- Myrbäck, K. (1949). *Ergebn. Enzymforsch.* **10**, 168.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Pollock, G. E. & Holmstrom, C. D. (1951). *Cereal Chem.* **28**, 498.
 Stein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
 Spiegelman, S. & Nozawa, M. (1945). *Arch. Biochem.* **6**, 303.
 Spiegelman, S. & Reiner, J. M. (1947). *J. gen. Physiol.* **31**, 175.
 Trevelyan, W. E., Gammon, J. N., Wiggins, E. H. & Harrison, J. S. (1952). *Biochem. J.* **50**, 303.
 Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
 Trevelyan, W. E. & Harrison, J. S. (1954*a*). *Biochem. J.* **57**, 556.
 Trevelyan, W. E. & Harrison, J. S. (1954*b*). *Biochem. J.* **57**, 561.
 Trevelyan, W. E., Mann, P. F. E. & Harrison, J. S. (1954). *Arch. Biochem. Biophys.* **50**, 81.
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
 Yemm, E. W. & Folkes, B. F. (1954). *Biochem. J.* **57**, 495.

Studies on Yeast Metabolism

6. EFFECTS OF THIAMINE ON NITROGEN ASSIMILATION DURING FERMENTATION, AND ON FERMENTATION KINETICS

By W. E. TREVELYAN AND J. S. HARRISON

Research and Development Department, The Distillers Co. Ltd., Epsom, Surrey

(Received 10 March 1955)

Trevelyan & Harrison (1954*a, b*) showed that the addition of thiamine brought about a rapid lowering in the steady-state concentration of pyruvate in the yeast cell during fermentation, accompanied by only a slight immediate increase in fermentation rate. They suggested that carboxylase could therefore be considered to be one of the factors limiting fermentation rate only when thiamine was omitted from the medium, and that some other enzyme system or systems limited the fermentation rate of thiamine-saturated yeast.

Thiamine, in an appropriate medium, is known to promote an increase in fermentation rate which continues for several hours. This long-term action of thiamine, which was discovered by Atkin, Schultz & Frey (see their review in Anderson, 1946), is considered in the present paper in relation to the short-term effect on pyruvate levels mentioned above.

While this work was in progress, a paper by Maesen (1953) appeared in which it was reported that the long-term increase in fermentation rate mediated by thiamine was accompanied by a proportionate increase in the carboxylase activity of the yeast cells (determined after disruption by freezing and thawing). Rate increases were observed only when assimilable nitrogen (ammonium sulphate) was supplied to the yeast, but the rate of its incorporation into yeast protein was unaffected by thiamine. Thiamine thus appeared to have a specific effect on carboxylase synthesis, and this enzyme system was, Maesen concluded, the limiting factor of fermentation under his experimental conditions.

METHODS

Fermentation technique

Yeast was taken as required from a master sample of D.C.L. pressed baker's yeast stored in the refrigerator over a period of not more than 3 weeks. When necessary, the various master samples are distinguished by the letters A, B etc.

Full medium M1 contained sodium succinate buffer, pH 5.5 (0.05 M succinic acid partly neutralized with 75 mM-NaOH), 0.01 M-KH₂PO₄, 0.01 M-(NH₄)₂SO₄, 0.02 M DL-asparagine, 2.5 mM-MgSO₄, mM-ZnSO₄, mM-CaCl₂ and 0.1 μmole thiamine/g. yeast. Medium M2 contained sodium succinate buffer, ammonium sulphate and asparagine in identical concentration: the other constituents were 5 mM-MgSO₄, 2 mM-KH₂PO₄, 8 mM-KCl and 0.2 μmole thiamine/g. yeast. Essentially similar rates were obtained during fermentation for 2 hr. with the two media. Thiamine-free medium, N-free medium, P-free medium contained all constituents except thiamine, ammonium sulphate and asparagine, or phosphate, respectively. Asparagine-free medium had 25 mM-(NH₄)₂SO₄ as sole nitrogen source.

Fermentation was carried out at 30°, the suspension being stirred by bubbling N₂ through at about 150 ml./min. Variation of the gas flow between 100 and 200 ml./min. had no effect on the fermentation rate. Unless stated otherwise, the fermentable sugar was 0.3 M glucose. Usually the final volume of 100 ml. contained 2.5 g. (fresh wt.) of yeast, which had a dry-matter content of 27%. In previous studies of the short-term effect of thiamine, double this concentration of yeast was used (Trevelyan & Harrison, 1954*b*). Although the fermentation rate/g. of yeast was the same during the first 40 min. of fermentation, if 2.5 g. or 5 g. of yeast were used, it was found that a steady increase in rate after this time was observed only at the lower level of yeast concentration.

Fermentation rate, as an average over 10 or (normally) 20 min. intervals, was determined after absorption of CO₂ in alkali (Trevelyan & Harrison, 1954*a*).

Analytical methods

Results of estimations of yeast constituents are stated as $\mu\text{moles/g. original wt. of yeast}$, measuring changes in the whole culture, not in the individual cell.

Phosphate uptake. This was followed by the Gomori method (Gomori, 1942) applied to the supernatant after centrifuging off the cells. Preliminary experiments established that no phosphorus could be detected in the medium after exhaustion of orthophosphate.

Ammonia uptake. Diffusion into boric acid, using the Conway microdiffusion unit (Conway, 1947), was employed.

Cell counts. Triplicate counts, each of some 500 cells, were made with a haemocytometer.

Nucleic acid. Nucleic acids were determined in the cells, after three 20 min. extractions with chilled trichloroacetic acid (TCA), by the technique of Schneider (1945). This has been previously applied to yeast (DiCarlo & Schultz, 1948), though some doubt has been cast on the suitability of the method (Bourdet, Mandel & Guillemet, 1951).

Metaphosphate. 'Acid-insoluble' metaphosphate was calculated from the acid-labile phosphorus in the Schneider extract, as described by Wiame (1949).

Acid-soluble ribose. This was expected to give a rough idea of the level of coenzymes in the cell and was determined in the TCA extract of yeast (washed free from medium), using the method of Mejbaum (1939). Trehalose interfered, but after fermentation for 40 min. in N-containing media the trehalose level was low enough (Trevelyan & Harrison, 1956) for the ribose estimation to give reliable values.

Acid-insoluble nitrogen (AIN). After extraction by cold TCA the suspension, containing 50 mg. yeast, was centrifuged, and the cells were washed with water and transferred, using the minimum amount of water, to a Pyrex tube for Kjeldahl digestion according to the directions of Koch & McMeekin (1924).

Intracellular pyruvate. This was estimated as previously described (Trevelyan & Harrison, 1954a).

Intracellular amino acids. Yeast cells were centrifuged, washed twice by centrifuging with ice-cold water, and the drained cells were extracted by shaking for 30 min. at room temperature with 1 ml. of m TCA/g. of yeast. Paper chromatography of the supernatant was carried out by using the solvent and ninhydrin reagent described by Toennies & Kolb (1951); other solvents were butanol-acetic acid-water (100:30:20, v/v, top layer), and pyridine-butanol-water (100:150:65, v/v). As the amount of water remaining in the packed cells after centrifuging was unknown, spot intensities were compared with a standard prepared by extracting a duplicate sample of yeast with TCA to which had been added 200 μmoles of alanine. The comparison was made after cutting out spots from the paper and dissolving the coloured material in 75% (v/v) acetone, a solvent proposed by Naftalin (1948). Optical density at 600 $m\mu$. of the solutions was then measured.

RESULTS

Effect on fermentation rate of constituents of the medium

The first 40 min. of glucose fermentation by D.C.L. yeast may be considered as a lag period. In all media examined, fermentation rate rises during

this period. Yeast which has been adapted to the medium by a preliminary fermentation and which has been centrifuged and washed will begin fermentation when transferred to fresh medium without an induction period. After this time, fermentation rate may steadily increase, remain constant, or decline, according to the composition of the medium.

A steady increase in rate requires a source of assimilable nitrogen (Maesen, 1953). The necessity for adding to the medium other substances, such as thiamine or phosphate, appears to depend upon the type of yeast used, as may be seen by comparing the results of this paper (Figs. 1-4) with those of

Fig. 1. Fermentation rate of yeast A in medium M1. $\circ$, Full medium; upper curve, fresh yeast; lower curve, yeast stored for 13 days at 4°. $\square$, Asparagine-free medium; $\times$, thiamine-free medium; $\bullet$, thiamine added after fermentation for 95 min. in thiamine-free medium ($\uparrow$).

Maesen (1953) and Atkin *et al.* (cited in Anderson, 1946). Schultz, Atkin & Frey (1937) and Heyns (1939) found that thiamine could be replaced by 4-amino-5-ethoxymethyl-2-methylpyrimidine, the yeast being apparently capable of synthesizing the thiazole portion of the thiamine molecule. With D.C.L. yeast, on the other hand, thiamine (5 μM) could be replaced by high concentrations (100 μM) of 5- β -hydroxyethyl-4-methylthiazole: the pyrimidine part of the thiamine molecule (5 or 100 μM) was less effective than thiamine itself in promoting rate increases, as was the thiazole at the lower concentration.

In N-free medium, the fermentation rate (after the lag period) decreased with time. After addition of ammonium sulphate and asparagine at 107.5 min. the rate showed only a small immediate increase, after which the rate/time curve ran parallel to that given by yeast fermenting from the beginning in

full medium (Fig. 2). Supplementing full medium with nicotinamide resulted in a greater acceleration: adenosine, on the other hand, had the opposite effect (Fig. 2). Schultz, Atkin & Frey (1938) reported a smaller stimulatory effect of nicotinamide and found that adenylic acid was without effect.

Fig. 2. Fermentation rate of yeast E in medium M2. ○, Full medium; ●, full medium + nicotinamide; ×, full medium + adenosine; ■, thiamine-free medium for 107.5 min., then thiamine added (↑); □, N-free medium for 107.5 min., then ammonium sulphate + asparagine added (↑). The rates in asparagine-free medium + nicotinamide are not plotted: they were close to those in full medium unsupplemented with nicotinamide.

Yeast which had been pretreated with glucose in full medium, washed, and then transferred to N-free medium showed a greater decline in fermentation rate than untreated yeast, or yeast which had been pretreated in N-free medium (Fig. 3). Addition of ammonium salts to the medium did not restore the rate to the initial value.

The increase in fermentation rate induced by thiamine and assimilable nitrogen was not maintained unless phosphate was also present in the medium: in P-free medium the rate appeared to approach a limiting value (Fig. 4). Restoration of phosphate caused the rate to rise immediately to that of a control fermentation in full medium. As the first sample of yeast tested had an anomalously low fermentation rate, this observation was repeated on a fresh batch of yeast.

Glucose could be replaced by fructose, in full medium, without change in the fermentation rate/time curve (Fig. 5). Mannose was fermented

slightly more slowly and the general shape of the curve was altered (Fig. 5).

In thiamine-free medium, a constant fermentation rate was observed after the initial induction period. When, after 95 min. fermentation in thiamine-free medium, thiamine was added, the rate showed only a small immediate increase, after which the rate/time curve ran parallel to, but below, that for fermentation in full medium (Figs. 1, 2).

Fig. 3. Fermentation rates in N-free medium. □, Yeast G prefermented for 2 hr. in thiamine-free medium, thiamine added before fermentation in N-free medium (↑); ○, ●, yeast prefermented in full medium; ×, yeast prefermented in full medium + nicotinamide. In ●, 0.02M-NH₄Cl + 0.1M glucose added at 112.5 min.; in ○, ×, and □, NH₄Cl only (↑).

Fig. 4. Effect of phosphate on fermentation rate. ●, P-free medium, yeast C; ○, full medium: full line, yeast E; broken line, yeast D; ×, phosphate added after fermentation in P-free medium for 107.5 min. (↑).

A large number of experiments were made in which thiamine was added after fermentation for 107.5 min. in thiamine-free medium supplemented with (a) casein hydrolysate, or single amino acids; (b) alkaline or acid-hydrolysed ribonucleic acid, purines, pyrimidines, adenosine or uridine; (c) vitamins, singly or in combination; (d) formate, acetate or butyrate, as sources of 1- and 2-carbon

Fig. 5. Fermentation rate (yeast D, medium M2) of O, glucose, ●, fructose, and x, mannose. □, Mannose fermentation by yeast after pre-fermentation for 130 min. with glucose.

units used in synthetic processes. Rates were measured (i) at 95 min., to record the constant rate in thiamine-free medium, (ii) at 120 min., to determine the rapid increase in rate on addition of thiamine, and (iii) at 140 min., to determine, in conjunction with the 120 min. value, the rate of increase in full medium. The additions had little effect. Some of the rates recorded, in the order (i), (ii), (iii) above, were: control, 59, 76, 85 μmoles of CO₂/min./g.; most effective amino acids were 5 mM L-glutamine with rates of 63, 84, 92; and 0.01M DL-serine with rates of 54, 76, 90; inhibitory amino acids were 0.01M DL-cysteine with rates of 52, 65, 70 and 0.01M DL-methionine with rates of 62, 68, 72. Of group (b) adenine and adenosine were most active. These compounds slightly increased the rate in thiamine-free medium and also the immediate effect of thiamine, but decreased the subsequent increase in fermentation rate. For example, mM adenine had rates of 56, 79, 85. Vitamins had little effect. In full medium the rates at comparable times were 103, 112, 121. When air (150 ml./min.)

was passed through the fermentation vessel in place of nitrogen, the rates altered only slightly, to 104, 116, 125 respectively. A greater effect was shown on the rate in thiamine-free medium, and on the effect of restoring thiamine, typical figures being 69, 91, 101. Supplementation of the medium with casein hydrolysate, etc., again produced only minor effects.

Other (anaerobic) experiments showed (a) that destruction of added thiamine was not involved, since increasing the thiamine addition by a factor of 20 was without effect, and (b) that inhibitory

Fig. 6. Fermentation rate, 1.25% (w/v) yeast F in medium M2 containing 0.45M glucose. O, Full medium; ●, thiamine added after fermentation for 227.5 min. in thiamine-free medium (↑).

substances released into the medium during thiamine-free fermentation were not important, since after centrifuging and washing the yeast, and adding thiamine immediately on starting fermentation in fresh medium, the rate was still well below that in the full medium. In another experiment the yeast concentration was reduced to 1.25 g./100 ml., and the time of fermentation extended to over 4 hr. After 195 min. in thiamine-free medium the rate was 66 μmoles of CO₂/min./g., and the same rate was recorded at 215 min. Thiamine was added at 227.5 min.; at 240 min. the rate was 85 μmoles of CO₂/min./g., rising to 94 at 260 min. In full medium the rates at these times were over 160 μmoles of CO₂/min./g. (Fig. 6).

Effect of thiamine on the level of intracellular pyruvate

The fermentation rate of D.C.L. baker's yeast has been shown to be related to the intracellular pyruvate concentration (Trevelyan & Harrison, 1954a). This relation is described by an equation of the Michaelis-Menten type, with a K_m of 3 μmoles of pyruvate/g. of yeast. From the fermentation rate and intracellular pyruvate level may be calculated the fermentation rate at infinite pyruvate

concentration, which, it has been suggested, may be a measure of carboxylase activity *in vivo*. This theoretical maximum rate is increased by the addition of thiamine.

After fermenting glucose in thiamine-free medium for 100 min., the yeast was separated by centrifuging, washed, and a second fermentation under the same conditions was set up. After 5 min. the intracellular pyruvate reached the high level of $15.1 \mu\text{moles/g.}$ of yeast. Excess of thiamine was added at 7.5 min., so that the total period of thiamine-free fermentation was the same as in the experiments discussed in the preceding section. The pyruvate level fell rapidly to $2.5 \mu\text{moles/g.}$ at 10 min., 2.5 at 15 min., and 2.4 at 20 min. By extrapolation the fermentation rate immediately after addition of thiamine was found to be $79 \mu\text{moles of CO}_2/\text{min./g.}$ The calculated rate at infinite pyruvate concentration was $170 \mu\text{moles of CO}_2/\text{min./g.}$ after addition of thiamine. For the yeast before fermentation in thiamine-free medium it was found to be $160 \mu\text{moles of CO}_2/\text{min./g.}$

When the yeast fermented glucose in full medium, however, quite different results were obtained. Yeast thus treated was separated after 210 min. and transferred to thiamine-free medium. Its initial fermentation rate was $182 \mu\text{moles of CO}_2/\text{min./g.}$, which was unaltered by further addition of thiamine, which had, moreover, little effect on the intracellular pyruvate. This was $4.85 \mu\text{moles/g.}$ (original wt. of yeast) at 15 and 20 min. (thiamine-free fermentation) and at 30 min. after thiamine addition the level of pyruvate was 4.47 . Since both cell counts and optical density of the yeast suspension showed that the actual amount of yeast had doubled during the 210 min. fermentation, the level of pyruvate/g. actual wt. of yeast during the second fermentation was about $2.5 \mu\text{moles/g.}$, and the fermentation rate $90 \mu\text{moles of CO}_2/\text{min./g.}$ That is, during fermentation in full medium, the calculated maximum fermentation rate increased in parallel with the observed fermentation rate, so that the intracellular pyruvate concentration remained constant.

If the calculated maximum rate is equated with carboxylase activity, the results agree with those of Maesen (1953): that carboxylase is synthesized in proportion to the increase in fermentation rate, and that fermentation in the presence of thiamine increases the rate of synthesis. But, during fermentation in full medium, the pyruvate level is not high enough to support the contention that the carboxylase system is rate-limiting.

Effect of thiamine on some synthetic activities of yeast during fermentation

Results summarized below indicate that thiamine increased the extent of several general synthetic

processes, though its effect was not as marked as on fermentation rate.

Cell division. The mean rate of cell division during fermentation for 2 hr. in full medium was $13 \pm 3 \%/hr.$ The deoxyribonucleic acid content of the suspension increased by $12 \%/hr.$, but by only 4% in the absence of thiamine. After 4 hr. fermentations, microscopic examination showed obviously greater numbers of buds, and more frequent chains of cells, when thiamine had been added: the increase in cell count was approximately doubled by thiamine.

Nitrogen and phosphorus metabolism. D.C.L. yeast contained $1220 \mu\text{moles}$ of acid-insoluble nitrogen (AIN)/g. About $200 \mu\text{moles}$ were present as ribonucleic acid (RNA), corresponding to $50 \mu\text{moles}$ of RNA phosphorus. In full medium, AIN increased by $266 \mu\text{moles/g.}$ of yeast/hr., but by only 136 in thiamine-free medium. Omission of phosphate from the medium considerably lowered incorporation of nitrogen into AIN to 134 with, and 94 without, thiamine. Using the high-phosphate medium M1 (see Methods), thiamine was shown to increase the uptake of phosphate (Fig. 7). Most of the phosphate taken

Fig. 7. Effect of thiamine on phosphate uptake by yeast B from medium M1. □, Thiamine-free medium, with or without asparagine; ○, broken line, asparagine-free medium; full line, full medium.

up appeared in the hot TCA extract; after 2 hr. in full medium 285 μ moles of phosphate were taken up, the total phosphorus in the hot TCA extract increased from 52 to 226, and metaphosphate from 3 to 117 μ moles P/g. In full medium the rate of RNA formation was 21.7 μ moles of P/g./hr. and was reduced to 13.6 by omission of thiamine. With ammonium sulphate as the sole source of nitrogen the rates of formation with and without thiamine were 16.6 and 11.1 μ moles of P/g./hr. respectively. Uptake of ammonia during fermentation in medium M1 was increased by thiamine from 215 to 298 μ moles/g./hr. In view of the effect of thiamine on nucleic acid formation it is interesting to note that no effect on the level of acid-soluble ribose during fermentation for 2 hr. could be shown.

Amino acid metabolism

If addition of thiamine during fermentation were actually to stimulate overall protein synthesis, its effect could conceivably be either (a) direct, by virtue of some coenzyme requirement for amino acid synthesis or subsequent incorporation into protein, or (b) indirect, due to its marked influence on the intracellular pyruvate level, when one might expect an effect on the amino acid directly derived from pyruvate, namely alanine.

It was found that after fermentation of glucose for 4 hr. in full medium, the only amino acids detectable in the medium by paper chromatography [phenol-isopropanol-water (70:5:20, v/v) solvent] were asparagine and aspartic acid. When thiamine was omitted from the medium, however, an amino acid with the R_f of alanine appeared. This excretion of alanine was evidence of a high intracellular level during thiamine-free fermentation. The examination of instantaneous intracellular levels during fermentation, necessitating rapid destruction of enzyme action (e.g. by TCA), was not attempted in view of the difficulty of separating amino acids heavily contaminated by constituents of the medium. The pattern of amino acids extracted after washing the yeast free from medium, however, clearly showed the expected influence of thiamine. Fortunately, after paper chromatography alanine was well separated from the other amino acids present in TCA extracts of yeast. Detailed identification of the other amino acids was not attempted: according to Halvorsen & Spiegelman (1952, 1953*a, b*) glutamic acid and lysine are important constituents. With D.C.L. yeast, it was found that about one-quarter of the colour produced after spraying chromatograms with ninhydrin was due to alanine. Gale (1953) states that the amino acid generally present in highest concentration within bacterial cells is alanine.

After fermentation in N-free medium for 2 hr. of glucose, fructose or mannose the level of intra-

cellular amino acids decreased; maltose fermentation under the same conditions scarcely affected the amino acid pattern. Addition to the medium of ammonium sulphate counteracted the disappearance of amino acids during fermentation of glucose; the alanine spot on the chromatograms after ninhydrin treatment was slightly less intense than that given by the original yeast. Asparagine addition produced minor intensity differences, and caused

Fig. 8. Effect of thiamine on utilization of intracellular amino acids during fermentation in N-free medium. After applying ninhydrin to a paper chromatogram developed in butanol-acetic acid, the blue colour was eluted and compared with an alanine standard. O, Alanine spot; x, total amino acids; broken line, thiamine absent; full line, thiamine present.

the appearance of a new spot which reacted orange-brown with ninhydrin and was presumably due to asparagine itself. Omission of thiamine during fermentation in the presence of ammonium sulphate increased the intensity of spots due to minor components of the amino acid pattern, and most noticeably increased the amount of alanine present to 3.3 times that in either the original yeast or yeast which had fermented glucose in the presence of thiamine and ammonium sulphate.

Thiamine also influenced the utilization of intracellular amino acids during the first hour of glucose fermentation in N-free medium (Fig. 8). With thiamine, the intensity of the alanine spot rapidly diminished, whereas no net change occurred in the absence of thiamine. In the latter case, the intracellular pyruvate level has been shown to rise steadily during the first 40 min. or so of the fermentation (Trevelyan & Harrison, 1954*a*), thus increasingly favouring alanine formation.

DISCUSSION

It is clear from the results that baker's yeast, when fermenting glucose in a suitable medium, is capable of anaerobic growth for at least 3 hr. Growth includes cell division and also the formation of fermentation enzymes and coenzymes, the result of which is a steady increase in fermentation rate. The effect on fermentation rate of ammonium salts or asparagine is not immediate, but manifests itself gradually as the incorporation of nitrogen into cell substance proceeds. The effect of thiamine, which is also time-dependent, is therefore presumably on some synthetic process included in the term 'growth'. Reduction in the rate of protein synthesis when thiamine is omitted from the fermentation medium, it is suggested, is due to the interference with the incorporation of amino acids into protein by the high intracellular concentration of pyruvate or alanine. The magnitude of this interference may depend upon the thiamine present in the particular yeast culture used, since Maesen (1953) found no effect of additional thiamine on overall protein synthesis.

Moreover, this reduction in protein synthesis appears too small to explain the much more marked effect on fermentation rate increase and on carboxylase synthesis, whether the course of this process is followed by enzyme assay (Maesen, 1953) or by determination of intracellular pyruvate concentration. The pyruvate measurements suggest that factors other than carboxylase are involved.

The phosphate requirement again may depend on the amount present in the yeast culture. With D.C.L. yeast, which has a relatively low phosphate content, fermentation rate does not increase steadily unless the medium is supplemented with phosphate. Unlike assimilable nitrogen or thiamine, phosphate has a large immediate effect on fermentation rate. This is probably due to an increase in the intracellular concentration of phosphorylated substrates of the fermentation enzymes. It should be pointed out that, when the cells of a yeast suspension divide, the effectiveness of a fixed amount of any substrate or dissociable coenzyme decreases, since it is the intracellular

concentration which is kinetically relevant. Any cell division occurring in P-free medium would therefore tend to decrease the fermentation rate, and the shape of the rate/time curve (Fig. 4) reflects the opposition of some such factor to the effect of enzyme synthesis or other processes tending to increase fermentation rate.

As the substrate of carboxylase is non-phosphorylated, it would appear unlikely that this enzyme is the limiting factor during fermentation by D.C.L. yeast in phosphate-free medium.

Only in the case of fermentation in thiamine-free medium does carboxylase appear to be rate-limiting. The constant fermentation rate observed, though the cells are slowly dividing, argues for rate control by an enzyme rather than by a coenzyme or by substrate levels.

In general, rate limitation by a single enzyme system of the Embden-Meyerhof-Parnas scheme is likely to be found only in yeast nutritionally deficient in one or more factors. The reason for this is that such limitation implies a very high intracellular level of one particular substrate, a level which moreover changes considerably with a slight alteration in the overall rate of metabolism. The possibility of inhibition of other cellular activities is clear. Yeast fermenting in full medium maintains a constant intracellular pyruvate concentration about sufficient to half-saturate the carboxylase system.

The decrease of fermentation rate with time when yeast ferments in the absence of assimilable nitrogen also implies that the amount of enzyme cannot be the sole rate-determining factor in fermentation.

SUMMARY

1. Acceleration of the fermentation rate of baker's yeast by thiamine depends on synthesis of fermentation enzymes from nitrogenous constituents of the medium.

2. This synthesis occurs in phosphate-free medium, but its effect on rate is masked by the dilution of intracellular phosphorylated substrates due to cell division.

3. This synthesis is restricted in the absence of thiamine. Cell division, nucleic acid and overall protein synthesis are less affected. The high intracellular level of pyruvate and alanine may interfere with protein synthesis.

4. Carboxylase synthesis during fermentation occurs only in the presence of thiamine, but the low intracellular pyruvate level shows that carboxylase is not a limiting enzyme in fermentation by thiamine-saturated yeast.

5. Fermentation-rate control appears to be a complex process involving factors other than the level of fermentation enzymes.

The authors thank the Directors of The Distillers Co. Ltd. for permission to publish this paper. They would also like to record their indebtedness to their colleagues, particularly to Mr S. Burrows, for providing many suggestions during discussion.

REFERENCES

- Anderson, J. A. (1946). *Enzymes and their Role in Wheat Technology*. New York: Interscience.
- Bourdet, A., Mandel, P. & Guillemet, R. (1951). *C.R. Acad. Sci., Paris*, **232**, 756.
- Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 1st ed. London: Crosby Lockwood.
- DiCarlo, F. J. & Schultz, A. S. (1948). *Arch. Biochem.* **17**, 293.
- Gale, E. F. (1953). *Advanc. Protein Chem.* **8**, 287.
- Gomori, G. (1942). *J. Lab. clin. Med.* **27**, 955.
- Halvorson, H. O. & Spiegelman, S. (1952). *J. Bact.* **64**, 207.
- Halvorson, H. O. & Spiegelman, S. (1953a). *J. Bact.* **65**, 496.
- Halvorson, H. O. & Spiegelman, S. (1953b). *J. Bact.* **65**, 601.
- Heyns, K. (1939). *Biochem. Z.* **258**, 219.
- Koch, F. C. & McMeekin, T. L. (1924). *J. Amer. chem. Soc.* **46**, 2066.
- Maesen, T. J. M. (1953). *Biochim. biophys. Acta*, **12**, 445.
- Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
- Naftalin, L. (1948). *Nature, Lond.*, **161**, 763.
- Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
- Schultz, A. S., Atkin, L. & Frey, C. N. (1937). *J. Amer. chem. Soc.* **59**, 2457.
- Schultz, A. S., Atkin, L. & Frey, C. N. (1938). *J. Amer. chem. Soc.* **60**, 1514.
- Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
- Trevelyan, W. E. & Harrison, J. S. (1954a). *Biochem. J.* **57**, 556.
- Trevelyan, W. E. & Harrison, J. S. (1954b). *Biochem. J.* **57**, 561.
- Trevelyan, W. E. & Harrison, J. S. (1956). *Biochem. J.* **62**, 177.
- Wiame, J. M. (1949). *J. biol. Chem.* **178**, 919.

The Effect of Certain Vitamin Deficiencies on Glycine Biosynthesis

BY H. R. V. ARNSTEIN AND V. STANKOVIĆ*

The National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 24 June 1955)

Conclusive proof that glycine is a dispensable amino acid has been obtained in recent nutritional experiments with synthetic diets (Rose, Burr & Sallach, 1952), but it is noteworthy that, at least in the rat, the amount of glycine synthesized by the growing animal exceeds its probable requirements by only a small margin (Arnstein & Neuberger, 1953b). Since glycine is a precursor of many important nitrogenous compounds such as creatine, purines and porphyrins (for a recent review see Arnstein, 1954), any significant decrease in the ability of the animal to synthesize this amino acid would be expected to have a profound influence on general metabolism and growth. It was therefore of interest to study the effect of vitamin deficiencies on glycine biosynthesis, and the present paper records experiments with guinea pigs deficient in ascorbic acid and rats deficient in pteroylglutamic acid and vitamin B₁₂. Only pteroylglutamic acid deficiency was found to affect the rate of glycine biosynthesis to a significant extent.

Serine is an important precursor of glycine (Shemin, 1946), but an alternative mechanism of glycine synthesis from glyoxylic acid or a closely related two-carbon acid has also been demon-

strated (Weinhouse & Friedmann, 1951; Weissbach & Sprinson, 1953). Since pteroylglutamic acid, or more probably its tetrahydro derivative (Blakley, 1954), is required for the metabolic interconversion of serine and glycine (Elwyn & Sprinson, 1950), the decrease in glycine formation observed in pteroylglutamic acid deficiency may be used to assess the quantitative importance of serine as a glycine precursor.

Ascorbic acid increases the *in vitro* conversion of pteroylglutamic acid into N⁵-formyltetrahydropteroylglutamic acid (citrrovorum factor) (Nichol, 1953), possibly by facilitating the reductive step to tetrahydropteroylglutamic acid. This observation suggested that ascorbic acid deficiency might affect glycine biosynthesis indirectly and thus account for the marked decrease in muscle glycine of scorbutic guinea pigs (Christensen & Lynch, 1948). The present experiments show, however, that scorbutic guinea pigs are able to synthesize normal amounts of glycine.

Earlier experiments on the biosynthesis of choline from [α -¹⁴C]glycine by the rat indicated a possible requirement of vitamin B₁₂ for the formation of serine from glycine (Arnstein & Neuberger, 1952; Stekol, Weiss & Weiss, 1952). This possibility appeared to be supported by the subsequent observation that the conversion of glycine into the

* Visiting worker; permanent address: Department of Pathological Physiology, Veterinary Faculty, Zagreb, Yugoslavia.