Observations on the Assay of Vitamin B₆ with *Saccharomyces carlsbergensis* 4228

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SUMMARY: The strain of *Saccharomyces carlsbergensis* 4228 studied differed from those previously used for the assay of vitamin B₆ in that it required this vitamin for rapid growth in the absence of added thiamine. The rate, rather than the ultimate extent, of growth of the organism is proportional to the concentration of vitamin B₆, and the size of the inoculum and its physiological age, the duration and temperature of incubation and the degree of aeration, must be strictly controlled. Non-phosphorylated forms of the vitamin only are utilized, and natural materials are acid-extracted before assay. The organism responds a little less well to pyridoxamine than it does to pyridoxal and pyridoxine. A linear response curve is obtained over a range of pyridoxine concentrations (0.2 to 2×10^{-8} M) when incubation is in static culture at 37° (24 hr.) in a basal medium slightly modified from those previously used.

Although several accounts (Hopkins & Pennington, 1947; Snell, 1950; Fournier, Thomas & Seigel, 1951; Parrish, Loy & Kline, 1955, 1956) of the use of *Saccharomyces carlsbergensis* 4228 for the assay of the three 'free' forms of vitamin B₆ have appeared since the original observations of Atkin, Schultz, Williams & Frey (1943), the present authors encountered some initial difficulties with the method. In the first instance, of three strains of *S. carlsbergensis* 4228 obtained from various sources, no two had the same nutritional requirements. The strain eventually selected required vitamin B₆ for rapid growth under all conditions of culture tested, so differing from previously used strains of the organism (Rabinowitz & Snell, 1951).

Atkin *et al.* (1943) described a semi-defined medium of pH 5 containing thiamine, upon which their strain of *Saccharomyces carlsbergensis* 4228 grew only slowly in the absence of vitamin B₆; the requirement for vitamin B₆ was later found by Rabinowitz & Snell (1951) to be induced by the presence of thiamine. When incubated in shaken culture at 30° for 18 hr., excellent growth proportional to vitamin B₆ concentrations below 2×10^{-8} M was obtained. Growth of the organism was still proceeding rapidly at the end of incubation, i.e. the rate of growth and not its final extent was dependent upon the vitamin B₆ concentration. The need to ensure that growth did not continue while its extent was being measured was subsequently often emphasized, and various devices, e.g. steaming, refrigeration, addition of chlorothymol, recommended. Inactivity of the phosphorylated derivatives of vitamin B₆ necessitated hydrolytic extraction of natural materials before assay (Atkin *et al.* 1943). Disparity between the activities of the free forms of the vitamin in supporting the growth of various strains of the organism has been described, pyridoxal and pyridoxine usually being equally effective, with pyridoxamine

sometimes showing 10–30 % less activity (Snell, 1950). It was suggested that supplementation of the basal medium with DL-tryptophan (0.1 mg./ml.) would eradicate this discrepancy (Jones & Morris, 1950). Further addition to the medium of nicotinic acid (2.25 µg./ml.) accelerated growth of the organism in the presence of excess pyridoxine (Hopkins & Pennington, 1947).

Uniform and continuous agitation has commonly been reported to be essential, though it has been stated (without further details) that the assay can be carried out without shaking with 'only a slight decrease in the uniformity of the results' (Snell, 1950), the standing culture tubes being sloped to maintain good aerobic conditions.

Irregularities encountered during the attempted assay of vitamin B₆ according to previously recommended procedures necessitated the detailed study of the response of the selected strain of *Saccharomyces carlsbergensis* 4228 to vitamin B₆; this is the subject of the present paper. The modifications which have been adopted, though apparently slight, have enabled this strain to be used with complete satisfaction in a routine sensitive method for the determination of vitamin B₆ in extracts of various micro-organisms and culture media.

METHODS

Organism. Three cultures all reputed to have been derived from the same parent strain were obtained from different sources. All three were found to possess different nutritional properties. The strain finally selected for use and designated *Saccharomyces carlsbergensis* 4228c is no. 534 of the National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Surrey, England. This was chosen as it was dependent upon vitamin B₆ for rapid growth under all conditions of culture tested. The organism was maintained on slopes of medium M stored at 2° after 24 hr. growth at 30°. Stock cultures were subcultured monthly.

Media. Medium M contained (g./l. final vol.), malt extract ('John Bull Brand', Paine and Co. Ltd., St Neots, Hunts), 22.5; ammonium tartrate, 5; yeast extract ('Difco'), 5; sucrose, 5; agar, 20; NH₄NO₃, 1; MgSO₄·7H₂O, 0.5; NaCl, 0.1; CaCl₂, 0.05; Fe²⁺ (final conc. 2×10^{-5} M) from a solution of FeSO₄(NH₄)₂SO₄·6H₂O in twice its molar concentration of sodium citrate. The pH value was brought to 5.5 with N-NaOH. Medium A ultimately adopted for assays differed from that of Atkin *et al.* (1943) in that it contained nicotinic acid (2.5 mg./l.), and that the amount of acid-hydrolysed casein (vitamin-free, prepared by the method of Snell & Rannefeld, 1945) was decreased to the equivalent of 2 g. casein/l. The pH value was 5–5.2.

All media were prepared at double strength and brought to the desired volume with water and other experimental additions before autoclaving for 7 min. at 115° (unless otherwise stated).

Routine growth tests

(a) *Static culture.* Medium (2 ml.) in 150 × 19 mm. hard glass tubes was held in wire baskets sloped at 5° to the horizontal (unless otherwise stated).

(b) *Shaken culture.* Medium (5 ml.) was contained in optically matched $150 \times 150 \times 16$ mm. L-shaped tubes (Monod, Cohen-Bazire & Cohn, 1951) clamped to a rocking device (similar to that of van Heyningen & Gladstone, 1953) and shaken in a thermostatically controlled water bath at 36 oscillations/min., of excursion 10 cm.

Assessment of growth. The extent of growth was measured with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex, England) with a neutral density filter and 6 mm. sample tubes; the uninoculated medium was used to give the zero setting. The relation between 'EEL reading' and dry weight was determined and was found to be linear up to a reading of 35–40.

RESULTS

Growth characteristics of Saccharomyces carlsbergensis 4228c

Growth in static culture. In a preliminary investigation to see whether it would be possible to obtain reproducible growth in static culture, 2 ml. volumes of the basal medium of Atkin *et al.* (1943) were supplemented with pyridoxine (0 – 10^{-8} M), inoculated with a light suspension of the organism, and incubated at 30° for an arbitrary time of 40 hr. Excellent agreement between duplicate tubes was obtained (Table 1). This was despite the fact that the organisms settled out completely during growth.

Table 1. *Comparison between growth of Saccharomyces carlsbergensis in static and shaken culture*

Basal medium of Atkin *et al.* (1943) with pyridoxine as shown, was incubated at 30° for the time stated.

Pyridoxine conc. (M)	Growth (EEL reading)			
	Static 40 hr.		Shaken 16 hr.	
	0	0	0	0
Nil	—	—	6.0	6.5
1×10^{-9}	4.5	4.5	12.0	12.0
2×10^{-9}	—	—	18.0	17.5
3×10^{-9}	9.0	9.0	28.0	28.0
5×10^{-9}	—	—	36.5	38.0
7×10^{-9}	14.0	14.5	—	—
1×10^{-8}	20.5	19.5	—	—
1.5×10^{-8}	25.0	26.0	—	—
2×10^{-8}	—	—	—	—

Time of incubation. Photometric determinations of growth can be made so rapidly that no special precautions were necessary to prevent the continuance of growth outside the incubator as long as immediate readings were made. The extent of growth obtained with any concentration of the vitamin (below 10^{-8} M) increased with increasing time of incubation (Fig. 1). Measurable growth in the absence of added vitamin B₆ was only obtained by long incubation (100 hr.). To obtain reproducible standard dose-response curves, strict adherence to a selected time of incubation was essential. Even so, it was found necessary to construct a new standard curve in the course of each assay.

Degree of aeration. Good aeration is essential for the rapid growth of this yeast (Atkin *et al.* 1943; Snell, 1950) and most rapid growth was only achieved by constant agitation of the culture (Table 1). If the increased rate of growth under these conditions is a function of the increased aeration and was not due to the dispersal of the organisms then it would be expected that relatively small changes in the degree of aeration might be reflected in the growth response curve. This was found to be the case (Fig. 2) when such changes were produced by alteration of the surface area:volume ratio in static culture. An identical degree of sloping the culture tubes on each occasion of assay was ensured by using standard wire baskets sloped at 5° to the horizontal.

Fig. 1

Fig. 2

Fig. 1. Effect of increasing times of incubation on growth response of *Saccharomyces carlsbergensis* 4228c to pyridoxine. Basal medium of Atkin *et al.* (1943) supplemented with pyridoxine as shown, was incubated in static culture at 30° for the times indicated.

Fig. 2. Effect of increasing aeration on growth response of *Saccharomyces carlsbergensis* 4228c to pyridoxine. Basal medium A (2 ml.) with pyridoxine as shown, incubated for 40 hr. at 30° in variously sloped tubes or 25 ml. conical flasks. Vertical tubes (●), 5° slope (⊙) increased slope giving 20% increased surface area (○), flasks giving greatest surface area/vol. ratio (×).

Temperature of incubation. Though 25° has been suggested as the optimal temperature for growth of *Saccharomyces carlsbergensis* (Parrish *et al.* 1955) strain 4228c grows much more rapidly at 36–37° than at 30°, and incubation for 24 hr. at the higher temperature gave a steeper and more linear response over the range of pyridoxine concentrations used (Fig. 3a). Further work was carried out at 36–37°, though relatively small changes in incubation time now had a marked effect on the slope of the standard curve (Fig. 3b).

Maintenance of a uniform incubation temperature was particularly important when the assay was carried out at 37°, for temperatures only slightly higher (0.5–1°) than this were inhibitory. Although for convenience designated as 37°, the results here reported were obtained in an incubation room whose temperature fluctuated between 36.4° and 37°.

Preparation of the inoculum. To minimize transference of vitamin B₆ from medium M, a small inoculum was used. The organisms were taken from 24 hr.

30° slope culture on medium M; their behaviour did not differ during subsequent incubation in medium A from those harvested after 24 hr. growth at 37° on medium A supplemented with 10^{-7} M pyridoxine. To diminish carry-over still further, the harvested organisms were washed by centrifugation from 0.2 % sterile saline before suspension in fresh saline to a density equivalent to *c.* 4 μ g. dry wt./ml. The washed suspension (0.1 ml.) was used to inoculate the volumes (1.9 ml.) of basal medium plus supplements. No growth was

Fig. 3. Growth response of *Saccharomyces carlsbergensis* 4228c to pyridoxine after incubation for (a) 24 hr. at various temperatures, and (b) various times at 37°. Tested in basal medium A in static culture.

obtained in the absence of added vitamin B₆ under the conditions of incubation used. Variation in inoculum size caused a variation in the bulk of growth attained in the standard incubation period with a particular concentration of pyridoxine.

Modification of the basal medium. Better growth (20 %) of *Saccharomyces carlsbergensis* 4228c was obtained when the concentration of casein hydrolysate in the basal medium was made less than that recommended by Atkin *et al.* (1943), and an amount equivalent to 2 g. casein/l. medium was used. Nicotinic acid slightly increased growth (Fig. 4) and was added to the basal medium in relatively high concentration (2×10^{-5} M). As no growth was obtained (24 hr.) in the absence of vitamin B₆, (NH₄)₂HPO₄ previously advised to decrease growth in the blanks (Hopkins & Pennington, 1947), was not used.

Effect of thiamine. Rabinowitz & Snell (1951) reported that the growth of their strain of *Saccharomyces carlsbergensis* 4228 was not dependent upon added vitamin B₆ in the absence of added thiamine. In very small concentrations thiamine caused a growth inhibition which was overcome non-competitively but in a strictly quantitative fashion by pyridoxine. When their inoculum was grown in a thiamine-rich medium, sufficient was carried over endogenously to necessitate adding vitamin B₆ if growth were to be obtained subsequently in a thiamine-free medium. Their organism could

therefore only be used for the assay of vitamin B₆ in the presence of an excess of thiamine. The strain used in the present work showed an entirely different thiamine-vitamin B₆ relationship.

In the absence of both vitamins, or of vitamin B₆ only, no growth of our organism was obtained in 24 hr. incubation at 37° (Fig. 4). In the absence of added thiamine, ten times as much pyridoxine was required to support maximal growth as when a basal medium was used containing the concentration of thiamine (7.5×10^{-7} M) recommended by Atkin *et al.* (1943). Increasing the concentration much above this value had little stimulatory effect

Fig. 4

Fig. 5

Fig. 4. Effect of nicotinic acid and thiamine on the growth response of *Saccharomyces carlsbergensis* 4228c to pyridoxine. Medium A without nicotinic acid (---), with nicotinic acid, 2×10^{-5} M (—), without thiamine (○), with 7.5×10^{-7} M-thiamine (●), with 5×10^{-6} M-thiamine (●).

Fig. 5. Response of *Saccharomyces carlsbergensis* 4228c to various forms of vitamin B₆. Medium A supplemented with Seitz-filtered solutions of pyridoxine (●), pyridoxal (○), pyridoxamine (□), and pyridoxal-5'-phosphate (×). Incubated in static culture for 24 hr. at 37°.

(Fig. 4) so that the presence of even considerable concentrations of thiamine in natural extracts will not interfere with the assay of vitamin B₆, when using our strain 4228c. Similar results were obtained when this strain was incubated at 30° as in the experiments of Rabinowitz & Snell (1951).

Response to various forms of vitamin B₆. Pyridoxal was just as effective as pyridoxine in promoting growth of strain 4228c on medium A and the activity of both forms was independent of the pH value of the basal medium within the range pH 3.5 to 5.8. Pyridoxamine appeared to be somewhat less active, but it is uncertain to what extent this was due to partial decomposition of the available sample of pyridoxamine (Fig. 5 and Table 2). Addition of DL-tryptophan (0.1 mg./ml.) had no effect on the response of the organism to pyridoxamine alone, or to an extract of natural material (*Escherichia coli*) known from bioautography to contain pyridoxamine.

Method used for the routine assay of vitamin B₆

Extraction. Hydrolysis of samples of pyridoxal phosphate and pyridoxamine phosphate was complete after autoclaving in 0.055N-H₂SO₄ at 126° for 2 hr. With all samples of bacteria and growth media used, maximal extraction was always achieved by 2–3 hr. autoclaving.

Table 2. *Growth response of Saccharomyces carlsbergensis 4228c to various forms of vitamin B₆*

Activity expressed as percentage of growth achieved in medium A with equimolar pyridoxine. Incubation 24 hr. at 37° in static culture.

Form of vitamin B ₆	Activity
Pyridoxal	100
Pyridoxamine	80 to 100
Pyridoxal phosphate	2
Pyridoxamine phosphate	< 0.4

The material containing usually 1–10 μ mole vitamin B₆ was diluted to 40 ml. in a 175 × 32 mm. glass tube, acidified with 5 ml. of 0.5N-H₂SO₄ and autoclaved for 3 hr. at 126°. Alternatively, 10 ml. samples + 1.25 ml. 0.5N-H₂SO₄ were autoclaved in 150 × 19 mm. tubes with no loss of efficiency. After extraction, a sample was brought to pH 5 with a measured volume of N-NaOH (bromocresol green as external indicator), clarified by centrifugation and usually assayed immediately. When stored at 2° for any length of time before assay, the extracts were kept in the dark.

When using heavily buffered samples (e.g. of growth media) it was necessary to add more acid to bring the pH value to the required range of 1.6–2.0 (Rubin, Scheiner & Hirschberg, 1947). With most of the culture media tested, 40 ml. samples required the addition of approximately 8 ml. of 0.5N-H₂SO₄.

Assay procedure. The samples or appropriate dilutions, at four different concentrations within the assay range, were added in 0.1–0.8 ml. quantities to 150 × 19 mm. tubes containing 1 ml. double strength medium A. Standards containing 5, 10, 20, 30, 40 and 50 μ mole pyridoxine were included in each complete assay. After dilution to 1.9 ml. and autoclaving at 10 lb. sq.in. for 7 min., the tubes were inoculated with 0.1 ml. of a suspension containing equiv. c. 4 μ g. dry wt./ml. of *Saccharomyces carlsbergensis* 4228c. This was prepared by suspending organisms harvested from a 24 hr. culture on medium M in sterile 0.2% saline, centrifuging and resuspending in fresh saline to the required density. Incubation was for 24 hr. at 36–37° with the tubes sloped at 5° to the horizontal. Growth was assessed immediately on removal of the cultures from the incubator.

The useful range of the assay was from 5–40 μ mole pyridoxine corresponding to growth equiv. 0.2–2.0 mg. dry wt. yeast/ml. All tubes were set up in duplicate and exposed as little as possible to direct illumination.

Validity of assay of bacterial extracts. Extracts of various bacterial cultures might interfere with the assay of vitamin B₆ by stimulating or inhibiting

growth of *Saccharomyces carlsbergensis* 4228c in vitamin B₆-supplemented medium A. It was found with all extracts tested that: (a) the dose-response curves relating concentration of extract and resultant growth of the yeast were superimposable upon the standard curve obtained with pyridoxine; (b) the response curves obtained when a known concentration of pyridoxine was added to various concentrations of extract, and conversely, when a small

Fig. 6. Assay of vitamin B₆ content of extract of *Escherichia coli* B166. Growth of *Saccharomyces carlsbergensis* 4228c measured after 24 hr. static incubation at 37° in medium A with amounts of pyridoxine (●), extract (×), extract + 3×10^{-9} M pyridoxine (Δ), and pyridoxine + 0.2 ml. extract (○).

amount of extract was added to each of the standard tubes containing known concentrations of pyridoxine, both lay parallel to the standard curve; (c) pyridoxine added to the extract was quantitatively recovered (see below). Typical results obtained with an extract of an *Escherichia coli* mutant are given in Fig. 6. It is recommended that such control experiments be carried out upon each new material to be assayed.

Accuracy. The recovery of pyridoxine added to bacterial extracts was $100\% \pm 10\%$. When results obtained by this method, but in another laboratory, were subjected to statistical analysis, the s.d. was found to be 8.2% (Dr Joan F. Powell, Porton; personal communication).

DISCUSSION

It is recommended that the growth requirements of any strain of *Saccharomyces carlsbergensis* 4228 be well studied before it is used as an assay organism, for various strains differ markedly from each other, particularly in their response to thiamine and vitamin B₆. Thiamine must be present in excess when vitamin B₆ is assayed with the strain used by Rabinowitz & Snell (1951) or with that (4228c) described above, but for quite different reasons. With the first strain thiamine produces the requirement for vitamin B₆, with the second, thiamine diminishes the amount of pyridoxine needed for any given rate of growth.

As emphasized throughout, the fact that the method is a 'rate assay' exaggerates the need for strict adherence to the chosen test conditions, but it has the useful corollary that within limits the sensitivity of the assay can be adjusted by varying the time of incubation. Thus by extending the incubation period beyond 24 hr. at 37°, it is possible to assay with accuracy over a range of pyridoxine concentrations lower than 5×10^{-9} M. Growth in the complete absence of pyridoxine, i.e. in the 'blank', becomes progressively greater with these longer times of incubation.

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