Journal of Clinical Investigation
Vol. 41, No. 6, 1962

ERYTHROCYTE METABOLISM. VI. SEPARATION OF ERYTHROCYTE ENZYMES FROM HEMOGLOBIN *

By M. A. HENNESSEY, A. M. WALTERSDORPH, F. M. HUENNEKENS AND BEVERLY W. GABRIO

(From the Department of Biochemistry, University of Washington, and the King County Blood Bank, Seattle, Wash.)

(Submitted for publication December 26, 1961; accepted February 5, 1962)

Studies of individual enzymes and metabolic pathways in the erythrocyte are hampered by the fact that in lysates, or even in partially purified systems, hemoglobin is present in overwhelming amounts compared to the various enzymic proteins. Although carbonic anhydrase (2), peptidase (3, 4), methemoglobin reductase (5), erythrocuprein (6), nucleoside phosphorylase (7–9), adenosine triphosphatase (10), and glutathione reductase (11) have been isolated from hemolysates, some special characteristic of the protein in each instance has made it possible to remove hemoglobin in a single operation.

The present paper describes a method which can be used for the separation of many erythrocyte enzymes from hemoglobin by treatment of the hemolysate at pH 7.0 with diethylaminoethyl (DEAE) cellulose; under these conditions, proteins having an isoelectric point below 7.0 are adsorbed onto DEAE, while hemoglobin, having an isoelectric point near neutrality, is not adsorbed. DEAE cellulose has been used widely for the fractionation of proteins (12–15), and as applied specifically to red cell enzymes, Kirkman (16) has utilized this exchanger in the purification of glucose-6-phosphate dehydrogenase.

The hemoglobin-free, protein fraction, called hereafter "enzyme protein fraction," serves as a useful starting material for the further purification of many erythrocyte enzymes and, furthermore, can be used with proper supplements for studying metabolic sequences and regulatory mechanisms. These latter findings will be reported in a separate communication.

EXPERIMENTAL

Materials. DEAE cellulose (capacity of 0.6 to 0.8 mEq per g) and semicarbazide were obtained from Eastman Organic Chemicals; Carbowax (polyethylene glycol compound 20-M) from Union Carbide Chemicals Company; Sephadex G-25 from Pharmacia Laboratories, Inc.; DPN,[1] TPN, DPNH, TPNH, sodium pyruvate, and glucose-6-phosphate, from Sigma Chemical Company; AMP, ADP, and ATP from Pabst Laboratories; lactic dehydrogenase and pyruvic kinase from C. F. Boehringer and Sons; inosine, oxidized glutathione, and dehydrated firefly tails from Schwarz BioResearch, Inc.; catalase and xanthine oxidase from Worthington Biochemical Corporation; crystalline bovine serum albumin from Armour and Company; sodium formate from Allied Chemical Co.; and glycylglycine from California Corporation for Biochemical Research.

Methods. Protein was determined by the biuret method with crystalline bovine serum albumin as the standard. Hemoglobin was measured by the procedure of Evelyn and Malloy (17) and by its absorbancy at 410 mμ. With the latter method, the concentration of hemoglobin was calculated, using a millimolar extinction coefficient of 120 (18). Spectrophotometric assays at a single wave length were carried out in a Beckman spectrophotometer, model DU. Absorption spectra over a large wave length region were obtained with the Beckman recording spectrophotometer, model DK-1. Inorganic and organic phosphate fractions were measured by a method described previously (19). ATP was assayed by the bioluminescent reaction of firefly extracts containing luciferin and luciferase (20, 21), with the use of the G. K. Turner fluorometer. ADP was measured with the coupled reaction, pyruvic kinase-lactic dehydrogenase (22). Nucleotide profiles (23) were obtained by the ion-exchange chromatography method of Hurlbert, Schmitz, Brumm and Potter (24).

* This work was supported by grants from the Office of the Surgeon General, Department of the Army (Contract DA-49-007-MD-508) and from the National Heart Institute, United States Public Health Service (H-4614). A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists, Chicago, Ill., April, 1960 (1).

[1] The following abbreviations have been used: AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate, respectively; DPN, and TPN, di- and triphosphopyridine nucleotide; DPNH, TPNH, reduced DPN and TPN; GTP, guanosine triphosphate; CMP, cytidine monophosphate; and UDP, uridine diphosphate.

Assays for the following enzymes were performed essentially according to published methods: nucleoside phosphorylase (7), catalase (25), glucose-6-phosphate dehydrogenase (26), TPNH-methemoglobin reductase (5), formate-activating enzyme (27), glutathione reductase (28), and adenylic deaminase (29). The methods used for the assay of the various glycolytic enzymes were the same as those described in the preceding paper (30), except that enolase was assayed by the method of Warburg and Christian (31); the reaction mixture contained 5×10^{-3} M 2-phosphoglycerate and 3×10^{-3} M $MgCl_2$. Continuous readings at 240 mμ were made for 10 minutes in the Beckman recording spectrophotometer, model DK-1. The amount of phosphoenolpyruvate produced was calculated, using a millimolar extinction coefficient of 1.4 (value determined in our laboratory at pH 8.1 in 0.05 M glycylglycine buffer).

Preparation of hemolysate. Fresh human blood was used in all experiments. The blood was collected in acid citrate dextrose (ACD, National Institutes of Health, Formula A) and centrifuged at 1,900 G for 30 minutes. All centrifugations were carried out at 4° C in the International refrigerated centrifuge, model PR-2. The ACD-plasma and buffy coat were discarded, and the erythrocytes were washed once by suspension and centrifugation (1,900 G, 20 minutes) with 2 vol of cold 0.15 M NaCl. Approximately 99 per cent of the plasma proteins was removed after the first washing. The cells were suspended in 1 vol of water, and the hemolysate was prepared by freezing and thawing the preparation three times. The pH of the hemolysate was 7.0, and the stromal fraction was not removed.

Preparation of DEAE cellulose. Forty g of commercial DEAE cellulose was suspended in 1 L of 1 M K_2HPO_4 (pH 9.1), stirred mechanically for 1 hour, and filtered with suction. This step was repeated several times until the filtrate was colorless. The adsorbent was washed further with water until the pH of the filtrate was approximately 7.0. An aqueous suspension (ca 8 g per 100 ml) of the adsorbent was adjusted to pH 7.0 and stored at room temperature.

Preparation of the enzyme protein fraction. A convenient amount of hemolysate (20 ml), derived from 10 ml of erythrocytes as described above, was divided equally among four 50-ml Lusteroid tubes. The following procedure represents the treatment of one of the 5-ml samples, carried out at 4° C without transfer of the material from the tube. To 5 ml of hemolysate was added 5 ml of DEAE cellulose suspension, and the mixture was allowed to stand at 4° C for 20 minutes, with manual stirring approximately every 4 minutes. The unadsorbed material in the supernatant fraction, principally hemoglobin, was separated by centrifugation for 15 minutes at 1,900 G, and residual hemoglobin was removed by washing the adsorbent 5 times with a total of about 175 ml of 0.003 M phosphate buffer, pH 7.0. The final wash was colorless. The phosphate buffer was more effective than 0.003 M Tris buffer, pH 7.0, or water in removing the residual hemoglobin.

The enzyme protein fraction was desorbed from DEAE cellulose in the following manner. Ten ml of 0.5 M KCl [2] was added to the adsorbent, and the mixture was stirred magnetically for 1 hour in an ice bath. The supernatant fluid (extract I) was collected by centrifugation at 1,900 G for 15 minutes. Serum albumin in a final concentration of 10 mg per ml was added immediately to the extract in order to stabilize the dilute enzymes. To the adsorbent was added an additional 5 ml of 0.5 M KCl, and the mixture was stirred magnetically for 15 minutes. The supernatant fluid (extract II) was collected by centrifugation, as above, and supplemented with serum albumin. The latter step was repeated once more (extract III), and the three extracts were combined to yield the enzyme protein fraction. Continuation of this procedure shows that 97 per cent of the total extractable protein is removed by the first three extractions. No additional protein was obtained when the KCl-treated adsorbent was extracted further with 0.5 M phosphate buffer, pH 9.5.

Although, for most purposes, salts may be removed from the enzyme protein fraction by dialysis, certain labile enzymes [3] are inactivated by this treatment. An alternate, but more laborious, procedure involves desalting the fraction by the method of molecular sieving with Sephadex (32). For example, 30 ml of enzyme protein fraction (in 0.5 M KCl) containing 50 mg of protein was passed through a 1.5×55 cm column of Sephadex with 0.05 M NaCl. The resulting protein solution was more dilute (0.7 mg per ml) than the starting material, but a tenfold concentration of the dilute solution could be achieved in about 2 hours by surrounding a dialysis bag containing the solution with Carbowax.

RESULTS AND DISCUSSION

Characteristics of the enzyme protein fraction. In a typical experiment, 145 mg of enzyme protein was obtained from 20 ml of hemolysate which contained 3,040 mg of hemoglobin. The total protein is 3,185 mg, and the enzyme protein fraction, therefore, comprises about 4.6 per cent of the original hemolysate. These values do not include the serum albumin added to the enzyme protein fraction. Similar analyses of 18 separate blood samples yielded an average value of 43.0 mg of enzyme protein per g of hemoglobin. Although

[2] Although 0.5 M potassium phosphate buffer, pH 7.0, was recommended previously for the desorption step (1), the present investigation has shown that KCl is preferable, since $HPO_4^=$ at high concentrations inhibits glycolysis [cf Table II in the preceding paper (30)].

[3] The conversion of fructose-1,6-diphosphate to lactate by the enzyme protein fraction, for example, is depressed by 25 per cent after 6 hours' dialysis of the fraction against 0.1 M KCl buffer, pH 7.0 (unpublished results, this laboratory).

the above data are based upon the amount of enzyme protein fraction recovered, these results give a good approximation of the relative amounts of hemoglobin and nonhemoglobin protein in the soluble fraction of the red cell, and are in general agreement with the data of Ponder (33), who suggested on the basis of interferometric measurements that the amount of red cell interior protein was 7 per cent of the hemoglobin concentration.

Since nucleotide coenzymes are also adsorbed and eluted from DEAE cellulose under conditions used for the isolation of the enzyme protein fraction, the latter preparation was analyzed chromatographically for nucleotides. The following data represent average values (based upon 10 ml of original red cells) obtained from nucleotide profiles of the acid-soluble fractions derived from three different enzyme protein preparations: 4.3 μmoles of ATP, 1.2 μmoles of ADP, approximately 0.1 μmole of AMP, and trace amounts of GTP, DPN, CMP, UDP, and uric acid. In a separate experiment with an acid-soluble portion from the enzyme protein fraction, ATP and ADP were measured by the enzymatic methods described in the Experimental section; 5.0 μmoles of ATP and 0.84 μmole of ADP were found. Thus, in a typical enzyme protein preparation, there is present, per milligram of protein, about 0.034 μmole of ATP, 0.007 μmole of ADP, and negligible amounts of other nucleotides. Comparison of these data with the results of chromatographic analysis of an acid filtrate of 10 ml of the original red cells indicated that only about 50 per cent of the ADP and ATP was recovered in the enzyme protein fraction. After dialysis of this fraction for 24 hours, 90 per cent of the recovered ATP is lost. Chemical analysis of the enzyme protein fraction for inorganic and organic phosphate revealed that, while 95 per cent of the phosphate originated from the procedure itself, the remaining 5 per cent (0.13 μmole per mg of protein) could be accounted for as nucleotide phosphates. From these data it is apparent that no detectable sugar phosphates are present, despite the fact that the amount of 2,3-diphosphoglycerate in the original hemolysate is about fourfold greater than the amount of ATP.

The absorption spectrum of the enzyme protein fraction shows a major peak at 260 mμ and a smaller peak at 410 mμ. The absorption at 260 mμ is due primarily to the contribution of adenine nucleotides which are present in the fraction. The small peak at 410 mμ is due to the heme components of catalase and methemoglobin reductase. Parenthetically, it is of interest to note that catalase can be desorbed from DEAE cellulose with a KCl concentration as low as 0.01 M, and thus can be separated from many other proteins in the fraction which require a higher salt concentration for desorption.

Glycolytic enzymes in enzyme protein fraction. Data on the levels of the glycolytic enzymes in the enzyme protein fraction are presented in Table I. Each enzyme has been assayed at pH 8.1, the over-all pH optimum for glycolysis in the intact cell and in hemolysates (30), in order that the individual steps may be compared with the over-all sequence. All of the glycolytic enzymes are present in the fraction, although the levels of phosphoglucose isomerase, aldolase, and phosphoglyceric kinase are considerably depressed relative to the original activities in the hemolysate [cf Table III, preceding paper (30)]. Thus, in order to achieve the glycolytic rate of the original hemolysate, the enzyme protein fraction must be partially supplemented with enzymes from other sources, as well as with the necessary cofactors (unpublished results, this laboratory).

TABLE I

Activities of glycolytic enzymes in the enzyme protein fraction from hemolysates *

Enzyme	Substrate converted
	mμmoles/hr/mg protein
Hexokinase	710
Phosphoglucose isomerase	920
Phosphofructokinase	1,730
Aldolase	500
Triose isomerase	168,000
Phosphoglyceraldehyde dehydrogenase	24,700
Phosphoglyceric kinase	700
Phosphoglyceric mutase	18,900
Enolase	3,180
Pyruvic kinase	4,700
Lactic dehydrogenase	91,200

* The enzyme activities were measured by the methods described in the Experimental section. All assays were performed at 25° C in 0.05 M glycylglycine buffer, pH 8.1, and with concentrations of protein from 0.002 to 0.02 mg per ml.

TABLE II

Activities of miscellaneous enzymes in the enzyme protein fraction from hemolysates *

Enzyme	Substrate converted per hour		
	Enzyme protein fraction		Hemolysate
	mμmoles/mg protein	*μmoles/ml cells*	
Catalase	230,000 × 10³	3,220 × 10³	3,230 × 10³
Nucleoside phosphorylase	115,000	1,610	1,600
Glucose-6-phosphate dehydrogenase	5,300	74	73
Glutathione reductase	1,850	26	28
Adenylic deaminase	1,640	23	24
TPNH-methemoglobin reductase	550	7.7	7.8
Formate-activating enzyme	540	7.6	10

* Concentrations of protein from 0.002 to 0.2 mg per ml were used in the assays performed on the enzyme protein fraction; and in experiments with hemolysates, an amount of enzyme source equivalent to 0.005 to 0.5 mg of hemoglobin was added.

Miscellaneous enzymes in the enzyme protein fraction. The enzyme protein fraction contains a number of miscellaneous enzymes, as listed in Table II. There is essentially a complete recovery of each enzyme.

The level of glucose-6-phosphate dehydrogenase reported in Table II is comparable to the values of Bock (34), Heller (35), Marks (36), and Kirkman (37), and their co-workers. Nucleoside phosphorylase activity is the same as that reported previously by this laboratory for the hemolysate (7, 38), and somewhat higher than the values of Sandberg, Lee, Cartwright and Wintrobe (9) and of Tsuboi and Hudson (8). The activity of glutathione reductase in the enzyme protein fraction is the same as that found in the hemolysate, and agrees with the value of 29.5 μmoles per hour per ml of erythrocytes given by Löhr and Waller (39). The value for adenylic deaminase activity in Table II is about the same as that reported by Conway and Cooke (40). Approximately 80 per cent of the formate-activating enzyme was recovered in the enzyme protein fraction when compared with values obtained in our laboratory and reported by Bertino, Simmons and Donohue (41).

Catalase activity in the enzyme protein fraction was found to be 230,000 μmoles of H_2O_2 decomposed per hour per mg of protein. When converted to the Kat $F_{(rbc)}$ units used by Beutler and Blaisdell (42), the above figure corresponds to a value of 76, and this may be compared to the former investigators' value of 66. Alternatively, recalculation of the present data for catalase into the units reported by Richardson, Huddleson, Bethea and Trustdorf (43) yields a value which is 91 per cent of the activity reported by these authors. Thus, the recovery of catalase in the enzyme protein fraction from the erythrocyte would appear to be essentially complete. The activity of TPNH-methemoglobin reductase (500 mμmoles TPNH oxidized per hour per mg of protein), when compared with the activity recorded previously (4) for the hemolysate, indicates complete recovery of this heme-containing enzyme in the enzyme protein fraction.

SUMMARY

1. A method, using diethylaminoethyl cellulose, is described for the isolation of a hemoglobin-free, enzyme protein fraction from erythrocyte hemolysates.

2. The isolated fraction contains approximately 140 mg of protein derived from 10 ml of packed red cells. In addition, the presence of ATP and ADP and small amounts of other nucleotides is demonstrated. The absorbancy at 260 mμ in the enzyme fraction can be accounted for by the nucleotides, and that at 410 mμ is attributed to the heme components of catalase and TPNH-methemoglobin reductase.

3. Various glycolytic enzymes and a number of miscellaneous enzymes (catalase, TPNH-methemoglobin reductase, nucleoside phosphorylase, glucose-6-phosphate dehydrogenase, glutathione reductase, adenylic deaminase, and formate-activating enzyme) are recovered almost quantitatively in the enzyme protein fraction.

REFERENCES

1. Hennessey, M. A., Haffner, A. M., and Gabrio, B. W. The separation of erythrocyte enzymes from hemoglobin. Fed. Proc. 1960, **19**, 65.
2. Keilin, D., and Mann, T. Activity of carbonic anhydrase within red blood corpuscles. Nature (Lond.) 1941, **148**, 493.
3. Adams, E., and Smith, E. L. Peptidases of erythrocytes. II. Isolation and properties of prolidase. J. biol. Chem. 1952, **198**, 671.
4. Adams, E., Davis, N. C., and Smith, E. L. Peptidases of erythrocytes. III. Tri-peptidase. J. biol. Chem. 1952, **199**, 845.
5. Huennekens, F. M., Caffrey, R. W., Basford, R. E., and Gabrio, B. W. Erythrocyte metabolism. IV.

Isolation and properties of methemoglobin reductase. J. biol. Chem. 1957, **227**, 261.

6. Markowitz, H., Cartwright, G. E., and Wintrobe, M. M. Studies on copper metabolism. XXVII. The isolation and properties of an erythrocyte cuproprotein (erythrocuprein). J. biol. Chem. 1959, **234**, 40.
7. Huennekens, F. M., Nurk, E., and Gabrio, B. W. Erythrocyte metabolism. I. Purine nucleoside phosphorylase. J. biol. Chem. 1956, **221**, 971.
8. Tsuboi, K. K., and Hudson, P. B. Enzymes of the human erythrocyte. I. Purine nucleoside phosphorylase; isolation procedure. J. biol. Chem. 1957, **224**, 879.
9. Sandberg, A. A., Lee, G. R., Cartwright, G. E., and Wintrobe, M. M. Purine nucleoside phosphorylase activity of blood. I. Erythrocytes. J. clin. Invest. 1955, **34**, 1823.
10. Caffrey, R. W., Tremblay, R., Gabrio, B. W., and Huennekens, F. M. Erythrocyte metabolism. II. Adenosinetriphosphatase. J. biol. Chem. 1956, **223**, 1.
11. Huennekens, F. M., and Gabrio, B. W. Unpublished results.
12. Peterson, E. A., and Sober, H. A. Chromatography of proteins. I. Cellulose ion-exchange adsorbents. J. Amer. chem. Soc. 1956, **78**, 751.
13. Sober, H. A., Gutter, F. J., Wycoff, M. M., and Peterson, E. A. Chromatography of proteins. II. Fractionation of serum protein on anion-exchange cellulose. J. Amer. chem. Soc. 1956, **78**, 756.
14. Rackis, J. J., Sasame, H. A., Anderson, R. L., and Smith, A. K. Chromatography of soybean proteins. I. Fractionation of whey proteins on diethylaminoethyl-cellulose. J. Amer. chem. Soc. 1959, **81**, 6265.
15. Speer, R. J., Prager, M. D., Kelley, T. F., and Hill, J. M. Protein fractionation. I. Chromatography of human serum proteins on DEAE-cellulose with special reference to blood group antibodies. J. Lab. clin. Med. 1959, **54**, 685.
16. Kirkman, H. N. Characterization of partially purified glucose-6-phosphate dehydrogenase from normal and primaquine-sensitive erythrocytes. Fed. Proc. 1959, **18**, 261.
17. Evelyn, K. A., and Malloy, H. T. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. biol. Chem. 1938, **126**, 655.
18. Sidwell, A. E., Jr., Munch, R. H., Barron, E. S. G., and Hogness, T. R. The salt effect on the hemoglobin-oxygen equilibrium. J. biol. Chem. 1938, **123**, 335.
19. Gabrio, B. W., and Finch, C. A. Erythrocyte preservation. I. The relation of the storage lesion to *in vivo* erythrocyte senescence. J. clin. Invest. 1954, **33**, 242.
20. Chase, A. M. The measurement of luciferin and luciferase. V. Applications of bioluminescent systems. I. Applications based on firefly luminescent system *in* Methods in Biochemical Analysis, D. Glick, Ed. New York, Interscience Publishers, 1960, vol. 8, p. 111.
21. Strehler, B. L., and McElroy, W. D. Assay of adenosine triphosphate *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1957, vol. 3, p. 871.
22. Kornberg, A., and Pricer, W. E., Jr. Enzymatic phosphorylation of adenosine and 2,6-diaminopurine riboside. J. biol. Chem. 1951, **193**, 481.
23. Gabrio, B. W., Finch, C. A., and Huennekens, F. M. Erythrocyte preservation: A topic in molecular biochemistry. Blood 1956, **11**, 103.
24. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. Nucleotide metabolism. II. Chromatographic separation of acid-soluble nucleotides. J. biol. Chem. 1954, **209**, 23.
25. Beers, R. F., Jr., and Sizer, I. W. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. biol. Chem. 1952, **195**, 133.
26. Kornberg, A., and Horecker, B. L. Glucose-6-phosphate dehydrogenase *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1955, vol. 1, p. 323.
27. Whiteley, H. R., Osborn, M. J., and Huennekens, F. M. Purification and properties of the formate-activating enzyme from *Micrococcus aerogenes*. J. biol. Chem. 1959, **234**, 1538.
28. Racker, E. Glutathione reductase (liver and yeast) *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1955, vol. 2, p. 722.
29. Nikiforuk, G., and Colowick, S. P. 5'-Adenylic acid deaminase from muscle *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1955, vol. 2, p. 469.
30. Chapman, R. G., Hennessey, M. A., Waltersdorph, A. M., Huennekens, F. M., and Gabrio, B. W. Erythrocyte metabolism. V. Levels of glycolytic enzymes and regulation of glycolysis. J. clin. Invest. 1962, **41**, 0000.
31. Warburg, O., and Christian, W. Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Z. 1941, **310**, 384.
32. Porath, J. Gel filtration of proteins, peptides and amino acids. Biochim. biophys. Acta 1960, **39**, 193.
33. Ponder, E. Haemoglobin and "apparent protein" in the human red cell. Nature (Lond.) 1959, **183**, 1330.
34. Bock, H. E., Karges, O., Löhr, G. W., and Waller, H. D. Fermenteigenschaften roten Blutzellen bei Lebererkrankungen. Klin. Wschr. 1958, **36**, 56.
35. Heller, P., Weinstein, H. G., West, M., and Zimmerman, H. J. Glycolytic, citric acid cycle, and hexosemonophosphate shunt enzymes of plasma and

erythrocytes in megaloblastic anemia. J. Lab. clin. Med. 1960, **55**, 425.
36. Marks, P. A. Red cell glucose-6-phosphate and 6-phosphogluconic dehydrogenases and nucleoside phosphorylase. Science 1958, **127**, 1338.
37. Kirkman, H. N., Riley, H. D., Jr., and Crowell, B. B. Different enzymic expressions of mutants of human glucose-6-phosphate dehydrogenase. Proc. nat. Acad. Sci. (Wash.) 1960, **46**, 938.
38. Gabrio, B. W., and Huennekens, F. M. The role of nucleoside phosphorylase in erythrocyte preservation. Biochim. biophys. Acta 1955, **18**, 585.
39. Löhr, G. W., and Waller, H. D. Biochemie und Pathogenese der enzymopenischen hämolytischen Anämien. Dtsch. med. Wschr. 1961, **86**, 27.
40. Conway, E. J., and Cooke, R. The deaminases of adenosine and adenylic acid in blood and tissues. Biochem. J. 1939, **33**, 479.
41. Bertino, J. R., Simmons, B., and Donohue, D. M. Purification and properties of the formate-activating enzyme from human erythrocytes. Fed. Proc. 1961, **20**, 65.
42. Beutler, E., and Blaisdell, R. K. Iron enzymes in iron deficiency. II. Catalase in human erythrocytes. J. clin. Invest. 1958, **37**, 833.
43. Richardson, M., Huddleson, I. F., Bethea, R., and Trustdorf, M. Study of catalase in erythrocytes and bacteria. II. Catalase activity of erythrocytes from different species of normal animals and from normal humans. Arch. Biochem. 1953, **42**, 124.