vitamin-B_{12} assays by the *Euglena gracilis* method; Glaxo Laboratories for financial aid; Dr. E. Lester-Smith for generous quantities of radioactive vitamin B_{12}; Lederle Laboratories for supplies of intrinsic factor; and Mr. D. Hart for technical assistance.

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Preliminary Communications

A Spectrophotometric Method for Estimating Formimino-glutamic and Urocanic Acid

Formimino-glutamic acid, an intermediary in the pathway of histidine breakdown (Borek and Waelsch, 1953), may be excreted in the urine (Bakerman, Silverman, and Daft, 1951) when there is interference with the function of the folic-acid coenzymes. Paper electrophoresis is adequate for identifying this product in the urine of patients excreting relatively large amounts (Knowles, Prankerd, and Westall, 1960; Kohn, Mollin, and Rosenbach, 1961). However, until now there has been no relatively simple sensitive and quantitative method for estimating this material. This communication describes a new method based on that of Silverman, Gardiner, and Condit (1958) for estimating formimino-glutamic acid. Some of the results in patients with folic-acid deficiency are illustrated.

MATERIALS

Principle.—A crude liver-enzyme preparation (Futterman, 1957) is employed firstly to reduce folic acid to the active form, and, secondly, to transfer the formimino group of formimino-glutamic acid to this active form of folic acid. The end-product of the reaction is converted to 5-10-methenyl-tetrahydrofolic acid with HCl and read spectrophotometrically.

Water.—All water used was passed through an ion-exchange resin.

Substrate Solution.—KH_2PO_4, 20 m-mole (2.72 g.); citric acid, 2 m-mole (0.42 g.); $MgSO_4.7H_2O$, 2 m-mole (0.49 g.); TPN, 12 μmole (0.01 g.); folic acid, 55 μmole (0.024 g.). The KH_2PO_4 and citric acid were brought into solution and the *p*H was adjusted to 6 with KOH. The folic acid was brought into solution with the minimum amount of KOH. The TPN and $MgSO_4$ were added, the volume was made to 100 ml., and the solution was stored at −20° C.

Enzyme Preparation.—All operations were carried out at 2–4° C. Frozen chicken liver was sliced into 2-mm. slices with a scalpel, added to 1.3 volumes of 0.1 M potassium phosphate buffer *p*H 6, and homogenized for 15 seconds in an M.S.E. homogenizer. The homogenate was centrifuged for 10 minutes at 33,000 *g.* and the supernatant dialysed against distilled water overnight. The *p*H was adjusted to 6 and the insoluble residue removed by centrifugation. The extract was stored at −20° C.

Standard Solutions.—Formimino-glutamic acid solution containing 0.5 μmole/ml. at *p*H 6 was prepared. Alternatively, urocanic acid, which is fully active and more readily available than formimino-glutamic acid, can be used as a standard instead of formimino-glutamic acid. 5-Formyl-tetrahydrofolic acid (folinic acid) was available in ampoules containing 3 mg. (6.35 μmole). This was further diluted to contain 0.5 μmole/ml. and the *p*H adjusted to 7.

Urine Samples.—Urine was collected direct into "polythene" containers, containing 5 ml. of N HCl. Collection was restricted to eight hours after an oral dose of 5 to 15 g. of histidine hydrochloride.

METHOD

One part of liver extract was mixed with two parts of substrate solution: 3 ml. of the solution was distributed into 12 × 125-mm. glass tubes and incubated at 37° C. for two hours. The urine being tested was added to each tube. The amount of formimino-glutamic acid in the sample should not exceed 0.2 μmole, the urine volume varying from 0.01 to 0.4 ml. Distilled water was added to make the volume 3.4 ml., the tube being gently inverted three times and incubated for a further two hours. At the end of this time 1.5 ml. of 3N HCl was added to each tube, mixed, and allowed to stand for 15 minutes. The mixture was filtered and the clear filtrate read on a Unicam spectrophotometer at 350 mμ, using a 1-cm. light path.

The test and controls are set out in Table I. Controls are treated in the same manner as the test. The tube containing the test urine (A) is read against the enzyme blank (B). The corresponding urine blank (C) is read against water, and this value is subtracted from the test (A). The amount of formimino-glutamic acid (A-C) is calculated from the reading of the standard formimino-glutamic acid solution (D).

TABLE I.—*Tests and Controls in Estimating Formimino-glutamic Acid*

	A Test	B Enzyme Blank	C Urine Blank	D Standard
Substrate solution	Yes	Yes	No	Yes
Liver enzyme	,,	,,	,,	,,
Urine	,,	No	Yes	No
Formimino-glutamic acid	No	,,	No	0·05 and 0·1 μmole
Water	Volume to 3·4 ml. with water			

RESULTS

Fig. 1 shows the extinction at 350 mμ after the addition to the enzyme system of increasing amounts of formimino-glutamic acid contained in 0.2 ml. of water and urine.

Reproducibility on repeated assay is shown in Table II. These tests were carried out with different enzyme preparations.

Recovery of formimino-glutamic acid added to urine was from 97% to 110% with 1 μmole/ml. and from 91% to 97% with 2 μmole/ml.

TABLE II.—*Results of Repeated Assay for Formimino-glutamic Acid*

Specimen	Assay (mg./8 Hours*)		
	1	2	3
1	640	650	
2	360	366	352
3	160	152	
4	120	113	
5	31	34	
6	16	15	15
7	9	5	

* 1 m-mole formimino-glutamic acid = 174 mg.

Clinical Results.—Normal subjects excrete less than 17 mg. of formimino-glutamic acid in the urine after the oral dose of histidine. Fig. 2 shows the pattern of formimino-glutamic acid excretion in the urine after 15 g. of histidine in a patient with myeloblastic leukaemia.

Fig. 3 shows the pattern of formimino-glutamic excretion in a patient with megaloblastic anaemia of pregnancy treated with 5 mg. of folic acid daily by mouth. There is a characteristic rise in the excretion of formimino-glutamic acid 48 hours before the reticulocyte peak and corresponding to the period of most intense marrow activity.

The fall of urinary formimino-glutamic acid excretion in a patient with idiopathic steatorrhoea treated by a gluten-free diet alone is shown in Fig. 4.

The last case (Fig. 5) is that of a patient with Addisonian pernicious anaemia. She was first treated with tetracycline 250 mg. six-hourly. There was no haematological response, and although an initial fall occurred in the formimino-glutamic acid excretion it returned to the earlier levels. After an injection of vitamin B_{12} the reticulocytes reached 26% and formimino-glutamic acid excretion returned to normal levels.

DISCUSSION

Two sensitive methods are available for estimating formimino-glutamic acid (Tabor and Wyngarden, 1958;

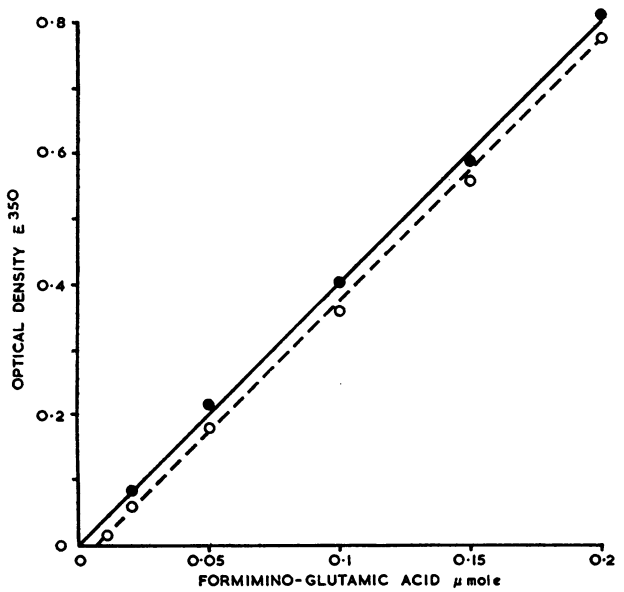

FIG. 1.—Optical density obtained when increasing amounts of formimino-glutamic acid were added to water (continuous line) and to urine (interrupted line).

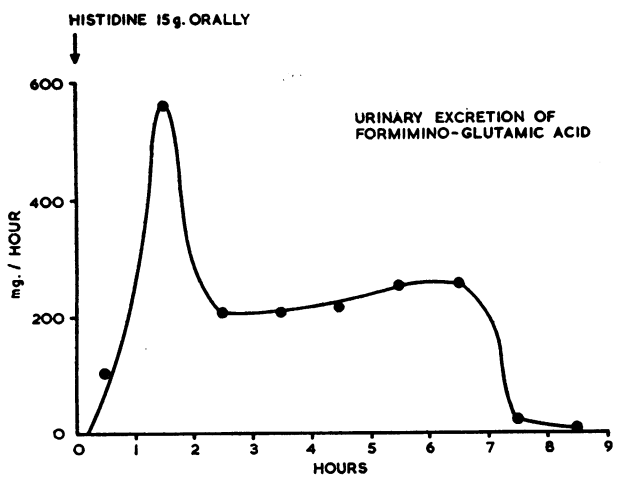

FIG. 2.—Pattern of urinary excretion of formimino-glutamic acid in a patient with acute myeloblastic leukaemia given a single oral dose of 15 g. of histidine hydrochloride.

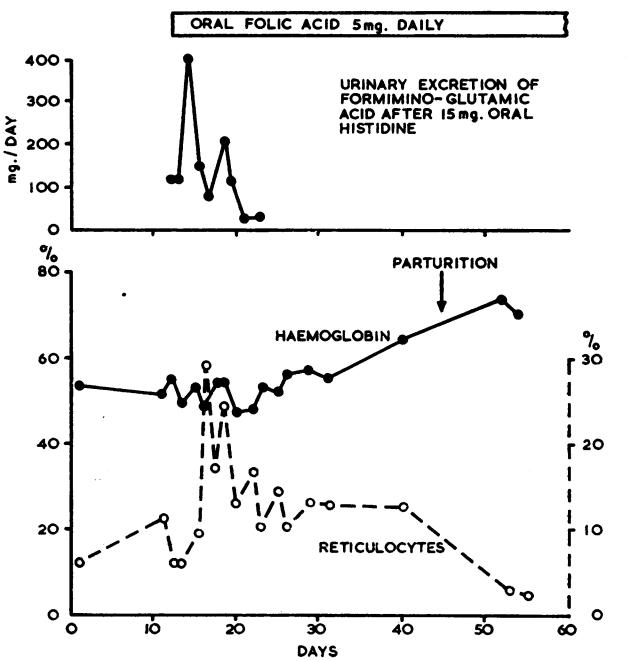

FIG. 3.—Pattern of urinary excretion of formimino-glutamic acid in a patient with sickle-cell thalassaemia complicated by megaloblastic anaemia of pregnancy. With treatment there is a steep rise in the amount of formimino-glutamic acid excreted preceding the reticulocyte peak. The excretion was within normal limits by the eighth day after oral folic acid.

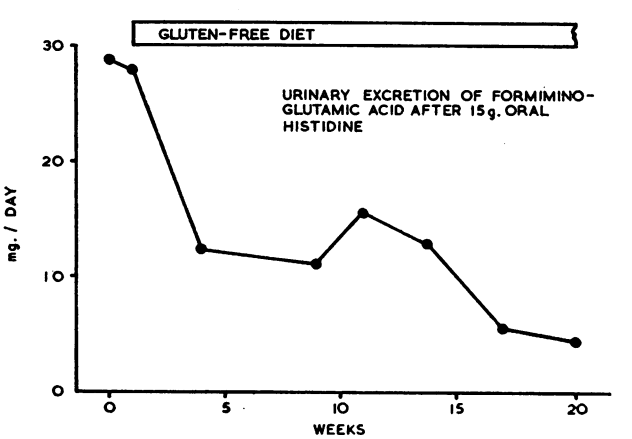

FIG. 4.—Return of urinary excretion of formimino-glutamic acid to within normal limits after a gluten-free regime in a patient with idiopathic steatorrhoea.

Silverman *et al.*, 1958). The first has the disadvantage of requiring the highly unstable tetrahydrofolic acid as a starting-point but the virtue that the end-product is read spectrophotometrically. The second overcomes the need for tetrahydrofolic acid as a starting material by using a crude liver extract which, containing folic acid reductase and a TPN regenerating system, reduces folic acid to the active form. The end-product, however, is assayed microbiologically, and this not only adds two days to the time of the test but makes it difficult to achieve quantitative results.

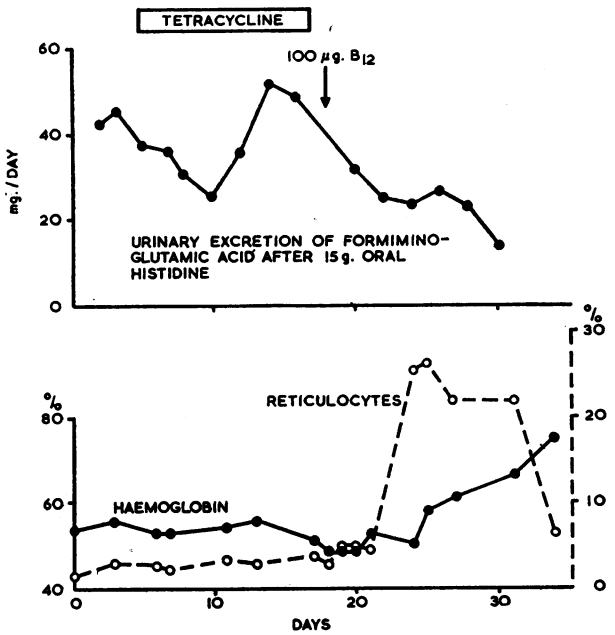

FIG. 5.—Increased urinary excretion of formimino-glutamic acid in a patient with Addisonian pernicious anaemia persisting after a course of tetracycline but disappearing after a single injection of vitamin B_{12}.

The method described in this paper combines the virtues of these two methods while eliminating their vices. Folic acid is used as a starting material, and the end-product is read spectrophotometrically.

The crude liver extract employed contains urocanase (Silverman *et al.*, 1958), so that urocanic acid, which is the precursor of formimino-glutamic acid in the pathway of histidine breakdown, is converted to formimino-glutamic acid and is therefore fully active in this system. The method therefore estimates the combined urocanic and formimino-glutamic acid excretion. This is of considerable importance, since urocanic acid may comprise more than 80% of the histidine derivatives appearing in urine in patients with folic-acid deficiency (Bennett and Chanarin, 1961). Urocanic acid may be estimated separately by destroying formimino-glutamic acid by autoclaving the urine at alkaline *p*H. Urocanic acid is not detected by other techniques for estimating histidine derivatives (Tabor and Wyngarden, 1958; Knowles *et al.*, 1960; Kohn *et al.*, 1961), so that results in clinical material may be completely misleading.

In comparing the extinction at 350 mµ of formimino-glutamic acid added to water and to urine, it was found that the values with urine were less than those obtained in water (Fig. 1). The enzyme mixture appeared to remove some material from urine which absorbed in the region of 350 mµ. This is apparent when testing urines containing no formimino-glutamic acid. Under these circumstances the urine blank may give a higher reading than the urine–enzyme mixture. In practice the error involved is equivalent to 2–5 mg. of formimino-glutamic acid in an eight-hour urine sample, which is well within the experimental error of the technique.

Folinic acid is also converted to 5-10-methenyl-tetrahydrofolic acid by HCl. With the best enzyme preparations there was 100% conversion of formimino-glutamic acid, so that the extinction at 350 mµ equalled that obtained with an equimolar amount of folinic acid treated in the same way. We have found this to be the most satisfactory method of assessing the activity of different enzyme preparations.

SUMMARY

A quantitative spectrophotometric method for the measurement of formimino-glutamic acid and urocanic acid is described. Both these substances may appear in the urine when there is interference with the function of the folic-acid coenzymes. The method is sensitive enough to detect these substances in the urine not only of patients with folic-acid deficiency but also of normal subjects given loading doses of histidine.

We are grateful to Dr. H. Tabor for a gift of formimino-glutamic acid and to Drs. W. D. W. Brooks, Carmichael Young, and T. A. Kemp for allowing us to study their patients.

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Amended Regulations dealing with ambulance arrangements in certain factories come into force on January 19. They impose a requirement that a responsible person shall always be readily available during working hours to summon an ambulance or other means of transport if needed in cases of accident or illness. The premises to which these Regulations apply are blast furnaces, copper mills, iron mills, foundries and metal works, sawmills, factories in which articles of wood are manufactured, and chemical works. They replace a requirement that occupiers of certain factories in these categories shall provide and maintain an ambulance unless they have made arrangements for one to be obtained. Except in the case of chemical works the earlier requirement applied only to factories at which 500 or more persons were employed. The new requirement is applicable to all factories of the kinds mentioned irrespective of the numbers employed. (The Blast Furnaces and Saw Mills Ambulance (Amendment) Regulations, 1961, S.I. 1961, No. 2434, H.M.S.O., price 3d. net. The Chemical Works Ambulance (Amendment) Regulations, 1961, S.I. 1961, No. 2435, H.M.S.O., price 3d. net.)