The measurement of serum pyridoxal by a microbiological assay using *Lactobacillus casei*

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SYNOPSIS A new method has been developed for the assay of serum pyridoxal using *L. casei*. Bound pyridoxal phosphate in serum is converted by acid hydrolysis to pyridoxal for which this organism is specific. This method proved to be considerably more sensitive than other methods so far reported in the literature.

Serum pyridoxal concentrations were measured in 151 control subjects aged 17 to 80 years. The range of concentrations found was 1.5 to 13.5 ng/ml which compared well with values obtained by most workers measuring pyridoxal phosphate by enzymatic methods. A marked fall with age was confirmed, and levels in women of childbearing age were lower than in men of comparable age. Subnormal serum pyridoxal concentrations were found in 62% of patients with sideroblastic anaemia and in the majority of patients with rheumatoid arthritis, Crohn's disease, coeliac disease, and in pregnant women at term.

Pyridoxal phosphate is the principal coenzyme form of vitamin B₆² and is presumably therefore the main B₆ compound present in the body. There was little definite information about the form of B₆ in blood until Kelsay, Baysal, and Linkswiler (1968) isolated pyridoxal from acid hydrolysates of whole blood by chromatographic separation and subsequent microbiological assay with *Saccharomyces carlsbergensis*. Acid hydrolysis converts the phosphate forms of B₆ to the free forms, and, as Kelsay *et al* (1968) could isolate only pyridoxal, it would appear that pyridoxal phosphate is the naturally occurring form of B₆ in blood. Therefore, the direct measurement of pyridoxal, phosphate or the measurement of pyridoxal after hydrolysis should provide a true assessment of the level of B₆ in blood.

Several workers have used various enzymatic methods for directly measuring pyridoxal phosphate in whole blood or plasma (Boxer, Pruss, and Goodhart, 1957; Wada, Morisue, Sakamoto,

and Ichihara, 1957; Wachstein, Kellner, and Ortiz, 1960; Hamfelt, 1962; Walsh, 1966; Hines Love, and Peart, 1969). We have measured pyridoxal by a microbiological assay method using *Lactobacillus casei*. A microbiological method deals easily with large numbers of specimens, requires very little serum, and is probably simpler to carry out than enzymatic methods. *L. casei* was chosen as, unlike other microorganisms, it responded only to pyridoxal and not to the other B₆ compounds, in the amounts likely to be found in body fluids under physiological conditions (Snell and Rannefeldt, 1945). It was also far more sensitive to B₆ than other microbiological or enzymatic methods. However, *L. casei* has appeared in the past to be unsatisfactory for assay of pyridoxal in serum and whole blood because falsely high values were obtained with the prolonged acid hydrolysis which was thought to be necessary to liberate pyridoxal (Storvick and Peters, 1964). A possible explanation for this was suggested by Haskell and Wallnöfer (1967) who demonstrated that if serum were acid hydrolysed for longer than two hours L-alanine in serum was converted to D-alanine.

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²The general term 'B₆' refers to all forms of the vitamin, and is used when no particular form is specified.

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Control Serum	Pyridoxal Concentration (ng/ml)		
	Range	Mean	SD
A	0.7- 2.0	1.4	0.4
B	3.4- 4.9	4.2	0.4
C = B + 10 ng/ml	12.4-16.5	14.2	0.9

Table I Summary of 30 consecutive assays on three control sera

Serum	Pyridoxal Standards
1 Add 16.5 ml 0.2N HCl to universal container Add 0.25 ml serum	Add 16.5 ml 0.2N HCl to each of seven universal containers. Add 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml respectively of working solution ¹
2 Autoclave at 15 psi for one hour and cool	Proceed exactly as for serum.
3 Add 2 ml 0.5M sodium phosphate buffer pH 6.4 Add 0.5 ml 8N KOH	
4 Check pH 6.4 with micro combination electrode	
5 Make volume up to 20 ml with distilled water	
6 Steam for three minutes and cool	
Thereafter use sterile precautions	
7 Filter through sterile no. 42 Whatman filter paper	
8 Assay filtrate	

Table II Preparation of serum and standards by acid hydrolysis

¹If serum pyridoxal concentration less than 3 ng/ml use 0.5 ml serum, and if greater than 10 ng/ml use 0.1 ml or less serum.
²Use pyridoxal HCl (Sigma) (121.8 mg equivalent to 100 mg pyridoxal). Two stock solutions, 500 µg/ml and 2.5 µg/ml pyridoxal in 0.02N HCl, which may be kept for one year and one month respectively at 4°C in dark bottles. Freshly made working solution 5 ng/ml in distilled water.

Step No.	Procedure
1	Sterilize 6½ in. Pyrex tubes held in aluminium racks and closed with Oxoid caps
2	Add 3 ml assay medium ¹ Add 2 ml filtrate (serum or standards) in duplicate tubes for each hydrolysate
3	Inoculate each tube with one drop of inoculum ² and shake
4	Incubate at 37°C for 20 hours
5	Cool tubes at 4°C for minimum of ½ hour
6	Mix contents on Whirlimixer
7	Measure growth turbidity in Unicam SP 1300 colorimeter in a 1 cm cell using violet filter, Ilford no. 621 (370-515 mµ), using uninoculated serum and standard tube contents as blanks respectively.
8	Plot standard curve on linear graph paper (see Fig. 2A) Final concentration of pyridoxal standards 0, 1, 2, 4, 6, 8 and 10 ng/100 ml Final dilution of serum 1 in 200
9	Read serum value from standard curve and multiply by 2 to give ng/ml Assay sera in two different assay batches and take mean Run control sera in every batch.

Table III Assay procedure using sterile precautions for steps 1-4

¹Make double strength medium to 6/5 volume with distilled water and sterilize in bulk at 10 psi for eight minutes (medium autoclaved before dispensing to avoid differences in individual assay tubes due to uneven heating in the autoclave).
²(a) Incubate a disc culture of *L. casei* in 10 ml sterile inoculum broth (Difco Bactola cto-bacilli broth AOAC-0901-15) at 37°C for 16 hours. (b) Transfer 1 ml into 10 ml sterile inoculum broth and incubate at 37°C for six to eight hours. (c) Dilute 1 ml in 20 ml sterile single-strength medium for inoculum.

and the concentration of this material increased with length of autoclaving. D-Alanine can replace pyridoxal for growth of *L. casei* (Snell, 1945), and this almost certainly explains the falsely high values obtained.

The purpose of the work described in this paper was to investigate the use of *L. casei* as an assay organism for pyridoxal in serum, and in particular to study whether a full extraction of pyridoxal from serum could be obtained by acid hydrolysing for a shorter period and hence preventing the production of D-alanine. In view of the importance of alanine in the function of B₆ in lactic acid organisms (Snell, 1945), the effect on growth of the addition to the assay medium of the different forms of alanine was studied.

As a result of these studies a specific, sensitive and accurate assay for serum pyridoxal was developed using *L. casei*. This enabled us to measure accurately the serum pyridoxal concentrations in control subjects and in patients with different diseases. Preliminary results are reported.

Materials and Methods

The assay organism used was *Lactobacillus casei* NCIB 8010 (ATCC 7469). It was maintained as dried gelatin discs (Stamp, 1947; Chanarin, Anderson, and Mollin, 1958). Glass-distilled water produced by the Loughborough all-glass still was used throughout. All glassware and pipettes underwent the rigid cleaning procedure necessary for microbiological assay as described by Waters and Mollin (1961).

COLLECTION OF BLOOD SAMPLES

Disposable syringes and needles were used to collect venous blood from subjects at least two hours after food. The blood was immediately put into a sterile universal container wrapped in silver paper to protect it from light, allowed to clot, and the serum separated in subdued light. The serum was stored protected from light at - 20°C until assayed.

CONTROL SERA

Three control sera with pyridoxal concentrations covering the range found in health and disease were set up in every assay batch. A large amount was collected for each control serum and stored at - 20°C. in aliquots sufficient for two assays. Details are given in Table I.

METHOD

Details of the extraction of serum pyridoxal and the assay procedure are given in Tables II and III respectively. As pyridoxal is sensitive to light

all procedures were carried out in subdued light, ie, without artificial lighting and screened from direct daylight.

ASSAY MEDIUM

The composition of the medium used (Table IV) is a modification of that used by Waters and Mollin (1961) to measure serum folate. The

Reagent	Concentration (per litre)
Enzymatic casein hydrolysate ¹	200 ml
Adenine	10 mg
Guanine	10 mg
Uracil	10 mg
Xanthine	20 mg
L-Asparagine	600 mg
L-Cysteine hydrochloride	500 mg
L-Tryptophan	100 mg
Riboflavin	1 mg
p-Amino-benzoic acid	2 mg
Pteroylglutamic acid	0.02 mg
Thiamine hydrochloride	0.4 mg
Calcium pantothenate	0.8 mg
Nicotinic acid	0.8 mg
Biotin	0.02 mg
D-Glucose	40 g
Tween 80 solution (0.5%)	20 ml
Glutathione (reduced)	5 mg
Salt solution ²	10 ml
Sodium acetate (anhydrous)	40 g
K ₂ HPO ₄	1 g
KH ₂ PO ₄	1 g
L-α-Alanine	1 g
MnSO ₄ ·H ₂ O (added after pH adjusted to 6.8 with 8N KOH or 6N HCl)	200 mg

Table IV Double-strength assay medium (pH 6.8)¹

¹The following ingredients were stored as solutions in dark bottles at 4°C: (1) adenine, guanine, and uracil, (2) xanthine, (3) asparagine (4) vitamins at pH 4.5, (5) MnSO₄·H₂O (6), salt solution.

²Sterile 5% solution of enzyme-hydrolysed vitamin-free casein (Nutritional Biochemicals Corporation, Cleveland, Ohio, or Calbiochem Ltd, 10 Wyndham Place, London, W1).

³One millilitre contains 40 mg MgSO₄·7H₂O; 2 mg NaCl; 2 mg FeSO₄·7H₂O·H₂O; 1.5 mg MnSO₄·H₂O.

modifications were as follows. (1) Pyridoxine was replaced by pteroylglutamic acid. (2) L-tryptophan was added routinely because the amount in different batches of casein hydrolysate varied (Waters, personal communication). (3) L-Alanine was added for reasons given later. This medium contained enzymatic casein hydrolysate, and particular care was taken to test new batches of this, as growth of *L. casei* varied considerably from batch to batch of casein. This variation was particularly noticeable before L-tryptophan and L-alanine were included in the medium. Furthermore, traces of pyridoxal were found in some batches of casein. These were submitted to ultra-violet light for two to three hours to destroy the pyridoxal before the addition to the medium. Because riboflavin is extremely light sensitive the medium was at all times protected from light (Anderson and Cowan, 1968).

Investigations into the Assay Method

The following special aspects of the assay method were investigated.

STABILITY OF *L. casei* IN DISC FORM

Growth in standards using *L. casei*, which had been maintained and subcultured into liquid broth for three months, was compared with growth using a culture from a newly made disc of *L. casei*, and there was no significant difference. In addition, growth in standards using two *L. casei* disc cultures, one 11 months and the other 20 months old, was compared with growth using *L. casei* from a newly made disc and again there was no significant difference.

ASSAY MEDIUM

As B₆ is known to be involved in the metabolism of alanine in lactic acid organisms (Snell, 1945) the effect of adding alanine to the medium was studied.

D- and DL-alanine When 0.2 g of either form was included in 100 ml of assay medium, there was uniformly high growth of *L. casei* throughout, whether pyridoxal was present or not.

L-alanine

In striking contrast, when 0.2 g of L-alanine was included in 100 ml of assay medium there was virtually no increase in growth in the absence of pyridoxal. But there was a marked increase in growth when pyridoxal was present and this increase was proportional to the concentration of pyridoxal (Figure 1). The slight increase in the blank (Fig. 1), where no pyridoxal was added, was probably due to traces of pyridoxal in the casein hydrolysate in the medium. The same effect was produced with 0.1 g of L-alanine per 100 ml of medium as with 0.2 g, and this amount was added to the medium in subsequent assays.

It has been found that varying amounts of L-tryptophan in different batches of enzymatic casein hydrolysate cause varying growth responses of *L. casei* when assaying folate (Waters, personal communication) and this was confirmed when assaying pyridoxal. It seemed probable that variations in the amount of L-alanine provided L-tryptophan was added to the medium might also explain variations in batches of casein. Figure 1 illustrates the effect of the addition of L-alanine to the assay medium on *L. casei* growth in standards using two different batches of casein hydrolysate. Casein A had promoted very poor growth and casein B apparently good growth but when L-alanine was added to the medium the growth was markedly increased in both.

It was also observed that the assay values for serum hydrolysates were significantly higher when

Fig. 1 The effect of the addition of L-alanine to the assay medium on growth of *L. casei* in pyridoxal standards comparing two enzymatic casein hydrolysates which had promoted (A) poor growth and (B) apparently good growth when L-alanine was not added to the medium.

● — — — ● No L-alanine in assay medium
● — — — ● L-alanine (0.2 g per 100 ml) added to medium.

L-alanine was not added to the medium, especially when using casein A, which was the more deficient in L-alanine (Figure 1). This is probably because some L-alanine is provided by the serum itself and takes effect particularly in sera with high pyridoxal concentrations where growth is high and hence L-alanine in the medium is exhausted. The recovery of pyridoxal added to serum was also falsely high.

Effect of heating

Equal quantities of medium were autoclaved by steaming for three minutes and at 10 psi for three, eight, and 15 minutes respectively. Growth in these four media decreased with length of heating. Up to eight minutes of heating the curves were parallel, the lowest growth blank being in the latter. The serum values were unaffected. Growth appeared to be depressed when medium was heated for 15 minutes suggesting some destruction of the nutrients. The most suitable length of

autoclaving where the growth blank was reduced was at 10 psi for eight minutes.

PYRIDOXAL STANDARDS

Effect of storage

Four separate stock solutions of 500 µg/ml in 0.02N HCl kept at 4°C were each tested after 13, 14, 15, and 16 months respectively and no deterioration was detected as measured by growth of *L. casei*. Similarly, different solutions of 2.5 µg/ml were tested after one, three, and five months and no deterioration was detected.

Effect of light

Pyridoxal standards were exposed to ordinary daylight on a dull day for three and a half hours, and, when compared with standards protected from light, more than half the pyridoxal activity as measured by *L. casei* was destroyed.

Effect of heating with assay medium

Pyridoxal standards were added to the tubes before and after autoclaving with the medium at 10 psi for three minutes. Half the activity of the pyridoxal was destroyed when it was autoclaved together with the medium.

Effect of acid hydrolysis

Pyridoxal standards were hydrolysed as described in Table II and growth was compared in these standards with aqueous pyridoxal standards, both sets being added after the medium had been sterilized. The growth was higher in the hydrolysed pyridoxal (Fig. 2). This effect was not due to autoclaving. If different concentrations of HCl were used, the growth increased within limits in proportion to the concentration of HCl used. However, the precise factor responsible for the increased growth is difficult to assess, because the concentration of HCl used also determined the amount of KOH subsequently required to adjust the pH to 6.4.

SERUM PYRIDOXAL

Effect of storage

Serum was stored protected from light and at -20°C for as long as two years and there was no detectable deterioration in the pyridoxal concentration.

Hydrolysates of the control sera were stored as above for several weeks and then assayed together with fresh hydrolysates of the same sera. Hydrolysates which had been stored for up to six weeks showed no significant deterioration but after longer storage the pyridoxal concentration gradually decreased.

Effect of different conditions of hydrolysis

(1) The concentration of HCl used was varied

Fig. 2 Comparison of growth of *L. casei* in (A) pyridoxal standards which had been diluted in HCl/KOH mixture as described for preparation of standards (the growth was the same whether hydrolysed or not) and (B) aqueous pyridoxal standards not heated at any stage.

and 0.05, 0.1, and 0.2N were compared. (2) The temperature of autoclaving at one hour was varied and pressures of 15 psi (121°C) and 20 psi (126°C) were compared. (3) The time of autoclaving at 15 psi was varied and one half, one, and two hours were compared. (4) The final pH to which the hydrolysate was adjusted was varied, and pH 5, 6, 6.4, and 6.8 were compared.

None of these variations had any significant effect on the serum pyridoxal assay value or on the recovery of pyridoxal added to serum, but the effect of the HCl/KOH mixture on growth of *L. casei* was the same as for the standards.

Dilution agreement

Assay values were the same when (1) different amounts of serum were hydrolysed (0.5 ml. and less); (2) different amounts of the hydrolysate were assayed. In view of the growth effect of the HCl/KOH mixture used in hydrolysis it was essential that the assay values for serum were read from standards containing the same volume of the HCl/KOH mixture.

In practice, the volume of hydrolysate assayed was fixed at 2 ml and the dilution was varied by

changing the volume of serum hydrolysed, eg 0.25 ml of serum hydrolysed gives a final dilution of 1 in 200; 0.5 ml serum gives a final dilution of 1 in 100.

IDENTIFICATION OF *L. casei* GROWTH FACTOR IN SERUM

The assay medium contained no pyridoxal, but as far as is known contained all the other factors likely to be found in body fluids, including the other vitamins which were necessary for the growth of *L. casei*. To confirm that the growth factor in serum hydrolysates was pyridoxal the following experiment was done.

Gel filtration using Sephadex G-10 was carried out on two 5 ml concentrates from pooled hydrolysates of 2.5 ml of normal serum (1) alone and (2) with added pyridoxal. Fractions, each of 6 ml, were eluted with a 0.1M phosphate buffer (pH 6.0). All fractions up to and including the last peak of ultraviolet absorbancy at 280 mμ were assayed with *L. casei*.

In the serum hydrolysate with no added pyridoxal, a small peak of growth was associated with the 'run-through' peak, but most of the growth factor was held on the column and eluted fairly sharply in the region of substances having the molecular weight of pyridoxal (167). In the hydrolysate to which pyridoxal had been added the small 'run-through' peak was slightly increased, but the rest of the increase in growth factor for *L. casei* eluted in exactly the same position as in the serum sample without added pyridoxal. This identity of molecular size determined by gel filtration strongly suggested that the growth factor in serum hydrolysates was B₆ and hence pyridoxal, being the only form to which *L. casei* responds at these levels. This was further supported by subjecting the peak fractions before assay to ultraviolet light exposure sufficient to destroy B₆ and finding that only a very small percentage (<5%) of the growth remained in these fractions. It was also shown that the addition of ³H labelled pyridoxine to a serum hydrolysate resulted in the appearance of a sharp peak of counts which eluted in the same position as did the growth factor for *L. casei*.

ACCURACY OF METHOD

Reproducibility

The three control sera mentioned under Materials and Methods were assayed in 30 consecutive assay batches and during that period four different workers carried out the assays. The results are summarized in Table I. A serum was extracted and assayed 12 times in the same batch and the reproducibility was good. The range was 8.5-10.4 (mean 9.5 and SD ± 0.6 ng/ml).

Recovery of added pyridoxal

The recovery of pyridoxal added to serum (10 ng/ml) ranged from 82 to 120% (all but five

fell between 90 and 110%). The mean recovery was 100.1% and the SD $\pm 7.7\%$.

RESPONSE OF *L. casei* TO DIFFERENT B₆ COMPOUNDS

The growth response of *L. casei* to different forms of B₆ is summarized in Table V. Pyridoxal was the only compound to which *L. casei* responded at physiological levels. *L. casei* was about 200 times as sensitive to pyridoxal as to pyridoxamine and was far less sensitive to any other form of B₆.

The compounds were acid hydrolysed as described in Table II, and only in the case of pyridoxal-5'-phosphate did this cause an increase in growth response of *L. casei*. In three experiments the recovery of pyridoxal from pyridoxal-5'-phosphate, as measured by growth of *L. casei*, was 80, 85, and 95% respectively.

	Aqueous ¹ (ng/ml)	Acid Hydrolysed ¹ (ng/ml)
Pyridoxal	0.005	0.005
Pyridoxal-5'-phosphate	5.0 ²	0.005 ²
Pyridoxamine	1.0	1.0
Pyridoxamine-5'-phosphate	10.0	10.0
Pyridoxine	20.0	20.0

Table V An approximate quantitative growth response of *L. casei* to different forms of B₆

¹The lowest concentration for which *L. casei* shows a measurable growth response.

²In terms of pyridoxal.

Serum Pyridoxal Concentrations in Control Subjects and in Patients with Various Conditions

SUBJECTS STUDIED

Two groups were studied: (1) 151 control subjects (75 men and 76 women) aged from 17 to over 60 years. The majority of subjects under 60 years were members of the staff and students. The 54 subjects over 60 years were chosen at random from a national geriatric survey. (2) 134 patients with the following conditions: 26 with sideroblastic anaemia, ie, with ring sideroblasts in the bone marrow and varying degrees of anaemia; 31 with rheumatoid arthritis, many receiving drug therapy; 48 with active Crohn's disease of varying degrees of severity; 16 with coeliac disease of whom four had been on a gluten-free diet; 13 pregnant women near term. These were selected for reasons that will be discussed.

SERUM PYRIDOXAL CONCENTRATIONS IN CONTROL SUBJECTS

The serum pyridoxal concentrations in 151 control subjects measured by *L. casei* are shown in Fig. 3, plotted according to age and sex. The mean concentration was calculated in men and women separately in the three age groups 20 to 29, 30 to 39, and 60 years and over. In the other

Fig. 3 Serum pyridoxal concentrations measured by *L. casei* microbiological assay in 151 control subjects. The means for men and women are shown in three age groups where justified by numbers.

● Men ○ Women

age groups there were not sufficient subjects to justify calculating the mean.

Sex differences

The mean serum pyridoxal concentrations were higher in men than in women in the three age groups and the means were as follows: 20 to 29 years 9.1 and 6.5 ng/ml, and the difference was highly significant ($P < 0.001$); 30 to 39 years 6.4 and 5.3 ng/ml, but there were too few subjects to prove these significantly different; 60 years and over 3.9 and 3.5 ng/ml, and these were not significantly different ($0.4 > P > 0.3$).

Age differences

There was a steep fall in the mean serum pyridoxal concentrations from the age of 20 to over 60 years (Fig. 3). The means in the three age groups decreased as follows: men 9.1 to 6.4 to 3.9 ng/ml, and the differences were highly significant ($P < 0.001$); women 6.5 to 5.3 to 3.5 ng/ml, and the difference between the last two means was statistically significant ($0.01 > P > 0.001$).

As there was an approximately even distribution of men and women in all age groups except the first, the mean value was calculated for

Fig. 4 The ranges and means of serum pyridoxal concentrations in different age groups measured by *L. casei* in 144 control subjects (74 males, 70 females), together with statistical analysis.

the combined sexes in the other age groups, and once more there was a progressive fall with age (Fig. 4).

COMPARISON OF SERUM PYRIDOXAL WITH PLASMA PYRIDOXAL PHOSPHATE MEASUREMENTS IN CONTROL SUBJECTS

These results in control subjects are compared with those obtained by another worker (Hamfelt, 1964), who measured plasma pyridoxal phosphate in control subjects using an enzymatic method (Table VI). The means are compared in the age groups which were used by him. His numbers were smaller and he did not take into consideration the sex, but there is a very good agreement between the values by the different methods in each age group.

Measurement	Method	Series	Pyridoxal Concentration (ng/ml)		
			20-29 Years	30-59 Years	60 Years and Over
Plasma pyridoxal phosphate	Enzymatic	Hamfelt (1964)	7.6 (13) ¹	4.8 (11)	2.3 (21)
Serum pyridoxal	Microbiological (<i>L. casei</i>)	Present authors	7.7 (53)	5.1 (22)	3.6 (54)

Table VI Comparison of serum pyridoxal with plasma pyridoxal phosphate measurements in control subjects

¹The number of subjects studied in each age group.

SERUM PYRIDOXAL CONCENTRATIONS IN PATIENTS WITH VARIOUS CONDITIONS

The serum pyridoxal concentrations in various conditions are plotted in relation to age in Fig. 5, where they are compared with the concentrations found in control subjects in corresponding age groups.

The majority of the patients studied had serum pyridoxal concentrations below the lower limit found in control subjects. In the 26 patients with sideroblastic anaemia 62% had subnormal concentrations and 92% had concentrations below the means found in control subjects. In the 36 patients with rheumatoid arthritis all but one that is 97%, had concentrations which were either borderline or subnormal but mainly well below the control range. In the 48 patients with untreated active Crohn's disease all but four patients that is 92%, had concentrations that were either borderline or subnormal, but mainly well below the control range. The former four patients were all older than 50 years. The younger patients, in particular those in the 20-to-29-year group, had consistently very low concentrations and all but two out of 18 in this group were below 2 ng/ml. Twelve of the 16 patients with coeliac disease were untreated and all but two of these patients that is 83%, had borderline or subnormal concentrations, but these were not as consistently low as in Crohn's disease. The four patients who had been on a gluten-free diet had concentrations well within the control range. In the pregnant women all 13 had subnormal concentrations.

Discussion

ASSAY METHOD

The present observations confirmed those of Snell (1945) that D-alanine could replace pyridoxal for growth of *L. casei* when the assay medium contained an enzymatic casein hydrolysate. However, contrary to observations by other workers, the addition of L-alanine to the medium also enhanced growth, but only in the presence of pyridoxal, the increase being in proportion to the pyridoxal concentration. As with *Streptococcus faecalis* R therefore, *L. casei* appears to require B₆ as the coenzyme for the racemase that converts L- to D-alanine, which then becomes available to the organism (Storvick and Peters, 1964). It is clear that L-alanine is an important ingredient in the assay medium, and variations in the amount of L-alanine in different batches of enzymatic casein hydrolysate were responsible for widely different growth responses even when adequate amounts of L-tryptophan had been added. No mention has been made previously of the importance of ensuring that the medium contains adequate amounts of L-alanine and the addition of this has greatly improved the

Fig. 5 Serum pyridoxal concentrations measured by *L. casei* microbiological assay in 134 patients with various conditions.

● Men ○ Women

The hatched areas represent the ranges with means for each age group found in 144 control subjects (74 males, 70 females). The plots crossed with lines in coeliac disease represent patients who have been on a gluten-free diet.

sensitivity, accuracy, and reproducibility of the method.

The method of maintenance of the organism in disc form (Stamp, 1947; Chanarin *et al*, 1958) is to be highly recommended in preference to a continuous subculturing method. It is simpler and there is less likelihood of contamination. There was no evidence that the organism was affected if maintained in disc form for up to 20 months.

Pyridoxal, whether in aqueous solution or in serum, remained stable, provided that it was protected from light, and this was a great advantage in the assay of serum B₆. It was important not to autoclave pyridoxal solutions with the assay medium, for the presence of amino acids appears to result in the transfer of amino groups to pyridoxal which converts it to pyridoxamine (Snell, 1944). This form of B₆ does not support growth of *L. casei* at levels normally found in the body. The fact that pyridoxal in serum hydroly-

sates deteriorated after storage for six weeks may be due to the contact with amino acids resulting from the hydrolysis of the serum.

The conditions of acid hydrolysis chosen for extraction of free pyridoxal from serum involved a smaller amount of serum (0.25 ml) than had been used by others and also a small volume of acid. The time of autoclaving could also be reduced to one hour, as there was no difference in the assay value when autoclaved at 15 psi over a range of one half to two hours. This is important, for the use of *L. casei* as assay organism for serum pyridoxal (Storvick and Peters, 1964) was invalidated by the production of D-alanine due to a hydrolysis process lasting longer than two hours (Haskell and Wallnöfer, 1967).

Using the present method of hydrolysis to extract serum pyridoxal, the recovery of pyridoxal added to serum was 100% and assay values were reproducible in spite of a high final dilution of 1 in 200 or 1 in 100 in the assay tube. However,

the conversion of pyridoxal phosphate standards to pyridoxal was not quite complete under these conditions and averaged 87%. Varying the conditions of hydrolysis did not improve this. It might be argued therefore that as pyridoxal phosphate is probably the main form of B₆ in serum this compound should be used in the standards instead of pyridoxal. However, the present results show that whether pyridoxal or pyridoxal phosphate is used as the standard the difference in the assay values would be very small and would mainly affect the higher serum B₆ concentrations.

NATURE OF THE *L. casei* GROWTH FACTOR IN SERUM

The present observations using Sephadex gel filtration have shown that the *L. casei* growth factor in an acid hydrolysate of serum was eluted in the same position as pyridoxal. Presumably the form of B₆ in the hydrolysate is pyridoxal, since it is the only form of B₆ which promotes growth of *L. casei* at physiological levels. However, the residual growth factor remaining after exposing the eluate to ultraviolet light may not be pyridoxal. On the other hand, a small amount of pyridoxal may have been protected from destruction by ultraviolet light, for this residual factor was slightly greater in the hydrolysate to which large amounts of pyridoxal had been added. In any case the amount was negligible and represented less than 5% of the total.

In preliminary studies in this laboratory using *Streptococcus faecium*, which measures both pyridoxal and pyridoxamine (Gregory, 1959), assay values were the same as with *L. casei* which suggests that pyridoxamine is not present in an acid hydrolysate of serum under normal conditions. These observations are consistent with those of Kelsay *et al* (1968), who could isolate only pyridoxal in acid hydrolysates of whole blood by ion exchange chromatography.

As the present assay results based on acid hydrolysis of serum are similar to those obtained by the majority of enzymatic methods of measuring pyridoxal phosphate in serum or plasma (eg, Table VI), this supports the fact that the naturally occurring form of B₆ in serum is pyridoxal phosphate and that there are no significant amounts of free pyridoxal.

CLINICAL SIGNIFICANCE OF SERUM PYRIDOXAL CONCENTRATIONS

Control subjects

The range of serum pyridoxal concentrations found in control subjects was very similar in terms of free pyridoxal to the ranges found by three other groups of workers who measured plasma pyridoxal phosphate by enzymatic methods (Wachstein *et al*, 1960; Hamfelt, 1964;

Walsh, 1966) but Hines *et al* (1969) reported considerably higher values by an enzymatic method. Baker, Frank, Ning, Gellene, Hutner and Leevy (1966) used the far less sensitive assay organism, *Tetrahymena pyriformis*, and also obtained higher values.

The present observations have shown that both age and sex affect the serum pyridoxal concentration. When equal numbers of men and women were grouped together according to age there was a marked fall in the serum pyridoxal with increasing age and the mean concentrations for corresponding age groups were similar to those reported by Hamfelt (1964). Walsh (1966) also observed a marked fall with increasing age, but even earlier Boxer *et al* (1957) had suggested this effect of age based on his observation that leucocyte pyridoxal phosphate was higher in children than in adults. Furthermore, Ranke, Tauber, Horonick, Ranke, Goodhart, and Chow (1960) found increased xanthurenic acid excretion after tryptophan loading and decreased serum GOT levels with increasing age, which disappeared when B₆ was given.

When the serum pyridoxal concentrations were analysed with regard to sex, the mean concentrations in men were higher than in women. This was most obvious between the ages of 20 and 40 years, but was not significant after the age of 60 years. It is not possible from our figures to tell whether the difference between sexes persisted until the menopause. Wachstein *et al* (1960) also observed that the mean plasma pyridoxal phosphate concentration in women was lower than that in men, though they made no mention of the age of the subjects.

The explanation for the marked difference in the levels of young men and women is uncertain, but could possibly be related to an increased requirement for B₆ in women of childbearing age. Furthermore, the possible effect of oral contraceptives has not been investigated in the present study.¹ The reason for the fall in the serum pyridoxal with increasing age is also uncertain. This has been discussed at length by Hamfelt (1964), who mentioned various possibilities, such as defective nutrition, absorption or phosphorylation of the vitamin to its active form, or increased urinary loss. Another possibility is a decrease in the transport protein to which the compound is bound. However, further studies are needed to elucidate these observations.

Patients with various conditions

Serum pyridoxal concentrations were measured in sideroblastic anaemia because some patients with this condition have been found to respond to treatment with pyridoxine. Sixteen of the 26 patients studied (62%) had subnormal levels.

Sideroblastic anaemia has also been reported in rheumatoid arthritis (MacGibbon and Mollieny, 1965), and there is other evidence of possible

¹See Addendum.

deficiency, such as abnormal tryptophan loading tests (Bett, 1962; Flinn, Price, Yess, and Brown, 1964; Pinals, 1964) and decreased excretion of B₆ compounds in the urine (McKusick, Sherwin, Jones, and Hsu, 1964). The present study provides further evidence of possible B₆ deficiency in this condition, in that 30 of the 31 patients (97%) had borderline or subnormal serum pyridoxal levels.

Crohn's disease, like rheumatoid arthritis, is an inflammatory disorder. A high incidence of subnormal serum folate levels has already been demonstrated in this condition (Hoffbrand, Stewart, Booth, and Mollin, 1968), and in the present study 44 of the 48 patients studied (92%) had borderline or subnormal pyridoxal concentrations. In particular very low levels were found in the young patients.

Sideroblastic anaemia with pyridoxine deficiency has been reported in an adult with coeliac disease, and this completely disappeared when the patient was put on a gluten-free diet (Dawson, Holdsworth, and Pitcher, 1964). Baker and Sobotka (1962) mentioned 'low circulating levels of vitamin B₆' in this condition (non-tropical sprue). The tryptophan loading test may also be abnormal in this condition (Kowlessar, Haefner, and Benson, 1964), and evidence of malabsorption of B₆ has been reported by Brain and Booth (1964) using tritiated pyridoxine. In this study, 10 of the 12 (83%) untreated patients with coeliac disease had subnormal pyridoxal levels, while four other patients who had been treated with a gluten-free diet all had normal levels.

Finally, pregnant women were studied because low plasma and leucocyte pyridoxal phosphate concentrations were found in the last trimester by Wachstein *et al* (1960), and abnormal loading tests have also been reported (Wachstein and Gudaitis, 1952; Brown, Thornton, and Price, 1961). Further, Karlin, Croizat, Revol, Pommatau, Viala, and Dumont (1968) showed abnormal pyridoxine load tests in postpartum subjects, using *S. carlsbergensis* to assay serum before and after a dose of pyridoxine. The 13 pregnant women in the present study all had subnormal levels.

The cause of the subnormal serum pyridoxal concentrations in these various conditions is not certain. An increased requirement for B₆ may be reflected in the low serum levels of the patients with rheumatoid arthritis, Crohn's disease, and in pregnancy, while malabsorption probably played a part in coeliac disease. Furthermore, an inadequate dietary intake of B₆ may have been a contributory factor in all these groups. However, the clinical significance of a subnormal serum pyridoxal concentration is still uncertain, but the fact that B₆-responsive anaemias have been associated with most of these conditions suggests that an alteration in B₆ metabolism may occasionally produce serious haematological effects.

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Addendum

Recently Brown, Rose, Price and Wolf (1969) and previously Rose (1966) showed evidence of disturbed B₆ metabolism, on the basis of the tryptophan loading test, in subjects taking oral contraceptives. We did not specifically question our female control subjects about taking oral contraceptives but in view of the above observations, this cannot be overlooked as a possible reason for the lower serum pyridoxal levels found in young women.

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