2. The tea oxidase, unlike the polyphenol oxidase of other tissues, is normally insoluble in water or buffer solution. The oxidase activity is associated with the chloroplasts of the leaf. If the chloroplasts are broken so as to release the grana it may be shown that the oxidase is associated with the grana.

3. Removal of chlorophyll from the grana by extraction with acetone leaves the enzyme activity of the preparation unaffected. Soluble oxidase may be extracted in small quantities from dried grana preparations.

4. The tea oxidase does not appear to participate in the normal leaf respiration of the tea plant.

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The Assay of the Vitamin B_6 Complex

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It is now known that pyridoxal, pyridoxamine and pyridoxal 'phosphate' (the co-enzyme of certain amino-acid decarboxylases) occur naturally and contribute to the 'vitamin B_6' effect by which pyridoxin was originally recognized. These compounds, closely chemically related to pyridoxin, give quantitatively very different responses with certain micro-organisms when these are used as the biological test object, while they have approximately equal potencies for the rat and the chick (Snell & Rannefeld, 1945; Luckey, Briggs, Elvehjem & Hart, 1945), and, presumably, for human beings. It is therefore convenient, if not essential, to have a microbiological assay method in which the test organism responds equally to all the compounds of the vitamin B_6 complex, so that assays of food-stuffs may be made which will give values proportional to their total content of vitamin B_6-active substances.

The yeast *Saccharomyces carlsbergensis* 4228, used by Atkin, Schultz & Frey (1943) for the microbiological determination of pyridoxin appears to fulfil this condition. Snell & Rannefeld (1945), using this yeast, found that the relative activities of pyridoxin, pyridoxal and pyridoxamine were 1·00 : 1·16 : 1·09, while Miller & Baumann (1945) found the activities equal within ± 5%. The method of assay with *S. carlsbergensis* has been used by us with slight modifications and tested for a variety of foodstuffs. The existence of an unknown member or members of the vitamin B_6 complex postulated by Melnick, Hochberg, Himes & Oser (1945) has not been confirmed by data presented here. The better-known chemical methods for the determination of vitamin B_6 have been tested and some criticisms are offered.

EXPERIMENTAL

Methods

The procedure of Atkin *et al.* (1943) was followed closely, only departures from it are stated here.

Culture. We are indebted to Dr Frey for a culture of *S. carlsbergensis* 4228. Another strain of *S. carlsbergensis* (Frohberg strain) kept in these laboratories was only stimulated slightly by pyridoxin.

Standards. Pyridoxin hydrochloride was kindly supplied by the Medical Research Council (a commercial sample was

found to possess only 92% of the activity of this); for pyridoxal, pyridoxamine and 4-pyridoxic acid we are greatly indebted to Mr R. T. Major of Messrs Merck and Co. Inc., Rahway, N.J., U.S.A.

Basal medium was as described by Atkin *et al.* (1943), ultimately modified by the addition of nicotinic acid in the same concentration as the calcium pantothenate (2·25 μg./ml.).

Method of assay. To a series of flat-bottomed specimen tubes, 10 × 1·5 cm. (internal), each containing 1·25 ml. of the medium, was added 0·2, 0·4, 0·5, 0·6, 0·8 or 1·0 ml. of the standard pyridoxin solution (0·01 μg./ml.) or 0·3, 0·6 or 0·9 ml. of the test extract calculated from a preliminary trial to contain an amount of vitamin B_6 falling within the range of the standard curve. The contents of each tube were then made up to 2·25 ml. with distilled water. Yeast suspension, 0·25 ml. (equivalent to 25 μg. of pressed yeast), was added with precautions to secure accuracy and equality of seedings. All tubes were set up in triplicate. After 16 hr. shaking in an incubator at 30° the tubes were placed for 10 min. in the refrigerator and the final suspension was diluted with 10 ml. of ice-cold water. All tubes were then shaken vigorously 20 times, and each inverted again 30 sec. before reading in a Pulfrich nephelometer or Spekker absorptiometer. If the extract to be tested was turbid, it was filtered before addition to the growth tube. Owing to the great dilution interference with the turbidimetric measurements by colour in the extracts was rarely encountered. In such cases the tubes were rapidly centrifuged after incubation, the clear coloured liquid poured off and an equal volume of dilute saline added, followed by shaking. This procedure was found reliable when tested on colourless suspensions.

Important precautions. Adequate, uniform and continuous shaking is essential and is facilitated by the use of small volumes of culture medium in the growth tubes. Temperature variation in different parts of the incubator, if allowed, is a serious source of discrepancy between triplicates.

The tubes employed for the growth tests were also used for reading in the nephelometer, having been tested for uniformity by the reading in them of standard suspensions of kieselguhr, a deviation between tubes of ±1% being allowed. For reading, the tubes were always turned into the same position. With prepared yeast suspensions nephelometer readings varied almost linearly with yeast concentration. Similar standardizations were performed for the Spekker instrument.

Improvement of the basal medium

Nicotinic acid in the same concentration as calcium pantothenate (2·25 μg./ml.) was found to increase growth by about 50% in a 16 hr. period (see Table 4), to a rate as rapid as in malt wort (15% malt), which is an ideal medium for brewers' yeasts. Asparagine, cystine, tryptophan, xanthine, choline, riboflavin, and *p*-aminobenzoic acid when added singly at levels much higher than would be present in the test extracts did not appreciably stimulate growth. It was later found that the addition to each tube of 10 mg. $(NH_4)_2HPO_4$ as a solution decreased the blank reading while slightly increasing the readings at the higher levels of pyridoxin.

Preparation of extracts

Materials were suitably sampled, finely ground if solid, and a portion containing *c.* 1 μg. of vitamin B_6 was autoclaved for 1 hr. at a pressure of 15 lb./sq. in. with 25 ml. of 2 N-H_2SO_4; after filtering and adjusting to pH 5·0 with NaOH, the volume was made up to 100 ml. It was found that a concentration of Na_2SO_4 equal to that present in the tubes when 0·9 ml. of the extracts was added did not influence the growth of the yeast. The use of 2 N-H_2SO_4 was recommended by Siegel, Melnick & Oser (1943) on the basis of results obtained with rice-bran concentrate. Increasing the time of autoclaving to 2 hr. had no significant influence, but, for a few materials, a 30 min. treatment as suggested by Siegel *et al.* (1943) gave slightly lower results. Similar treatment with 0·1 N-H_2SO_4, or the procedure of Bina, Thomas & Brown (1943) involving digestion with papain and takadiastase, gave for all materials listed in Table 3 vitamin B_6 values from 10 to 40% lower, with two exceptions mentioned below. A mixture of equal parts of commercial preparations of papain and takadiastase was found to have a vitamin B_6 content of about 2 μg./g. The above procedure did not give optimum results for yeast and dried milk. The values obtained for these materials by different extraction procedures are given in Table 2 and their significance discussed below.

With rare exceptions, which were rejected, the readings of the triplicate tubes agreed within ±7%. Vitamin B_6 values calculated for the different levels of added extract were within ±5%. Recovery of added pyridoxin was quantitative except with an extract of mixed invert sugar as used in a brewery, for which it was low, indicating the presence of inhibiting substances apparently produced by the autoclaving with acid. Hydrolysis with papain and takadiastase was used in this case; recovery was normal but the vitamin B_6 value was probably too low.

RESULTS

The figures in Table 1 confirm that the activities of pyridoxin, pyridoxal, and pyridoxamine for *S. carlsbergensis* 4228 are equal. Furthermore, the growth-time curves were identical with each compound. The very slight activity of 4-pyridoxic acid was possibly due to the presence of traces of one or more of the active compounds as impurity. The fact that the response of the organism to the known compounds possessing vitamin B_6 activity is the same as that of the rat is at present the best indication of the validity of the method. Comparison of results reported for similar materials by different methods is, for 'vitamin B_6', of doubtful value. Apart from inevitable variations in the materials, extraction procedures differ and the relative response to the

Table 1. *Comparative activity of pyridoxin, pyridoxal, pyridoxamine and 4-pyridoxic acid for* S. carlsbergensis 4228

Compound (0·002 and 0·004 μg./ml.)	Relative turbidity after 16 hr. growth
Pyridoxin	1·00
Pyridoxal	1·02
Pyridoxamine	0·99
4-Pyridoxic acid	0·0004

results by the *Neurospora* method, which show agreement with those obtained by the yeast method.

Yeast and dried milk gave low results when 2 N-H_2SO_4 was used for the extraction, although excellent recoveries (100 ± 7 %) of added pyridoxin, pyridoxal and pyridoxamine were secured. However, Table 2 shows that the destructive effect of 2 N-acid was not observed when the sample was first subjected to milder hydrolytic procedures. This is in

Table 2. *Apparent vitamin B_6 content of yeast and dried milk measured microbiologically after various extraction procedures*

Extraction procedure: Autoclaving with H_2SO_4 at 120° — Concentration of acid	Time (hr.)	Alternative to autoclaving	Pyridoxin (μg./g.): Pressed bakers' yeast (moisture 75 %)	Food yeast (moisture <10 %)	Dried milk
0·1 N	1	—	—	20·0	—
0·1 N	2	—	6·0	24·5	4·6
0·1 N	3	—	—	26·0	—
0·1 N	5	—	—	28·6	—
2·0 N	1	—	3·4	11·4	—
2·0 N	2	—	—	12·2	3·6
0·1 N, 2·0 N	2 followed by 1	—	6·2	26·5	5·5
2·0 N, 0·1 N	1 followed by 1	—	4·9	—	4·1
None	—	Papain and takadiastase digestion at 40° for 24 hr.	—	26·7	—
None, 2·0 N	— followed by 1	Do.	—	26·3	—

Table 3. *The vitamin B_6 content of various products measured by the* S. carlsbergensis *assay*

Product	Vitamin B_6 content expressed as pyridoxin (μg./g.)
Flour (85 % extraction)	1·7
Dried milk	5·5
Pressed bakers' yeast	6·2
Brewers' yeast	5·7
Food yeast	28·6
Shropshire barley (13·9 % moisture)	3·2
Shropshire barley after malting (4·1 % moisture)	6·0
Malt rootlets	10·2
Mixed invert sugar from a brewery (determined after enzyme extraction)	0·14
Rose hips	1·7
	(μg./ml.)
Whitbread's ale	0·52
Whitbread's stout	0·67

components of the complex may vary between one method and another. Comparable results with identical samples have been obtained on several occasions, and a few of these are given in Tables 5 and 6.

The results in Table 5 were obtained with two samples of beer which had not been pretreated with acid. Free pyridoxin only therefore was measured. We are indebted to Dr E. Barton-Wright for the

accordance with the fact that treatment with 2 N-H_2SO_4 for 2 hr. gives values as high as, or slightly higher than, treatment for 1 hr. On the other hand, if treatment with 2 N-acid precedes the treatment with 0·1 N-acid, results are again low. These observations seem to suggest that the vitamin is destroyed more readily by 2 N-H_2SO_4 when it is in a 'bound' state. The fact that these results were obtained with yeast, which contains mainly pyridoxamine (Snell, 1945), may mean that this component is more susceptible to destruction in this way. These results also show that 100 % recoveries are not infallible criteria of the reliability of an extraction procedure.

Melnick *et al.* (1945) also obtained low values for the vitamin B_6 content of yeast after treatment with 2 N-H_2SO_4 and ascribed this to the destruction of an unknown member or members of the vitamin B_6 group. However, in view of the above results it is unnecessary to postulate their existence.

The possibility was considered that other compounds may be present in natural materials to which the organism may adapt itself in time, so as to utilize them as vitamin B_6 when suboptimal amounts of the vitamin are present. However, when the incubation time was increased from 16 to 40 hr. the results obtained with yeast, beer and dried milk were not significantly different.

Few investigations of the stability of vitamin B_6 in foodstuffs have been reported. We found no significant change in samples of beer and dried yeast during 4 months' storage in the dark at room temperature. A standard solution of vitamin B_6 (100 μg./ml.) in N/50 HCl stored in the refrigerator was unchanged in potency after 8 months.

Use of the organism for other assays

S. carlsbergensis 4228 has been used also for assaying pantothenic acid. We investigated its dependence upon four other vitamins, both in the medium used above and in one in which the casein hydrolysate was replaced by $(NH_4)_2HPO_4$ (4 mg./ml.). The results in Table 4 indicate that it may also be suitable for the assay of biotin and of inositol.

Table 4. *Response of* S. carlsbergensis 4228 *to six growth factors*

One growth factor at a time was omitted from the group of six named in column 1.

	Nitrogen source	
	Casein hydrolysate	$(NH_4)_2HPO_4$
Growth factors in medium	Yield of yeast, dry weight: mg./tube (2·5 ml.)	
All six	3·88	1·28
Inositol omitted	0·22	0·24
Pantothenic acid omitted	0·11	0·25
Nicotinic acid omitted	2·35	0·83
Biotin omitted	1·00*	0·07
Aneurin omitted	1·65	0·82
Pyridoxin omitted	0·25	0·12

* High result probably due to the presence of biotin in the casein hydrolysate.

Table 5. *Comparison of values obtained by means of methods involving the use of* S. carlsbergensis 4228 *and of* Neurospora

	Pyridoxin (μg./ml.)	
	Yeast method	*Neurospora* method
Beer, draught	0·24	0·26
„	0·25	0·28

Chemical determinations

A number of procedures described in the literature for the colorimetric method involving coupling with diazotized sulphanilic acid was found to be unsatisfactory. We found that, in addition to the variety of compounds known to couple with this reagent, biotin gives a yellow colour, which is quantitative. Bina *et al.* (1943) used alkaline ethanol elution from a 'Superfiltrol'* adsorbate to make the test specific for pyridoxin. However, we found with their procedure upon evaporating off the ethanol and carrying out the colorimetric test in aqueous solution that reddish brown interfering colours were present; the chromogenic impurities were not precipitated by aqueous $AgNO_3$ or $Ba(OH)_2$. The ethanol appears not to act altogether as a specific eluent but merely to suppress the development of interfering colours. Consequently the procedure of Bottomley (1944), involving removal of the ethanol and reaction in aqueous solution, is open to criticism. Furthermore, the method of Bina *et al.* (1943) failed to give consistent results even under fully standardized conditions with pure solutions of pyridoxin, the colour developed varying from yellow to pink. Appreciable blank readings were recorded, apparently not corrected for by these workers. Values obtained on worts and yeasts were abnormally high. The use of *p*-aminoacetophenone, α-naphthylamine, or *p*-chloraniline instead of sulphanilic acid was no more satisfactory; *p*-aminobenzoic acid was found to give the most consistent results, but only in aqueous solution. The use of a borate blank (see below) was possible only in aqueous solution.

The indophenol method adapted by Hochberg, Melnick & Oser (1944) by the use of a borate blank to measure only pyridoxin was satisfactorily applied to many materials, recovery of added pyridoxin being 90–100%. Modifications used by us were: (*a*) preliminary purification of the extract by addition of 2 ml. 15% sodium tungstate followed by 1 ml. H_2SO_4 (50% (v/v)); this reduced the blank readings, which were very high for cereal products, by about 25%; (*b*) the use of 'Superfiltrol' (at pH 3) instead of Lloyd's reagent for adsorption; (*c*) the reading of the colour developed 2 min. after mixing, when the intensity was a maximum, in the Pulfrich photometer with filter S 57.

Table 6. *Comparison of values obtained for identical samples by the chemical, indophenol, method and microbiologically*

	Pyridoxin (μg./ml.)	
	Indophenol method	Yeast method
Beer, draught bitter	0·34	0·60
Beer, draught mild A	0·37	0·51
Beer, draught mild B	0·44	0·48
Beer, draught mild C	0·32	0·51
Stout	0·29	0·87
	(μg./g.)	
Brewery mixed grist (malted barley)	5·0	5·5

The method was originally developed to respond only to pyridoxin but was later (Melnick *et al.* 1945) shown to respond to a lesser extent to pyridoxal and pyridoxamine. Consequently the results obtained by this method have only a rough significance and the majority is not reported here. However, a few are shown in Table 6 with values obtained with the

* A proprietary adsorbent manufactured by the Permutit Co. Ltd.

same samples by the yeast method. As expected, the chemical values are lower and may give a rough indication of the proportion of the total vitamin B_6 present as pyridoxin.

SUMMARY

1. The microbiological method for the determination of vitamin B_6 with *Saccharomyces carlsbergensis* (Atkin *et al.* 1943) was satisfactorily applied, with slight modifications, to a number of foodstuffs.

2. Two chemical methods were also used and criticisms of them are presented.

3. Data obtained upon the effectiveness of different methods of extraction of vitamin B_6 from natural materials can be explained without assuming the existence of unknown compounds possessing vitamin activity.

4. Indications were obtained that the particular strain of *S. carlsbergensis* used might also be used for the estimation of biotin and inositol.

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Intercellular Hormones

6. RELEASE AND SYNTHESIS OF FACTORS OF THE VITAMIN B COMPLEX BY DAMAGED LIVING CELLS

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When cells are damaged under conditions which leave them grossly intact, proliferation-promoting factors ('wound hormones') are released into the intercellular suspension medium (see Cook & Cronin, 1941; Loofbourow, 1942*b* for earlier references). The mechanism of the effect appears to involve (*a*) increase in cell-membrane permeability, (*b*) diffusion of non-protein substances through the more permeable membranes, and (*c*) partial replacement within the injured cells of some of the substances lost through diffusion (Loofbourow, 1942*b*).

It has been suggested that the substances responsible for the proliferation-promoting activity might be amino-acids or vitamin B complex factors (Davidson, 1940), purines or pyrimidines (Loofbourow, Cook & Stimson, 1938) or combinations of nucleotides with vitamin B complex factors (Loofbourow, 1942*c*). Duplication of the proliferation-promoting effect of damaged-cell products by combinations of B complex factors, amino-acids, and adenine nucleotides has afforded presumptive evidence that all of these substances are involved in the effect (Loofbourow, 1942*b*).

To determine the role of any substance in the 'wound hormone' phenomenon it is desirable to establish the presence and relative concentration of the substance in products from damaged cells, and then to demonstrate its proliferation-promoting activity under like conditions of concentration and combination with other substances. The present paper presents quantitative assays of damaged-cell and undamaged-cell preparations with regard to factors of the vitamin B complex, together with growth tests employing B complex factors in comparable concentrations. Subsequent communications will deal with the role of amino-acids, nucleotides and nucleosides in the 'wound hormone' phenomenon.

METHODS

Preparation of products from damaged and undamaged cells. After thrice washing, by emulsification in volumes of 400 ml. of water and centrifugation, 400 g. wet weight (about 112 g. dry weight) of Fleischmann starch-free baker's yeast was suspended in distilled water to make a total volume of 4 l. Equal 2 l. amounts were placed in Pyrex glass containers. The two suspensions were stirred constantly by motor-