be a less efficient precipitant in this complex, high dielectric solvent system (plasma).

FIG. 3. Protein composition of various fractions obtained during fractionation of citrate human plasma by glycine at 20°C. Paper electrophoresis: 16 hr, 2.5 ma, 78 V, in barbital buffer, pH 8.6, ionic strength 0.075, Brom phenol Blue stain; Analytrol tracings. A — $BaSO_4$ supernatant (0.006 ml); B — first glycine supernatant (0.006 ml); C — first glycine precipitate from A (0.02 ml); D — second glycine supernatant (0.02 ml); E — second glycine precipitate (fibrinogen) (0.02 ml). Vertical line indicates point of application of sample.

TABLE II. Properties of Glycine Precipitated Fibrinogen.*

Fibrinogen, % of total solids		20.3 %
Clottable protein, % of total protein		94.4 %
Glycine, % of total solids		13.0 %
Total nitrogen, % of protein†		16.25%
$E^{1\,percent}_{1\,cm}$, 280 mμ, pH 7.1‡		13.9
$E^{1\,percent}_{1\,cm}$, 282 mμ, alkaline urea§		17.65
Opacity: Absorbance (600 mμ)/g/l, pH 7.1		.010
Ratio: 600 mμ (0.121) / 350 mμ (0.051)		4.1
S20w Component 1–	82%	6.7S
Component 2–	18%	7.5S
Electrophoretic mobility, μ (cm²/volt/sec)		
Component 1–	98%	-5.8×10^{-5}
Component 2–	2%	-37.1×10^{-5}
Thrombin clotting time	No Ca^{++}	32″
	.003 M Ca^{++}	8″

* Lyophilized: .055 M sodium citrate solution (97.8% clottable before lyophilization).
† Values for other preparations 16.45%, 16.93%.
‡ In .055 M sodium citrate.
§ Values for other preparations 17.62, 17.55.

glycine solution contained 85% fibrinogen and 15% beta-globulin by paper electrophoretic analysis. Since solutions of this fraction were quite milky and opalescent and were considerably less soluble in the cold, the beta globulin in this fraction was presumed to be cold-insoluble globulin. The yield of this fraction was 55 mg/100 ml plasma.

Properties of fibrinogen precipitated by 2.2 moles of glycine at 20°C. Lyophilized fibrinogen (approximately 65% sodium citrate) was an amorphous, completely soluble white powder; however, after dialysis against distilled water much of fibrinogen was insoluble. When the lyophilized product was restored with distilled water to 0.55 M sodium citrate, the fibrinogen concentration was approximately 0.5% and glycine concentration 0.03 M. The properties of 98% clottable fibrinogen, listed in Table II, are generally similar to those reported in the literature; however, some deviations are to be noted. The ratio of absorbancies at 600 mμ and 350 mμ was approximately half the value of 8.6 reported by Ferry and Morrison(5), although opacity per unit concentration was comparable. Two components were observed on ultracentrifugation, and by moving boundary electrophoresis.

Solutions of fibrinogen were stable since they remained clottable after incubation at 38°C for 24 to 48 hours. Fibrin clots showed no evidence of lysis for 15 hours, suggesting the absence of plasmin; however, clots prepared after addition of streptokinase dissolved in 7 to 12 minutes, indicating the presence of plasminogen. Prothrombin could not be demonstrated by activation with thromboplastin and calcium. Clots formed in the presence of calcium ions and cysteine were not completely soluble in monochloracetic acid and presumably contain Fibrin Stabilizing Factor. The clotting time of a recalcified mixture of hemophilic A plasma was slightly shortened but not normalized by addition of fibrinogen solution; the AHG (VIII) content of a 0.5% fibrinogen solution was 3 to 4% of that of normal plasma in the TGT test.**

Discussion. The observation that glycine precipitates fibrinogen *from plasma* is new, although Edsall and Lever(14) observed that 1 M to 2 M glycine precipitated fibrinogen from aqueous solutions of Cohn Fraction I. A method of preparation based on this principle (precipitation) has not been reported. Earlier, the salting-in and salting-out properties of glycine for beta-lactoglobulin, hemoglobin and carboxy-hemoglobin were demonstrated by Richards(15), Cohn *et al.*(16), and Grönwall(17). Blombäck and Blombäck (1,3) utilized these properties of glycine, combined with the precipitation effect of ethanol, with great success to prepare from Fraction I of the Cohn Cold Ethanol Procedure a fibrinogen with 99% clottable protein, free of impurities except for a trace of plasminogen. The ease with which fibrinogen was separated from the plasma and the fact that an amino acid was the precipitant prompted the present investigation, especially in view of the fact that glycine has not been shown to precipitate other proteins although it does affect their solubility(15,16,17).

The glycine precipitation method provides a relatively simple technique for obtaining fibrinogen from plasma. It is less complicated than existing methods for the separation of fibrinogen(1,22,23), and approaches in its simplicity the freeze-thaw technique(24). It offers the average laboratory an opportunity

** We are indebted to Dr. R. R. Holburn and Miss M. DeSipin for the TGT assays.

to prepare fibrinogen on either a small or moderately large scale (from 50 to 1500 ml plasma), without elaborate facilities, at room temperature, and in good yield. The lyophilized product is stable and easily serves as a convenient starting point for further purification by procedures designed to remove trace protein contaminants such as plasminogen(25,26, 27). Where plasminogen is not an important parameter, glycine-precipitated fibrinogen has been used in this laboratory successfully in place of ordinarily available fibrinogens for chemical studies and for blood clotting assays. The trace amount of glycine present does not interfere with clotting in agreement with observations of others(14,28).

Glycine readily dissolves in human citrated plasma to give saturated solutions at 2.65 M at 20°C, just as it does in distilled water (2.68 M). Under these conditions fibrinogen precipitates from plasma; the amount precipitated was a function of glycine concentration (Fig. 1). In the range of 0.7 to 2 M glycine the concentration of fibrinogen (unprecipitated fraction) was logarithmically proportional to the molar concentration of glycine (Fig. 2). The data fit the solubility equation of Cohn(18):

$$\log S = \beta - K_s m$$

when S = g of fibrinogen/1000 ml solution, m is molar concentration of glycine, and where K_s, the salting-out constant, is obtained from the slope of the line and β from the intercept on the abscissa (Fig. 2). This observation supports the concept of the salting-out action of glycine for fibrinogen in a complex solvent system such as plasma.

For the separation of pure fibrinogen from plasma by precipitation with glycine it was necessary to remove prothrombin prior to precipitation to eliminate spontaneous activation to thrombin, and also to precipitate fibrinogen at 20°C with 0.78 saturated glycine in order to eliminate the beta globulin contaminant. The very great effect of temperature on solubility of glycine(13) demanded the use of a constant temperature for the controlled precipitation of fibrinogen.

Fibrinogen prepared by this method does not contain plasmin or prothrombin; however, plasminogen (and proactivator), fibrin stabilizing factor, and some Factor VIII have been found. The amount of Factor VIII present was not very effective in shortening the clotting time of hemophilia A plasma. The properties by which the fibrinogen differs from other preparations are mainly physicochemical ones. The Tiselius moving boundary method of electrophoresis disclosed 2 components, the main component accounting for 98% of the total protein. The extremely high mobility of the 2% component in our fibrinogen suggests that it is an extraneous protein, one perhaps not derived by degradation from fibrinogen during electrophoresis as observed for other preparations(1,29). Ultracentrifugal analysis disclosed the presence of 2 major components with S20W constants of 6.7s (82%) and 7.5s (18%). Heavier components of the 15S type regarded as representing early stages of polymerization of fibrinogen were not present. Fibrinogen with this order of clottability has not been reported to have a bimodal ultracentrifugal pattern and the significance of this observation is not clear.

It is of interest to note that the fraction of fibrinogen remaining in plasma solution at 20°C and 0.78 saturation (2.07 M), but which is precipitable at saturation (between 2.07 M and 2.65 M), contains an amount of beta globulin that corresponds to a calculated concentration of 8 mg/100 ml of original plasma, a value close to the 10 mg reported by Oncley (21) for cold insoluble globulin. The amount present in fibrinogen prepared at 0°C (saturated system) corresponded to only 4.7 mg/100 ml.†† The physical properties of the beta globulin-fibrinogen fraction suggests that it could well be cold insoluble globulin; however, one would expect greater precipitation of it at the colder temperature. The presence of glycine may possibly alter the cold precipitating property characteristic of this protein; until it is identified as cold insoluble globulin, this aspect of the problem remains unsolved.

Summary. A method was described for the preparation of fibrinogen with 98% thrombin

†† The preparation of fibrinogen at 0°C and glycine concentrations less than saturation was not investigated. It is possible that cold insoluble globulin may not be precipitated at some lower concentration of glycine.

clottable protein before, and 94% clottable protein after lyophilization. The fibrinogen was precipitated from citrated prothrombin-free plasma with glycine at 0.78 saturation, pH 6.1-6.3, 20°C and purified by reprecipitation with glycine. The biological trace contaminants were plasminogen, fibrin stabilizing factor and antihemophilic globulin; prothrombin and plasmin were not found. Although fibrinogen prepared by the glycine precipitation method was very pure by the criterion of clottability and its properties were similar to those of other fibrinogen preparations, moving boundary electrophoresis and ultracentrifugation nevertheless disclosed two components in different proportions. When fibrinogen was prepared by fully saturating plasma with glycine, a beta globulin, presumably cold insoluble globulin, was a major contaminant. The precipitation of fibrinogen from plasma between 0.75 and 2.0 M glycine followed the logarithmic solubility relationship of Cohn; the salting-out constant (Ks) was 0.68.

1. Blombäck, B., Blombäck, M., *Arkiv. f. Kemi.,* 1956, v10, 415.

2. Lever, W., Gurd, F., Uroma, E., Brown, R., Barnes, B., Schmidt, K., Schultz, E., *J. Clin. Invest.,* 1951, v30, 99.

3. Blombäck, B., *Arkiv. f. Kemi.,* 1958, v12, 99.

4. Seegers, W. H., Loomis, E. C., Vandenbelt, J. M., *Arch. Biochem.,* 1945, v6, 85.

5. Ferry, J. D., Morrison, P. R., *J. Am. Chem. Soc.,* 1947, v69, 388.

6. Lorand, L., Jacobsen, A., *J. Biol. Chem.,* 1958, v230, 421.

7. Tocantins, L. M., Ed., *Coagulation of Blood,* Grune and Stratton, N. Y., 1955, p100.

8. Biggs, R., MacFarlane, R. G., *Human Blood Coagulation,* 2nd Ed., C. C Thomas, Springfield, Ill., 1957.

9. Baumgarten, W., Cimminera, J. L., Cole, R. B., *Vox. Sang.,* 1960, v5, 416.

10. Surgenor, D. M., Noertker, J. F., *J. Am. Chem. Soc.,* 1952, v74, 3448.

11. Pennell, R. B., in *The Proteins,* F. W. Putnam, Academic Press, New York, v1, Chap. 2, p9.

12. Cohn, E. J., McMeekin, T. L., Edsall, J. T., Weare, J. H., *J. Am. Chem. Soc.,* 1934, v56, 2270.

13. Dalton, J. B., Schmidt, C. L. A., *J. Biol. Chem.,* 1933, v103, 549.

14. Edsall, J. T., Lever, W. F., *J. Biol. Chem.,* 1951, v191, 735. (Footnote 7, p747).

15. Richards, M. M., *ibid.,* 1937, v122, 727.

16. Cohn, E. J., McMeekin, T. L., Ferry, J. D., Blanchard, M. H., *J. Phys. Chem.,* 1939, v43, 169.

17. Grönwell, A., *Compt. rend. trav. lab. Carlsberg Ser. Chim.,* 1942, v24, 185.

18. Cohn, E. J., *Physiol. Rev.,* 1925, v5, 349.

19. Cohn, E. J., Ferry, J. D., in Cohn, E. J., Edsall, J. T., *Proteins, Amino Acids and Peptides,* 1943, Chap. 24, p586.

20. Edsall, J. T., Wyman, J., *Biophysical Chemistry,* Academic Press, New York, 1958, v1, 273, 319.

21. Oncley, J. L., in *Hormones in Human Plasma,* H. N. Antonaides, Ed., Little, Brown & Co., Boston, 1960, p13.

22. Morrison, P. R.. Edsall, J. T., Miller, S. G., *J. Am. Chem. Soc.,* 1948, v70, 3103.

23. Kekwick, R. A., MacKay, M. E., *Med. Res. Council Special Report,* Series 286, Her Majesty's Stationary Office, London, 1954.

24. Ware, A. G., Guest, M. M., Seegers, W. H., *Arch. Biochem.,* 1947, v13, 231.

25. Bergstrcm, K., Wallen, P., *Arkiv. f. Kemi.,* 1961, v17, 503.

26. Mosseson, M. W., *Biochim. et Biophys. Acta,* 1962, v57, 204.

27. Hink, J. L., McDonald, J. K., Johnson, F. F., *Vox. Sang.,* 1962, v7, 100.

28. Glazko, A. J., Greenberg, D. M., *Am. J. Physiol.,* 1940, v128, 399.

29. Caspary, E. A., Kekwick, R. A., *Biochem. J.,* 1957, v67, 41.

Received April 15, 1963. P.S.E.B.M., 1963, v113.