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Research Article

THE ASSAY AND NATURE OF FOLIC ACID ACTIVITY IN HUMAN SERUM *

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It has been generally accepted that the folic acid nutritional status of man could not be assessed by whole blood or serum sampling (1–3). Recently, however, the subject was reopened with the finding that serum folic acid activity, as measured by *Lactobacillus casei* (ATCC [1] no. 7469), is apparently an accurate measure of the nutritional status of man with respect to folic acid (4–7).

The present report indicates that the folic acid activity in human serum may be mainly due to one or more reduced triglutamyl (or higher) forms of folic acid. Some of the implications of this probability will be considered.

Incidental to these studies, the original assay method was further modified, and an explanation for the failure of prior studies by others was found in the lability of the serum folic acid activity measured. A preliminary report of these studies has been presented (8).

MATERIALS AND METHODS

Our standard method for assay of serum folic acid activity was as follows.

Preparation of serum samples. Blood was obtained from fasting subjects using either acid-washed sterile syringes or Vacutainers no. 3200 (Becton, Dickinson & Co., Rutherford, N. J.) to ensure freedom from contamination with traces of folic acid. The blood was allowed to stand for approximately 3 hours at room temperature (in the original Vacutainer or after transfer of blood from syringes to acid-washed screw-top tubes). The clots were "rimmed" with glass rods or wooden applicator sticks, the tubes centrifuged for 5 minutes at 3,000 rpm, and the supernatant serum aspirated and frozen at −20° C until assay. On the day of assay, the sera were thawed by standing at room temperature, or occasionally more rapidly by placing the tubes in warm water until almost thawed.

Serum aliquots were prepared for assay by diluting 1:10 with 0.05 M sodium phosphate buffer at pH 6.1 (9) in which ascorbic acid, in the concentration under study, had been freshly dissolved. The serum-buffer solution was autoclaved for 10 minutes at 118° C, without preliminary incubation. In the earlier method (5), preliminary incubation at 37° C for 90 minutes was part of the protocol; this has proven superfluous (10, 11). The white protein coagulum was then thrown down by centrifugation for 5 minutes at approximately 3,000 rpm and the clear supernatant assayed. This method of preparation of serum samples is similar to the method of preparation of whole blood by Toennies, Usdin and Phillips (12).

Maintenance of assay organisms and preparation of inocula. L. casei, Streptococcus faecalis (ATCC no. 8043), and *Leuconostoc citrovorum* (*Pediococcus cerevisiae,* ATCC no. 8081) were maintained in a medium previously described for maintenance of *L. casei* (5), and stored at 4° C. This medium proved excellent for maintaining all three microorganisms.

De-ionized water [2] was used for preparation of all media and buffers. It was free of folic acid and more easily prepared than distilled water. The heavy metal ions in distilled water have the undesirable ability to accelerate oxidation of the ascorbic acid in the buffer.

Five hundred ml of maintenance medium was generally prepared at one time and dispensed into 50 screw-capped tubes. The tubes, with screw caps loosely affixed, were autoclaved for 30 minutes at 118° C. They were then allowed to cool, the screw caps were tightened and the tubes incubated overnight at 37° C. The following morning they were examined for clarity (indicating absence of bacterial contamination) and, if sterile, were then stored at 4° C until used. At a maximal interval of once every 2 weeks, 1 drop of each stored liquid culture was added to 10 ml of fresh maintenance medium, incubated 18 hours at 37° C, and then stored at 4° C. During the afternoon of the day prior to an assay, 1 drop of the latest stored culture was added to 10 ml of maintenance medium, and incubated for 18 hours at 37° C. The next

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[1] American Type Culture Collection, Washington, D. C.

[2] Tap water passed through a Barnstead Bantam standard demineralizer cartridge no. 0802 (Barnstead Still and Sterilizer Co., Boston, Mass.).

TABLE I

Assay protocol finally adopted as the "standard method" of determination of folic acid activity in serum

Flask		Double-strength basal medium	Ion-free water	Buffer*	Folic acid standard	Serum diluted in buffer*
Number	Nature					
		ml	*ml*	*ml*	*ml*	*ml*
1, 8	Control	5	4	1	0	0
2, 9	Standard	5	3	1	1 (10^{-10} g/ml)	0
3, 10	Standard	5	1	1	3 (10^{-10} g/ml)	0
4, 11	Standard	5	3	1	1 (10^{-9} g/ml)	0
5, 12	Standard	5	1	1	3 (10^{-9} g/ml)	0
6, 13	Standard	5	3	1	1 (10^{-8} g/ml)	0
7, 14	Standard	5	1	1	3 (10^{-8} g/ml)	0
15, 16	Unknown serum 1	5	4	0	0	1
17, 18	Unknown serum 2	5	4	0	0	1

* Sodium phosphate buffer, 0.05 M, pH 6.1, containing 150 mg per cent ascorbic acid.

morning, 0.5 ml of this fresh 18-hour culture was added to 10 ml of maintenance medium and incubated for 6 hours at 37° C. The inoculum was prepared by adding 0.5 ml of this fresh 6-hour culture to 10 ml of single-strength basal medium. One drop of inoculum was added to each flask.

Basal media. The assay medium for *L. casei* has been previously described (5). This medium was almost identical with the basal medium used by Jukes (13) for *L. casei,* differing in only two respects, neither crucial. It should be noted that the *L. casei* basal medium of Jukes was only slightly modified from the medium for *S. faecalis* of the Association of Official Agricultural Chemists (14), which in turn derived from that of Teply and Elvehjem (15). In our basal medium: *1)* a commercial salt-free hydrochloric acid hydrolysate of casein (Hy-Case, salt-free; Sheffield Chemicals, Norwich, N. Y.) was used instead of preparing it in the laboratory. An almost identical simplification of *L. casei* assay media preparation had been used previously by others (12) who used a similar, but not salt-free, hydrolysate. *2)* The amount of para-aminobenzoic acid used was twice that used by Jukes. This was done to counteract any inhibitory effect on growth should any of the sera assayed contain appreciable amounts of sulfonamides. The reader is referred to prior publications for a tabular listing of the ingredients of the medium (5, 16) and for the method of preparing each ingredient prior to use (13, 14, 16). See the Appendix for a detailed summary.

Different commercial basal media for *S. faecalis* and *L. citrovorum* (17) were used in initial studies, but were discarded when it was found that our *L. casei* basal medium allowed as good or better growth of these two organisms as did the commercial media specially formulated for them.

Volatile preservative (VP) was added to all stored solutions of assay medium and of buffer by spraying a small aliquot from a wash bottle into each solution after each use. VP consisted of one part ethylene dichloride, one part monochlorobenzene, and two parts 1-chlorobutane (18).

Standards. Three standard solutions of folic acid (pteroylglutamic acid, PGA) were prepared: 10^{-8}, 10^{-9}, and 10^{-10} g per ml. Similar standard solutions of folinic acid (N^5-formyl-tetrahydrofolic acid, citrovorum factor) were prepared by diluting calcium leucovorin [3] to concentrations twice those of the folic acid standards. Since calcium leucovorin is a synthetic racemic form of folinic acid (citrovorum factor) (19), such dilution resulted in concentrations of folinic acid activity equivalent to the folic acid activity of the standard solutions of folic acid. Weight for weight, we found calcium leucovorin to be approximately half as active for *L. casei* as folic acid. Dawbarn, Hine and Smith (20) had found calcium leucovorin to be 38.3 per cent as active for *L. casei* as folic acid. It is possible that in our assay system, some of the inactive *d*L-isomer may be converted to the active *l*L-isomer. This has been reported to occur in the presence of dilute acid (21), and *L. casei* gradually makes its assay medium more acid as it grows and produces lactic acid.

Assay protocol. All glassware was rendered folic acid-free by rinsing thrice in tap water, boiling gently in Haemo-sol (Meinecke & Co., New York, N. Y.) solution for 0.5 hour, rinsing 12 times in tap water, thrice in de-ionized water, and then drying in air. The aluminum caps which covered the flasks were rendered folic acid-free by rinsing 12 times in tap water, then thrice in de-ionized water. They were not washed in detergent because it proved unnecessary (and it corroded the metal).

With an automatic pipet, 5 ml of double-strength basal medium was added to each 10 ml micro-Fernbach flask (18) (10 ml Erlenmeyer flasks are less expensive, equally good, but more susceptible to tipping over). From 1 to 4 ml of de-ionized water was next added to each flask, the volume being such as to bring the total volume in each flask to 10 ml when all ingredients had been added.

Table I illustrates the assay protocol of our standard method for the determination of folic acid activity in serum. After all ingredients had been added, the flasks

[3] Kindly provided by Drs. T. H. Jukes and E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y.

were autoclaved for 30 minutes at 105 to 110° C, allowed to cool, and inoculated.

One drop of inoculum was added to each flask, and the flasks incubated [4] 16 to 18 hours at 37° C. Each flask was then vigorously shaken and its content transferred to a Klett colorimeter tube. Growth density was then measured with a Klett-Summerson photoelectric colorimeter [5] using a red filter ($\lambda = 640$ to 700 mμ) to reduce error due to the color of the medium. The growth densities of the standards were plotted on semilogarithmic paper and serum values calculated from the curve (5). Usually the standards were run in duplicate, the results averaged, and the average values plotted. Duplicates rarely differed significantly.

Aseptic addition method. In experiments using unheated serum, 0.1 ml aliquots of serum were added aseptically to autoclaved solutions containing 5 ml of double-strength medium, 4 ml of de-ionized water, and 1 ml of stated concentrations of ascorbic acid in phosphate buffer. This is referred to below as the "aseptic addition method."

All experiments involving more than 350 mg per cent ascorbic acid [6] were done by the "aseptic addition method," because such amounts of ascorbic acid prevented serum protein from being thrown down in the centrifugation of the standard method. The dense heated protein markedly raised the background turbidity, masking that due to bacterial growth.

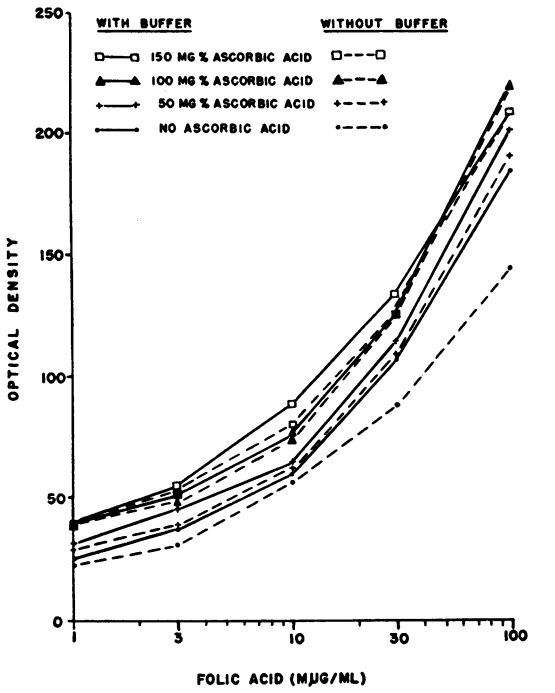

FIG. 1. ENHANCEMENT OF *L. casei* GROWTH BY ASCORBIC ACID AND PHOSPHATE BUFFER.

RESULTS

Effect of ascorbic acid, phosphate buffer, and heat

1. Effect on growth of L. casei standards. Employing our standard method, *L. casei* standard curves were obtained, adding 1 ml of varying concentrations of ascorbic acid with and without phosphate buffer. Figure 1 demonstrates that the growth of *L. casei* is stimulated by both ascorbic acid and phosphate buffer. Stimulation by ascorbic acid increased with increasing amounts of this agent up to 150 mg per cent. The addition of 1 ml of ascorbic acid solution in concentrations above 150 mg per cent and up to 1,500 mg per cent had no further marked stimulatory effect, and amounts in excess of 1,500 mg per cent *inhibited L. casei* growth (Table II). Adding the standard solutions of folic acid (PGA) to the assay medium after autoclaving did not result in any increase of *L. casei* growth over that obtained when folic acid was added before autoclaving (data not shown).

2. Effect on ability of human serum to stimulate growth of L. casei. Sera were prepared for assay by four different methods: Method 1, our standard method; Method 2, 1 ml serum was diluted to 50 ml with distilled water, and 5 ml of this diluted serum was added to 5 ml of double-strength medium which was then autoclaved and inoculated (this was the method used by Chanarin, Anderson and Mollin (22) for preparing serum for "folic acid" assay with *S. faecalis*); Method 3 same as Method 2, but with 9 ml of 150 mg per cent ascorbic buffer replacing 9 ml of the water used to dilute the serum; Method 4, the aseptic addition method, using 150 mg per cent ascorbic buffer. Since Methods 2 and 3 produced a serum protein precipitate in the incubation medium, non-inoculated control sera were run, and the optical density of the controls was subtracted from the optical density reading of the inoculated samples. To

[4] American standard incubator, A. H. Thomas Co., Philadelphia, Pa.

[5] Klett Manufacturing Co., New York, N. Y. The optical density units employed in the present paper are the scale readings of this instrument. The scale readings are directly proportional to concentration when Beer's law holds.

[6] "Mg per cent ascorbic acid" will always refer to mg per cent ascorbic acid in the 1 ml of buffer of column 5 or column 7 in Table I. Thus, *the actual concentration of ascorbic acid in 10 ml of finished medium is one-tenth the amount stated.*

TABLE II

Effect of increasing concentrations of ascorbic acid from 100 to 15,000 mg per cent on growth of L. casei in the presence of varying amounts of folic acid

	Growth of *L. casei* (in optical density units) in medium containing varying amounts of added ascorbic acid in 1 ml of phosphate buffer														
	Ascorbic acid (mg %)						Ascorbic acid (mg %)						Ascorbic acid (mg %)		
Folic acid	Expt. A	100	150	200	250	350	Expt. B	150	500	1,000	1,500	2,000	2,500	Expt. C	150	1,500	15,000
mμg/ml				*mu*						*mu*						*mu*	
0		44	49	47	48	50		38	39	31	33	32	29		37	39	8
0.1		49	56	50	53	51		47	46	43	47	43	36		48	49	7
0.3		50	61	61	61	67		67	67	64	67	62	52		62	67	8
1		65	80	80	81	84		111	115	115	116	103	90		94	95	8
3		98	110	113	105	109		163	165	160	160	155	133		140	138	9
10		163	165	172	170	165		228	228	226	230	213	210		204	205	9
30		221	195	211	209	196		250	248	252	245	255	235		226	222	9

this value was added the optical density reading of an uninoculated control flask containing no added folic acid or serum. The resulting figure was used to calculate folic acid activity. Chanarin and associates (22) do not record adding the reading of an inoculated control flask in their calculations. This may explain why they find *no* folic acid activity for *S. faecalis* in fasting serum, whereas we consistently find a small amount of such activity using their methodology, but compensating for growth occurring in the absence of added serum.

The "folic acid activity" of the sera was calculated against the appropriate standard curves (i.e., Methods 1, 3, and 4 were calculated against a standard with added ascorbic buffer, and Method 2 was calculated against a standard without added ascorbic buffer).

Table III shows results obtained with sera from patients with poor, marginal, and excellent folic acid nutritional status.[7] In the presence of 1 ml of 150 mg per cent ascorbic buffer, regardless of whether or not the serum was heated (Methods 1, 3 and 4), the serum folic acid activity for *L. casei* reflected the patient's nutritional status with respect to folic acid. On the other hand, when the sera were heated in the absence of ascorbic acid (Method 2), the folic acid activity for *L. casei* of sera from the patients with excellent and marginal folic acid nutrition fell as low as that of the patient with poor folic acid nutrition.

Incubation of serum from normal subjects in the absence of added ascorbic acid at 37° C for 18 hours also resulted in loss of all activity for *L. casei,* as measured by our standard method (data not shown).

Both the phosphate buffer and ascorbic acid protect serum folic acid activity for *L. casei* even when the serum is not heated ("aseptic addition method," Table IV; Figure 1 depicts the standard curves for Experiment A of Table IV).

Protection by ascorbic acid increases as its concentration rises to 150 mg per cent. Increasing the amount of ascorbic acid from 150 to 350 mg per cent slightly increased the protective effect over that obtained with 150 mg per cent ascorbic acid (Table IV, Experiment B; the standard curve for this experiment is recorded in Table II, Experiment B).

The serum folic acid activity is unaffected by raising the amount of added ascorbic acid from 1 ml of 500 mg per cent to 1 ml of 1,500 mg per cent (Table IV, Experiment C). There is an *ap-*

[7] Basis of classification: Poor = diet devoid of meat, fruit, vegetables, normal serum vitamin B_{12} level; megaloblastic anemia responsive to folic acid. Marginal = diet poor in sources of folic acid; normal serum vitamin B_{12} level; slight macrocytosis and increase in segmentation of nuclei of polymorphonuclear leukocytes without overt megaloblastic anemia. Excellent = good diet; no signs or symptoms of malabsorption; normal serum vitamin B_{12} level; no macrocytosis or increase in segmentation of nuclei of polymorphonuclear leukocytes.

TABLE III

Effects of heat and ascorbic acid on serum folic acid activity

Serum treatment			Folic acid nutritional status of serum donor		
Heat	Ascorbic acid	Method*	Poor	Marginal	Excellent
			Serum folic acid activity		
			mμg/ml		
Yes	Yes	1	2.1	6.3	29.0
Yes	No	2	2.3	1.8	2.9
Yes	Yes	3	3.3	4.1	28.0
No	Yes	4	2.1	4.0	24.0

* See text.

parent rise when the added ascorbic acid is raised to 1 ml of 2,500 mg per cent, but this is due to inhibition of the standard curve growth by this amount of ascorbic acid (indicated in Table II, Experiment B); this rise therefore is apparent but not real. It may be due to the presence in serum of various ascorbic acid-inactivating substances, which would reduce the actual ascorbic acid level in the sera below the toxic level in the standards. The inhibition of growth by large amounts of ascorbic acid was probably not due to lowering of pH, since in the experiments recorded in Tables II and III the ascorbic acid solutions were all titrated to pH 6.5 with 10 per cent KOH before they were used. However, "moderate changes in the buffering capacity of the medium, such as may result from the buffering power of the added analytical specimen, may significantly affect growth response" (23).

Substitution of dD-*iso-ascorbic acid or 2-mercaptoethanol for ascorbic acid.* One hundred fifty mg per cent D-iso-ascorbic acid (D-araboascorbic acid) (D-erythro-3-ketohexonic acid lactone) is an effective substitute for the same amount of ascorbic acid (or sodium ascorbate) in the buffer used to prepare sera for assay, as indicated by the fact that sera prepared with either agent assayed almost identically (Table V, Experiment D). However, D-iso-ascorbic acid provided less stimulus than ascorbic acid to growth of the *L. casei* standard curves, and was therefore not further studied as a possible replacement for ascorbic acid, because for assay purposes it was desired to provide, insofar as possible, an excess of every *L. casei* active material that human serum contains *except folic acid active materials.* Human serum ordinarily contains ascorbic acid.

TABLE IV

Protective effect of phosphate buffer and ascorbic acid

		Ascorbic acid	Serum folic acid activity	
			Serum diluent	
			Water	Buffer
		mg %	*mμg/ml*	
Expt. A	Serum 1	0	6	8
Aseptic		50	5	8
addition		100	7	10
method		150	14	15
Expt. B	Serum 2	100		7
Standard		150		13
method		200		15
		250		17
		350		17
Expt. C	Serum 3	500		8
Aseptic		1,000		8
addition		1,500		8
method		2,000		10
		2,500		14

Using the "aseptic addition method," it was found that 1 ml of 2 g per cent 2-mercaptoethanol substituted completely for ascorbic acid in protecting serum folic acid activity, whereas 1 ml of 0.2 g per cent 2-mercaptoethanol afforded only slight protection (data not shown).

Using the "standard method," 0.2 g per cent 2-mercaptoethanol protected serum folic acid activity (Table V, Experiment E). This concentration of 2-mercaptoethanol prevented protein precipitation, making necessary the use of a control (not inoculated) flask.

Unlike ascorbic acid (and D-iso-ascorbic acid, to a lesser extent), 2-mercaptoethanol did not stimulate *L. casei* growth. Addition of 1 ml of 2

TABLE V

Protection of serum folic acid activity on heating in the presence of 150 mg per cent ascorbic acid or D-*iso-ascorbic acid, or 0.2 g per cent 2-mercaptoethanol*

	Serum	150 mg % ascorbic acid	150 mg % D-*iso*-ascorbic acid	200 mg % 2-mercaptoethanol
		Serum folic acid activity		
	no.	*mμg/ml*	*mμg/ml*	*mμg/ml*
Expt. D	4	8.0	7.0	
	5	11.3	9.4	
	6	2.8	3.4	
	7	4.1	4.7	
Expt. E	8	12.5		13.4
	9	1.3		<1.0

TABLE VI

Similarity of the folic acid activity of serum and plasma

Subject	Serum Folic acid activity	Plasma
	mμg/ml	
10	3.7	3.6
11	4.4	4.0
12	6.9	5.3
13	10.3	12.5
14	15.8	14.3

g per cent 2-mercaptoethanol to the medium slightly inhibited *L. casei* growth in the presence of 1 ml or more of 10^{-8} g per cent PGA but not with lesser PGA concentrations (data not shown).

Folic acid activity of serum versus plasma. Table VI indicates that the folic acid activity of serum prepared as indicated in Materials and Methods is essentially the same as that of plasma obtained from the same subjects at the same time. The plasma was obtained by centrifugation of whole blood *immediately after withdrawal* from subjects into Vacutainers, no. 3204, containing dry balanced oxalate with immediate aspiration and freezing until assay of the plasma layer after centrifugation.

Activity of serum for L. casei versus S. faecalis versus L. citrovorum. Table VII shows that 1 ml of serum from normal subjects markedly stimulates microbiological growth (exceeding that produced by 5 mμg of folic acid) only when supplied to *L. casei*. The growth of *S. faecalis* and *L. citrovorum* is slight on all human sera studied so far, and neither of these microorganisms grows substantially better on sera from normal human subjects than on sera from folic acid-deficient subjects. This is true whether the sera are prepared for assay by autoclaving in the phosphate-ascorbic acid buffer or by aseptic addition to pre-autoclaved ascorbic acid-containing media (Table VII).

TABLE VII

Folic acid activity of sera determined by standard method or aseptic addition method with L. casei, S. faecalis and L. citrovorum

	Serum	*L. casei* Serum folic acid activity	*S. faecalis*	*L. citrovorum*
		mμg/ml		
Expt. F	15	1.6	1.6	1.6
Aseptic	16	2.7	4.1	2.5
addition	17	6.5	1.8	1.7
method	18	7.7	2.2	1.6
	19	12.2	1.9	1.6
Expt. G	20	2.9	1.1	1.0
Standard	21	6.5	1.0	<1
method	22	8.6	1.5	<1
	23	11.3	1.1	<1
	24	34.0	1.7	<1

DISCUSSION

L. casei growth is stimulated by both ascorbic acid and phosphate buffer. Therefore, serum "folic acid" values obtained by using ascorbic acid-phosphate buffer in the sera and not in the standards would give erroneously high results. In our prior studies of serum "folic acid" activity (4–6) this error was made. Fortuitously, the results obtained by that method compare fairly well with results obtained by the method here reported, because the lesser growth of *L. casei* in the standards in our prior studies (due to the absence of ascorbic acid) was counterbalanced by lesser growth in the sera, due to incomplete protection of serum folic acid activity by 50 mg per cent ascorbic buffer. The fact that ascorbic acid per se enhances growth of *L. casei* was previously observed by other workers (24, 25). This important consideration was not taken into account in our prior studies (4–6), or in most of the reported studies on the folic acid content of foods. For this reason, a significant amount of the reported "folic acid" content of diets in various studies may in fact be ascorbic acid content.

The studies here reported indicate the great lability of serum folic acid activity if it is not adequately protected by ascorbic acid. This protection appears to be afforded by the reducing ability of ascorbic acid. This is indicated by the fact that D-iso-ascorbic acid, which is biologically relatively inactive except as a reducing agent (26), affords protection similar to that provided by ascorbic acid. 2-Mercaptoethanol, a less potent reducing agent, also provides protection when it is in adequate concentration.

The lability of the serum folic acid activity was first suspected from initial studies in Boston, where we consistently obtained lower values for serum folic acid activity than we did in New York where a large steam autoclave with reliable temperature control was used. The difficulty was traced to the

use in Boston of a small electric autoclave that failed to produce a rapid rise to pre-set temperature and maintained poor temperature control thereafter. This resulted in a more prolonged exposure part of the time to higher temperatures. Recognition of the source of the difficulty led to the present use of 150 rather than 50 mg per cent ascorbic acid, which protects the serum folic acid activity against thermal vicissitudes. It is possible that a similar problem may have beset other workers (27) who were unsuccessful in an attempt to reproduce our results, using modification of our method. They autoclaved sera at 15 psi, which is 121° C when the autoclave is functioning properly and there is complete replacement of air by steam (17). At that temperature, serum folic acid activity is destroyed in only 50 mg per cent ascorbic acid. Their use of that pressure may have derived in part from the error in our paper (5), stating that autoclaving was performed at "118° C, 16 psi." The 118° C was correct; 16 psi was incorrect. At 16 psi, assuming a properly functioning autoclave and complete replacement of air by steam, the temperature would be 122° C (17). The finding by Cooperman, Luhby and Avery (27) of marked folic acid activity in sera from folic acid-deficient subjects may have been due to contamination of serum samples with folic acid activity. A major source of such contamination in our early studies was the use of non-acid-washed syringes.

The three changes from our original (5) protocol and the reasons therefore are: *1*) 1 ml of phosphate-ascorbic acid buffer is added to each standard because the buffer itself stimulates *L. casei; 2*) for the same reason 1 ml of serum diluted in buffer is used instead of the 0.5, 1, and 1.5 ml amounts previously used, because varying amounts of phosphate-ascorbic acid buffer provided varying stimulation of *L. casei; 3*) 150 mg per cent ascorbic acid is used because 50 mg per cent is not adequate to protect serum "folic acid" from accidentally higher than normal autoclave temperatures.

Toennies and co-workers (12) found that ascorbic acid in excess of 50 mg per cent interfered with protein coagulation of whole blood diluted 750-fold. Fortunately for our "standard method," amounts of ascorbic acid up to 350 mg per cent do not interfere with protein coagulation of serum diluted 10-fold.

During the course of a report (12) on precursors of the folic acid-active factors in blood, it was stated that normal plasma is nearly inactive for *L. casei.* Because of that statement, it seemed possible that what we refer to as *"serum* folic acid activity" was in fact derived from the large quantity of folic acid-active material in erythrocytes (12, 28, 29) during the 3 hour period the clotted blood stands at room temperature prior to removal of the serum. The present finding that *serum* folic acid activity and *plasma* folic acid activity are approximately the same indicates that this activity is not derived from the cellular components of blood *in vitro,* since the plasma was removed from whole blood immediately after venipuncture.

After ingestion of 15 mg of folic acid, a "labile citrovorum factor" appears in the urine (30). Like "serum folic acid activity," this material is inactivated by autoclaving in the absence of ascorbic acid, but is protected (presumably against oxidative destruction) by the presence of ascorbic acid. The material with these characteristics appears to be a derivative of N^{10}-formyltetrahydrofolic acid (30).

Serum folic acid activity differs from the "labile citrovorum factor" in urine in at least one important respect: it has very little growth activity for *L. citrovorum.* The evidence that serum folic acid activity may be due mainly to a derivative of N^{10}-formyltetrahydrofolic acid, and the seemingly opposed fact that despite autoclaving in the presence of ascorbic acid, which should have converted it to folinic acid (30), it has little growth activity for *L. citrovorum,* requires explanation. Serum protein binding appears to play no role in the relative unavailability of serum folic acid activity for *S. faecalis* and *L. citrovorum,* because these organisms do not grow markedly on human serum whether or not serum protein is precipitated by autoclaving prior to assay.

The most obvious explanation for *L. casei* activity at a higher level than *S. faecalis* or *L. citrovorum* activity is the presence of folic acid-active materials at the triglutamyl (or higher) level (29, 31, 32). The latter two organisms grow slightly or not at all on such materials. The folic acid-active diglutamate, pteroyl-γ-glutamyl-glutamic

acid, is active for both *L. casei* and *S. faecalis;* the diglutamate N^5-formyltetrahydrofolylglutamate is active for all three microorganisms; all the known folic acid-active monoglutamates are active for *L. casei* and for either or both *S. faecalis* and *L. citrovorum* (29, 31). The latter organism is unique in its requirement for a reduced form of folic acid (32). Folic acid-active polyglutamates isolated and characterized from natural materials appear to have three or seven glutamic acid residues (32). Since *L. casei* does not grow appreciably on heptaglutamates (31–33) it is reasonable to infer that serum folic acid activity is due mainly to *tri*glutamates.

It was previously suggested (5) that utilization of PGA polyglutamates may be the crucial property contributing to the superiority of *L. casei* over *S. faecalis* for the assay of serum folic acid activity. This superiority was assumed by comparison of our *L. casei* results with the *S. faecalis* results of Chanarin and co-workers (22). However, in light of the present studies of the lability of serum folic acid activity, the failure to find folic acid activity in fasting serum using *S. faecalis* may have been due to the failure to protect serum folic acid activity with ascorbic acid. The present study demonstrates that even with such protection serum folic acid activity is unavailable to *S. faecalis.* The present study therefore indicates that serum folic acid activity is due mainly to polyglutamates, but suggests that they are polyglutamates of reduced forms of PGA, rather than of PGA itself.

In cell-free bacterial enzyme systems, tetrahydrofolic acid (THFA, which has one glutamic acid residue) appears catalytically inactive as a one-carbon unit carrier; this pteridine coenzyme becomes catalytically active only when it has three glutamic acid residues—i.e., when it is a polyglutamate (34). This catalytic activity is dependent upon the presence of DPN, Mn^{++}, and pyridoxal phosphate (34). It is possible that in all bacterial enzyme systems a polyglutamyl derivative(s) is the true cofactor form of folic acid and that in the crude systems, when the monoglutamyl derivatives are active, a mechanism is available for the synthesis of the derivatives with more than one glutamic acid present (35).

In studies with partially purified cell-free systems of mammalian (rabbit) liver, THFA appears to be catalytically active as a one-carbon unit carrier in the presence of pyridoxal phosphate (36). The experimental data suggested, however, that conjugates of THFA possessing three or seven glutamic residues were even more active than THFA itself (36). One interpretation of these data might be that the partially purified cell-free mammalian liver preparations contained an enzymatic mechanism capable of converting THFA to a polyglutamate, and that only THFA polyglutamates are catalytically active. Conjugases for folic acid-active polyglutamates are distributed in animal tissues, especially liver and kidney (31, 37, 38). The conjugase in human blood appears able to split pteroylheptaglutamates mainly to triglutamates, and, to a lesser degree, to monoglutamates (39).

It may reasonably be inferred that an enzymatic system for synthesizing polyglutamates from monoglutamates occurs in man. Evidence favoring this possibility is the preliminary finding that when unconjugated PGA is administered parenterally to humans there is an immediate rapid rise in folic acid activity for both *S. faecalis* and *L. casei,* but within an hour the *S. faecalis* activity falls sharply while *L. casei* activity remains elevated (40).

Serum folic acid activity appears to be due mainly to triglutamyl derivatives of reduced forms of folic acid, but the precise characterization of the derivative(s) involved awaits isolation of the intact compound(s). Most of the serum folic acid activity may be due to triglutamyl derivatives of N^{10}-formyl THFA, $N^{5\text{-}10}$-methenyl THFA, and/or THFA carrying one-carbon units other than formyl. The heat instability of serum folic acid activity suggests that little or none of it is the triglutamyl derivative of folinic acid(41). Some sera may show more folic acid activity when assayed by the aseptic addition method than when assayed by the standard method (unpublished data). This suggests that a significant part of the activity of some sera may be due to the triglutamyl derivative of THFA carrying no one-carbon unit, since THFA itself assays higher by the aseptic addition method than by the standard method (42). The isolation of folic acid and citrovorum factor, rather than other THFA derivatives, from natural materials, is a reflection of the oxygen lability of THFA and its other derivatives, which are in fact probably the predominant (or only) forms in which the vitamin occurs in natural materials. By

the same token, the isolation of monoglutamates rather than polyglutamates probably reflects the instability of the latter.

Further study is needed to delineate how much of serum folic acid activity may derive from food absorbed as a polyglutamate and how much may be converted to the form or forms present in serum by enzymatic systems in intestine, liver and other tissues. The "folic acid deficiency" of patients with liver disease and idiopathic steatorrhea (7) may be related in part to inability to form folic acid-active polyglutamates due to deficiency or absence of the enzyme(s) involved in their synthesis from monoglutamates. This enzymatic system may reside not only in liver but also in intestine and other tissue.

SUMMARY

1. The previously reported serum "folic acid" assay with *L. casei* was modified by: addition of phosphate-ascorbic acid buffer to the *standards* as well as to the unknowns; use of *150* instead of 50 mg per cent ascorbic acid; use of *identical* aliquots of serum.

2. The maintenance medium and basal medium used for *L. casei* assay served equally well for *S. faecalis* and *L. citrovorum* assay.

3. The folic acid activity level of plasma is the same as that of serum.

4. Serum folic acid activity for *L. casei* appears to be due mainly to triglutamyl derivatives of reduced forms of folic acid. This is suggested by its relative unavailability to *S. faecalis* and *L. citrovorum,* and its protection during autoclaving by ascorbic acid, D-iso-ascorbic acid, or 2-mercaptoethanol. The metabolically active forms of the folic acid coenzymes in man may be reduced triglutamates rather than monoglutamates.

APPENDIX

The assay and maintenance media have previously been presented in tabular form (5), but without details of preparation. Such details are provided herewith in Tables VIII and IX. Preparation of the assay medium is tedious and fraught with possibilities for contamination with folic acid. Several investigators have been unsuccessful in preparing it. For these reasons, two firms were asked to prepare the assay medium in dehydrated form ("dry mix"). As of the present time, we have tested and found satisfactory the dry mix (lot 009-607)

TABLE VIII

Assay medium

Constituent	Amount for 4 L of double-strength medium	Concentration in final volume
		mg %
Hy-Case*	40 g	500
L-tryptophan	0.8 g	10
Adenine†‡	4 ml	0.5
Guanine HCl†‡	4 ml	0.5
Uracil†‡	4 ml	0.5
Xanthine†‡	8 ml	1.0
L-asparagine H_2O	2.4 g	30
L-cysteine HCl§	2 g	25
Riboflavin†‖¶	4 ml	0.05
Para-aminobenzoic acid†‖	8 ml	0.1
Pyridoxine HCl†‖¶	16 ml	0.2
Thiamine HCl†‖	1.6 ml	0.02
Ca pantothenate†	3.2 ml	0.04
Nicotinic acid†	3.2 ml	0.04
Biotin†¶**	1 ml	0.001
Glucose	160 g	2,000
Tween 80††	4 ml	0.005 ml
Glutathione (reduced)	20 mg	0.25
Salt mix‡‡	40 ml	0.5 ml
Na acetate (anhydrous)	160 g	2,000
K_2HPO_4	4 g	50
KH_2PO_4	4 g	50
$MnSO_4H_2O$§§	0.8 g	10

* Salt-free hydrochloric acid hydrolysate of casein, Sheffield Chemicals, Norwich, N. Y. Dissolve in 2 L of de-ionized water with gentle heating and stirring.

† California Foundation for Biochemical Research, Los Angeles, Calif.

‡ Solution (10 mg/ml) prepared by adding 1 g chromatographically pure reagent to 25 ml de-ionized H_2O, gently heating with added pellets of KOH until reagent dissolved, making up to final volume of 100 ml with de-ionized H_2O. Store in refrigerator with volatile preservative added.

§ Dissolve in KOH before adding.

‖ Solution (1 mg/ml) prepared by dissolving 0.1 g reagent in 100 ml de-ionized H_2O. Store in refrigerator with VP added.

¶ Store in amber polyethylene bottle (6026, Cole-Parmer Instrument and Equipment Co., Chicago, Ill.) to protect from light.

** Solution (0.08 mg/ml) prepared by dissolving 8 mg biotin in 100 ml de-ionized H_2O. Store in refrigerator with VP added.

†† Atlas Powder Co., Wilmington, Del. The viscous commercial preparation (specific gravity =1) is diluted 1:10 with water containing some ethanol to reduce foaming.

‡‡ One ml contains: $MgSO_4 \cdot 7H_2O$, 40 mg; NaCl, 2 mg; $MnSO_4 \cdot 4H_2O$, 2 mg; $FeSO_4 \cdot 7H_2O$, 2 mg. Make 500 ml by dissolving 20 g of $MgSO_4 \cdot 7H_2O$, 2 g of NaCl, 1 g of $MnSO_4 \cdot 4H_2O$, and 1 g of $FeSO_4 \cdot 7H_2O$ in de-ionized water, add 1 ml concentrated HCl, store with VP added.

§§ Added after pH of assay medium adjusted to 6.6 to 6.8 with 0.1 N H_2SO_4.

TABLE IX

*Maintenance medium**

Constituent	Amount for 500 ml medium	Concentration in final medium
		g/100 ml
Yeast extract (Difco)	3.75 g	0.75
Proteose peptone (Difco)	3.75 g	0.75
Glucose	5 g	1.0
KH_2PO_4	1 g	0.2
Tomato juice filtrate†	50 ml	10 ml
Tween 80‡	0.5 ml	0.01 ml
L-cysteine HCl	0.5 g	0.1

* Adjust to pH 6.8 to 7 with 1% KOH. This medium, without the L-cysteine, has been previously described (43).

† Canned or vacuum-bottled tomato juice passed through coarse filter paper; straw-colored filtrate adjusted to pH 7 with 10% KOH.

‡ See footnote††, Table VIII.

prepared by Baltimore Biological Laboratory, Baltimore, Md.

The sodium phosphate buffer (9) is prepared as follows: dissolve 27.6 g of $NaH_2PO_4 \cdot H_2O$ in 1 L distilled water (solution A); dissolve 71.6 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L distilled water (solution B). To 212.5 ml of solution A add 37.5 ml of solution B; dilute to 1 L with de-ionized water. The pH should be 6.1. Store solutions at room temperature, with volatile preservative added.

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