An Unidentified Erythropoietic Substance in Liver

By DANIEL L. HORRIGAN

IN A PREVIOUS publication,[1] two patients were described with hypochromic, microcytic anemia refractory to treatment with usual hematopoietic agents but responsive to the oral administration of crude liver extract (Liquid Extract of Liver, Valentine). Other findings in these patients included erythroblastic hyperplasia of bone marrow and markedly elevated serum iron concentration with almost complete saturation of the total iron binding capacity. In one of the patients, who was an alcoholic, a diagnosis of hemochromatosis was made by the pathologic changes revealed by needle biopsy of the liver.

Responses to therapy with oral crude liver in these patients were characterized by reticulocytosis and prompt rise in hematocrit levels, normoblastic maturation in bone marrow, and a fall in serum iron values to normal coincident with increasing erythrocytic regeneration.

Shortly before this publication, Harris et al.[2] reported the case of a patient with a similar type of anemia which responded to pyridoxine but was refractory to oral crude liver extract. Subsequent observations on the patients responsive to liver extract indicate that they, too, respond to pyridoxine given orally in doses of 5 to 10 mg. daily. This, of course, presents the question of the identity of the active substance in crude liver extract with pyridoxine.

The following observations indicate that this liver factor is not pyridoxine but apparently a previously unrecognized substance which stimulates erythropoiesis in human beings. Although the identity of this substance is not known, evidence is presented that it may be involved in metabolic pathways for erythropoiesis which also concern tryptophane and pyridoxine. These observations include hematologic responses of one of the previously reported patients to pyridoxine, to tryptophane and to an active fraction derived from crude liver extract which did not contain pyridoxine. Other than hypochromic anemia, no hematologic abnormalities have been apparent in this patient during five years of observation. Furthermore, spontaneous hematologic remissions have not been observed during this time, and the hematologic relapses after responses to courses of therapy have always been predictable.

The complete clinical report of this patient has been presented in the previous publication (reference 1: Case 1, P. J.). However, it is apropos to restate here that he presents no clinical nutritional deficiency. His diet is average, both in quality and in quantity; he is an abstainer from alcoholic beverages. Studies of hepatic and renal function have been normal.

From the Department of Medicine, Western Reserve University School of Medicine, and the Departments of Medicine, Crile Veterans Administration Hospital and the University Hospitals of Cleveland, Ohio.

This investigation was supported by research grant no. C-2501 from the National Institutes of Health, Public Health Service, and by a grant from Merck, Sharp, and Dohme, Inc.

Submitted June 29, 1960; accepted for publication Aug. 14, 1961.

Observations

Preparation of liver fraction: Details of extraction procedures involved in the derivation of the active material from crude liver extract will be published elsewhere. Briefly, starting material for the fraction used in these observations was 900 ml. of liquid extract of liver (Valentine), Control #20942. (The control number of the crude preparation is important since clinical activity may vary as much as 50 per cent from lot to lot.) Steps in the extraction procedures included precipitation with 70 per cent ethanol and extraction of the acidified 70 per cent ethanol-soluble fraction with ethyl acetate. The ethyl acetate extract was then subjected to anion exchange resin and paper chromatographic procedures which ultimately yielded approximately 10 mg. of solid material. Since the original liver extract was prepared so that one ounce was equivalent to 0.5 lb. of liver,* this 10 mg. fraction was derived from approximately 6.8 Kg. of fresh liver.

Response to the "liver factor": Figure 1 shows the hematologic response of the patient following the oral administration of this liver fraction in amounts of 2.0 mg. daily for 5 days. Reticulocytosis was apparent on the 4th day of therapy and reached a peak of 8.5 per cent on the 10th day. Prompt erythrocytic regeneration occurred; the hematocrit rose from 24 per cent to 29 per cent between the 4th and 8th days and continued a rapid rise to 45 per cent within 6 weeks. The duration of the remission following this 5 day course of treatment was about 9 months. The hematocrit fell very gradually from 46 per cent to 41.5 per cent between the 8th and 9th months after therapy. During the 9th month, there was a precipitous fall to 34 per cent in a 2-week period. During this remission the mean corpuscular hemoglobin concentration rose from 25.8 to 29.6 per cent.

Responses to pyridoxine: The responses to pyridoxine given orally in this patient were similar to that following the liver factor with prompt reticulocytosis and erythrocytic regeneration. However, the durations of the remissions produced were considerably shorter and not related to the dose of pyridoxine administered. Figure 2A shows the hematologic response following the oral administration of pyridoxine in doses of 5 mg. daily for 5 days. Reticulocyte response occurred with a peak of 6.8 per cent on the 7th day of therapy. The hematocrit rose from 30 per cent to 43 per cent in 28 days. A fall in hematocrit from 45 per cent to 31.5 occurred between days 112 and 145. On changing the dose of pyridoxine to 12.5 mg. orally per day for 5 days (fig. 2B), a peak reticulocytosis of 9.8 per cent occurred on the 5th day of therapy; the hematocrit rose from 25 per cent to 39 per cent in 14 days. This remission was of considerably shorter duration than those observed previously; a fall in the hematocrit became apparent after only 55 days. In neither of these responses to pyridoxine did the mean corpuscular hemoglobin concentration rise above 27.5 per cent.

Response to tryptophane: Figure 3 shows the hematologic observations made following the oral administration of a single dose of 4 Gm. of l-tryptophane. Although fluctuations in daily reticulocyte counts were observed during the three weeks after this tryptophane load, no clear-cut response occurred and the number of reticulocytes at no time exceeded 5 per cent.

Suboptimal but definite erythrocytic regeneration was observed following this exhibition of tryptophane. The hematocrit rose from 25 per cent to 36 per cent in 17 days. The duration of this suboptimal response was very short. Falling hematocrit levels became apparent after only 5 weeks. Further, no increase in the mean corpuscular hemoglobin concentration occurred.

Following the oral administration of tryptophane, the concentrations of kynurenine, kynurenic acid, acetyl kynurenine, hydroxykynurenine, anthranilic acid glucuronide, O-amino-hippuric acid, xanthurenic acid and N-1-methyl-2-pyridone-5-carboxamide were measured in the urine.† The results of these analyses were within the normal range.

---

*Personal communication with Mr. Granville Valentine, The Valentine Company, Richmond, Va.

†The author is grateful to Dr. James Price, Cancer Research Hospital, Medical School, University of Wisconsin, Madison, for performing these studies.

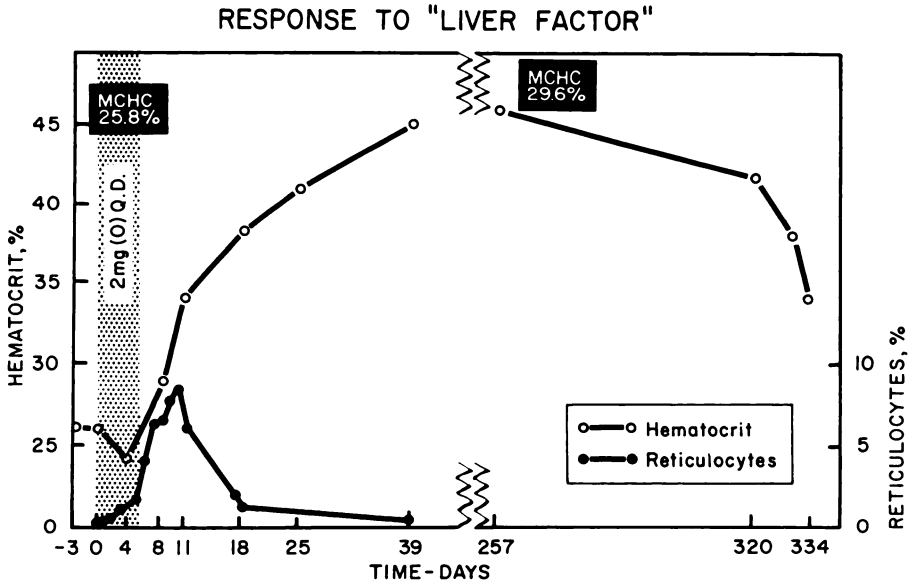

Fig. 1.—Hematologic response to the liver factor, 2.0 mg. by mouth daily for 5 days.

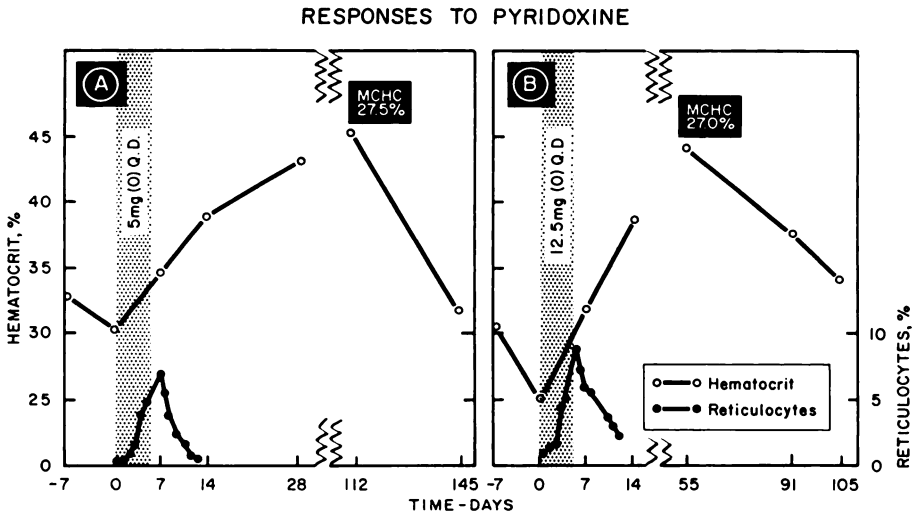

Fig. 2.—Hematologic responses to pyridoxine in doses of 5.0 mg. (A) and 12.5 mg. (B) by mouth daily for 5 days.

Characterization of the liver factor: Observations on the nature of the liver factor are preliminary at present. Chemical characteristics observed during its isolation from crude liver extract include (1) solubility in 70 per cent ethanol, (2) extractability into ethyl acetate from acid solution, and (3) marked avidity for adsorption on anion exchange resin. These observations suggest that the active factor is a non-proteinaceous substance of small molecular weight which contains one or more strongly acidic groups.

Further, chemical characterizations of all clinically active fractions so far derived have indicated the presence of the indole nucleus. These observations include (1) color de-

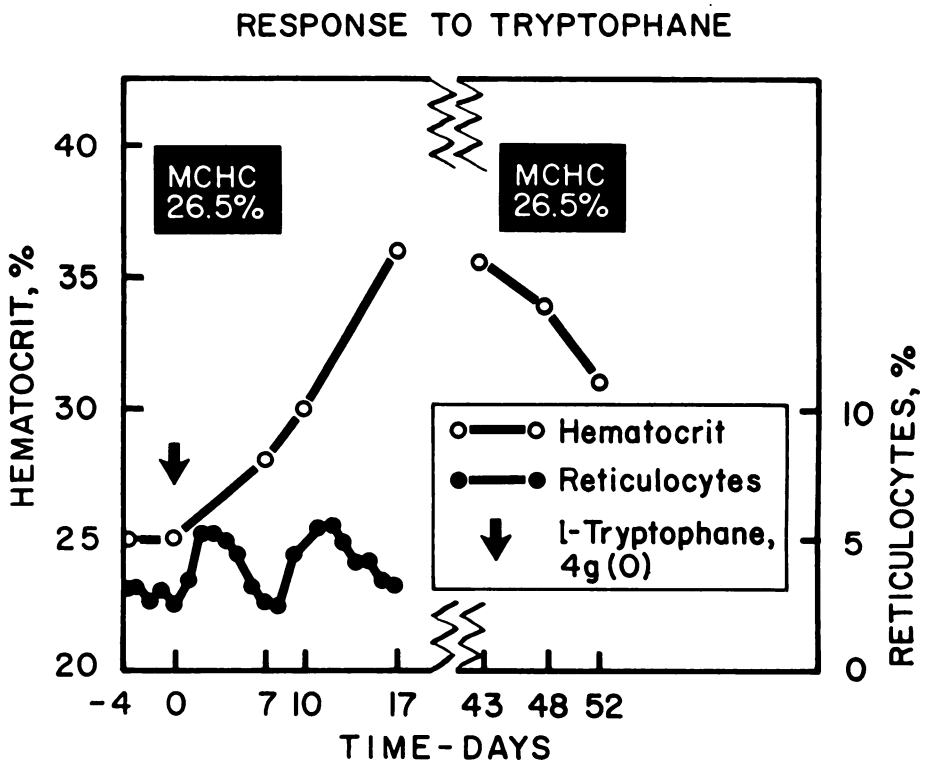

Fig. 3.—Hematologic response following the administration of a single oral dose of l-tryptophane, 4.0 Gm.

velopment on reaction of the fractions with Ehrlich's aldehyde reagent and (2) ultraviolet absorption spectra characteristic of indole derivatives with a peak of absorbance at 275–280 μ and a shoulder from 285 to 290 μ (fig. 4). The solubility characteristics described above are also in accord with an indolic acid structure.

Attempts to demonstrate a bacterial requirement for clinically active fractions have been unsuccessful. However, microbiological assays have shown that active fractions do not replace the growth requirement of S. *fecalis* for folic acid nor of *L. arabinosus* for pyridoxine.

Discussion

Numerous cases of hypochromic anemia with hypersideremia, exclusive of thalassemia, have been reported.[3-16] Since Harris' publication in 1957,[2] other reports of such anemias responding to pyridoxine have also appeared.[12-19] In most of these patients, administration of pyridoxine was followed by prompt hematologic responses with reticulocytosis and rapid rise in hemoglobin concentration. However, the hemoglobin levels reached were often suboptimal and erythrocytic hypochromia was not completely corrected. It would appear, therefore, that the erythropoietic deficiencies in these patients were only partially corrected with pyridoxine. Also, in some, pyridoxine deficiency was further demonstrated by abnormal urinary excretion of tryptophane metabolites following an oral load.[13,16,19] In others,[15,17] as in the presently reported patient, no such defect in tryptophane metabolism could be demonstrated. This

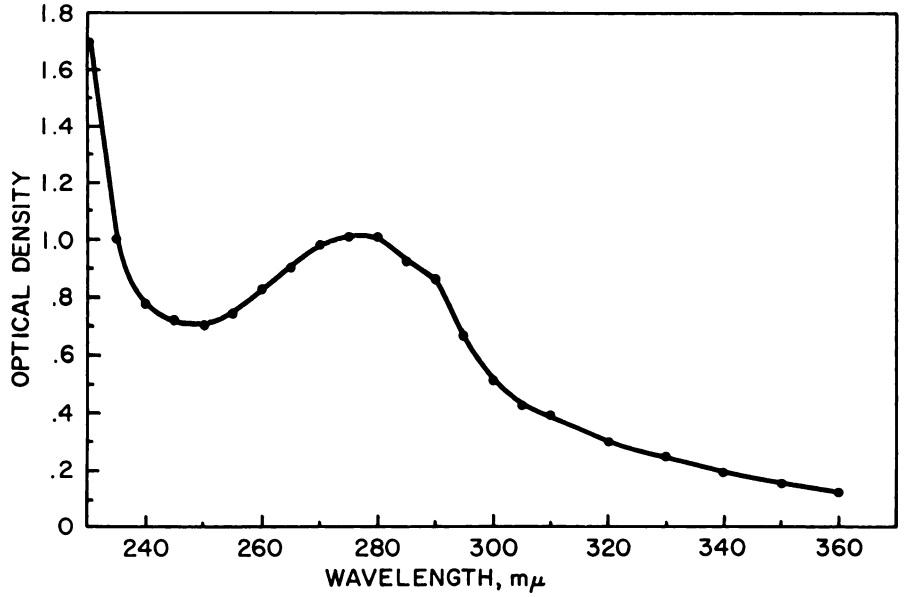

Fig. 4.—Ultraviolet spectrum of liver fraction producing hematologic response at dose level of 2.0 mg. daily.

apparent inconsistency remains unexplained. Might this result from abnormalities in the metabolism of pyridoxine manifest only in erythropoietic tissues, or does this represent a deficiency in some other substance or substances, the manifestations of which are corrected by the administration of pyridoxine? A situation analogous to the latter may be seen in the hematologic responses to folic acid in the vitamin B_{12} deficiency of pernicious anemia.

The possible role of the liver factor in the production of the anemias in these reported cases is difficult to assess. Some were not given trials of crude liver extract. In those who were, the variability of potency of liver extracts from the same source demonstrated in this study makes it impossible to interpret a negative result with a single trial of only one preparation.

In this regard, a case reported by Albahary and Boiron[12] is of particular interest. Their report concerns a 40 year old woman with refractory anemia and hemosiderosis who responded only partially after the administration of Valentine's liver extract in amounts of 45 ml. daily for 10 days. We have observed certain lots of this extract which were inactive at this dose level, but which produced optimal responses when given in larger amounts. These authors record two additional responses of their patient to an oral crude liver preparation of French origin. They also demonstrate a suboptimal response to pyridoxine in doses of 50 mg. daily for 15 days; subsequent exhibition of the vitamin at this dose level, however, was ineffective. On increasing the dose of pyridoxine to 250 mg. daily for a similar period, a suboptimal hematologic response was again obtained.[20]

Nutritional studies in animals also suggest the presence in liver of unidentified erythropoietic substances. In both swine[21] and dogs,[22] the anemia induced

by a pyridoxine-deficient diet responded only partially to the addition of pyridoxine. In the experiments on dogs, addition of liver extract to the diet resulted in further hematologic response. In similar studies in monkeys, Elvehjem and co-workers[23-25] have demonstrated the presence in liver of a "monkey anti-anemia factor" which is not identified with any of the known members of the vitamin B complex including vitamin B_{12} and folic acid.[25]

The observations recorded in the present report indicate the presence in crude liver extract of a previously unrecognized factor which stimulates erythropoiesis in a human being with hypochromic anemia, which also responds to pyridoxine. That the unidentified liver factor is not related to known derivatives of pyridoxine is indicated by chemical and physical characteristics of the active fraction of liver which have been determined in this study and also by the failure of active fractions of liver to replace the growth requirement of *L. arabinosus* for pyridoxine in microbiological studies.

The patterns of response in the patient to this liver factor, pyridoxine and tryptophane suggest an abnormality in metabolic pathways not previously known involving these substances in erythropoiesis. Chemical and physical characteristics of derivatives of liver containing the active factor suggest that the indole nucleus is a part of its structure. This, along with the hematologic response of the patient to tryptophane, may indicate that this amino acid is the precursor of this indolic structure.

The mode of action of this liver factor for erythrocytic regeneration remains unclear. However, the hematologic responses to the administered liver factor differed from those to pyridoxine. First, the amount of erythrocytic regeneration and the duration of the response following pyridoxine does not seem to be related to the dose given. This is unlike observations of responses of the patient to oral crude liver extract in which the amount of erythrocytic regeneration and the duration of responses could be titrated according to the amount of crude liver administered.[1] Second, the method of utilization of the liver factor for erythropoiesis appears different from that of pyridoxine. The duration of the remission after the administration of the liver factor in doses of only 2.0 mg. daily for 5 days was much longer than that following 5 or 12.5 mg. doses of pyridoxine given for a similar period. These observations suggest that during relapse, amounts of the administered liver factor exceeding the immediate requirements for erythropoiesis can be stored for subsequent utilization. Short remissions induced with pyridoxine, the duration of which are independent of dosage, suggest no such storage of this agent.

Additional observations on the hematologic responses of patients with hypochromic anemia and hyperferremia may help to clarify these apparent relationships of pyridoxine, tryptophane and the unidentified liver factor in erythropoiesis. Further studies on the purification and characterization of this liver factor are in progress.

Summary

Hematologic observations on a patient with hypochromic anemia and hyperferremia indicate (1) the presence in oral crude liver extract of an un-

identified erythropoietic substance, and (2) an apparent metabolic relationship of this liver factor with pyridoxine and tryptophane.

SUMMARIO IN INTERLINGUA

Observationes hematologic in un patiente con anemia hypochromic e hyperferremia indica (1) le presentia in oral extracto de hepate crude de un non-identificate substantia erythropoietic e (2) un apparente relation metabolic inter iste factor hepatic e pyridoxina e tryptophano.

REFERENCES

1. Horrigan, D. L., Whittington, R. W., Weisman, R., Jr., and Harris, J. W.: Hypochromic anemia with hyperferricemia responding to oral crude liver extract. Am. J. Med. 22:99, 1957.
2. Harris, J. W., Whittington, R. W., Weisman, R., Jr., and Horrigan, D. L.: Pyridoxine responsive anemia in the human adult. Proc. Soc. Exper. Biol. & Med. 91:427, 1956.
3. Goldish, R. J., and Aurderheide, A. C.: Secondary hemochromatosis. II. Report of a case not attributed to blood transfusions. Blood 8:837, 1953.
4. Garby, L., Sjölin, S., and Vahlquist, B.: Chronic refractory anemia with disturbed haem-metabolism. Brit. J. Haemat. 3:55, 1957.
5. Majoor, C. L., and Wijdeveld, P. G.: Hemachromatose met hypochrome Anemie. Nederl. tijdschr. geneesk. 101:1846, 1957.
6. Heilmeyer, L., Emmrick, J., Henneman, H. H., Schubothe, H., Keiderling, W., Lee, M. H., Bilger, R., and Bernauer, W.: Über eine neuartige hypochrome Anämie bei zwei Geschwistern auf der Grundlage einer Eisenverwertungsstörung; Anämie Sideroachrestica Hereditaria. Schweiz. med. Wchnschr. 87:1237, 1957.
7. Gelpi, A. P., and Ende, W.: An hereditary anemia with hemochromatosis: studies of an unusual hemopathic syndrome resembling thalassemia. Am. J. Med. 25:303, 1958.
8. Malassenet, R.: Les anémies hypochromes avec hypersidérémie. Sang 29:486, 1958.
9. Debray, J., and Conte, M.: Troubles de l'hémoglobinogenèse (hypochromie hypersidérémique) avec hémochromatose mortelli. Sang 29:481, 1958.
10. Dacie, J. V., Smith, M. D., White, J. C., and Mollin, D. L.: Refractory normoblastic anemia: A clinical and haematological study of seven cases. Brit. J. Haemat. 5:56, 1959.
11. Crosby, W. H., and Sheehy, T. W.: Hypochromic iron-loading anemia: Studies of iron and haemoglobin metabolism by means of vigorous phlebotomy. Brit. J. Haemat. 6:56, 1960.
12. Albahary, C., and Boiron, M.: Anémie primitive réfractaire avec hypersidérose sanguine médullaire et hépatique (case féminin). Acta med. scandinav. 163:429, 1959.
13. Gehrmann, G.: Pyridoxinemengal-Anämie beim Menschen. Folia haemat. (Frankf.) 2:225, 1958.
14. Foy, H., and Kondi, A.: Hypochromic anemias of the tropics associated with pyridoxine and nicotinic acid deficiency. Blood 13:1054, 1958.
15. Bishop, R. C., and Bethell, F. H.: Hereditary hypochromic anemia with transfusion hemosiderosis treated with pyridoxine: Report of a case. New England J. Med. 261:486, 1959.
16. Erslev, A. J., Lear, A. A., and Castle, W. B.: Pyridoxine-responsive anemia. New England J. Med. 262:1209, 1960.
17. Verloop, M. C., and Rademaker, W.: Anemia due to pyridoxine deficiency in man. Brit. J. Haemat. 6:66, 1960.
18. Leeming, J. T., and Wilkinson, J. F.: Hypochromic anemia in an adult responding to pyridoxine hydrochloride. Clin. Res. 7:208, 1959.
19. Raab, S., Cartwright, G. E., and Wintrobe, M. M.: Anemia partially responsive to pyridoxine. Clin. Res. 8: 104, 1960.
20. Albahary, C.: Personal communication.
21. Wintrobe, M. M., Follis, R. H., Jr.,

Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E.: Pyridoxine deficiency in swine with particular reference to anemia, epleptiform convulsions and fatty liver. Bull. Johns Hopkins Hosp. 72:1, 1943.

22. McKibben, J. M., Schaeffer, A. E., Frost, D. V., and Elvehjem, C. A.: Studies on anemia in dogs due to pyridoxine deficiency, J. Biol. Chem. 142:77, 1942.
23. Cooperman, J. M., Waisman, H. A., McCall, K. B., and Elvehjem, C. A.: Studies on the requirements of the monkey for riboflavin and a new factor found in liver. J. Nutrition 30: 45, 1945.
24. McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S.: A study of pyridoxine and pantothenic acid deficiencies in the monkey (Mucacca Mulatta). J. Nutrition 31:685, 1946.
25. Ruegamer, W. R., Sporn, E. M., Register, U. D., and Elvehjem, C. A.: Distribution and fractionation of the monkey anti-anemia factor. J. Nutrition 36:405, 1948.

Daniel L. Horrigan, M.D., Assistant Professor of Medicine, Western Reserve University School of Medicine; Assistant Physician, Department of Medicine, University Hospitals of Cleveland; Attending Physician in Hematology, Crile Veterans Hospital, Cleveland, Ohio.