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Studies on Yeast Metabolism

2. SYNTHESIS OF CELL CARBOHYDRATES DURING GLUCOSE FERMENTATION, AND ITS INHIBITION BY AZIDE

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The major products of the fermentation of glucose by yeast are ethanol and carbon dioxide, but under certain conditions an appreciable amount of the glucose metabolized is accounted for by synthesis of cellular carbohydrate (see review by Clifton, 1946). The nature, extent and status of carbohydrate assimilation appear, however, to be still undecided. This is partly due to the use of indirect methods of judging the extent of carbohydrate synthesis using, for example, cell carbon content, dry matter, opacity or, in many cases, relying simply on manometric data. We have therefore examined the applicability of the method of analysis of yeast carbohydrates previously described (Trevelyan & Harrison, 1952) to this problem, and further extended the method to an examination of the action of azide on fermentation, as this inhibitor has played an important part in assimilation studies.

METHODS

Overall carbohydrate balance after fermentation

Fermentations were carried out in an atmosphere of N_2 at 30° in the Warburg manometric apparatus. Carbohydrates were determined (Trevelyan & Harrison, 1952) on the yeast suspension prior to fermentation, and on the suspensions remaining in the flasks after exhaustion of fermentable sugar.

Pressed baker's yeast (Distillers Co. Ltd.) was suspended in $0.067\text{ M-KH}_2\text{PO}_4$ at pH 4.5. The substrate, in most cases glucose, was added from the side arm of the Warburg flask after temperature equilibration, and NaN_3 , when used, was dissolved in the substrate solution. The solubility of CO_2 in the medium was taken to be the same as in water, although according to graphical data presented by Van Slyke, Sendroy, Hastings & Neill (1928) the solubility in $0.067\text{ M-KH}_2\text{PO}_4$ at 38° is about 3% less than in water. This variation hardly affects our figures.

In anaerobic yeast fermentation CO_2 is formed by the decarboxylation of a C_3 compound, pyruvic acid, which is split to give CO_2 and a C_2 moiety. The nature or fate of the

latter does not affect the applicability of the Gay-Lussac equation, according to which 1 mg. of hexose is equivalent to almost exactly $250\text{ }\mu\text{l. of CO}_2$ at N.T.P. This value is used to calculate the 'hexose equivalent' of the CO_2 measured manometrically. Carbohydrate analyses are expressed 'as glucose', i.e. in terms of the colour produced in the anthrone reaction by a standard glucose solution.

Active fermentation studies

In studies of the changes occurring during fermentation, samples were withdrawn at different stages of active fermentation of glucose by yeast, and the progressive variations with time of some constituents of the yeast suspension were determined. As explained in a previous paper (Trevelyan & Harrison, 1952) all analyses are referred to the original weight of pressed yeast (24–26% dry matter).

Procedure A. Yeast suspension (yeast suspension is defined in Part 1) and glucose solution were prepared separately and chilled in ice. When cold, equal volumes were mixed and 2 ml. of the mixture were dispensed into each of six 15 ml. centrifuge tubes. Air was rapidly displaced from the tubes by N_2 , and they were stoppered and shaken in a water bath at 30° . (For details of amounts of yeast and glucose, see under separate experiments.)

Procedure B. This procedure was used to study fermentation in the presence of NaN_3 . Portions (1 ml.) of a yeast suspension were measured into centrifuge tubes, which were equilibrated at 30° while nitrogen was passed through the tubes. Glucose-azide solution, previously brought to 30° , was added and the tubes were stoppered and shaken. At various times after the commencement of fermentation tubes were removed, immediately centrifuged, and residual glucose determined by the anthrone method (Trevelyan & Harrison, 1952). In some experiments the ethanol content of the centrifugate was determined, using the microdiffusion method of Macleod (1949). Glycerol was estimated, after removing residual glucose by precipitation with CuSO_4 and Ca(OH)_2 according to Van Slyke (1917), by periodate oxidation and colorimetric determination of the resultant formaldehyde by the method described by Corcoran & Page (1947) for the estimation of mannitol in physiological fluids.

In repeat experiments, after withdrawing the tubes from the water bath, fermentation was arrested by the addition of trichloroacetic acid (TCA) to a final concentration of approx. 10% (w/v), and estimations of (a) TCA-extractable carbohydrate after bromine oxidation, and (b) total polysaccharide after KOH digestion of the extracted cells, were carried out as previously described (Trevelyan & Harrison, 1952). Production of CO_2 was followed on yeast-glucose preparations of identical concentration in a separate manometric experiment. In all cases the fermentation mixture was 0.067M with respect to KH_2PO_4 . The results, then, include analytical determinations carried out on samples from the same block of yeast (stored in the refrigerator) and used for centrifuge-tube fermentations performed over several days, and also manometric data for the same yeast. These results, although obtained under slightly varying conditions, may, because of their reproducibility and congruity, be taken as applying to a single fermentation without great error.

RESULTS

Autofermentability of yeast carbohydrate

In agreement with the results of Spiegelman & Nozawa (1945), it was found that yeast, in the absence of external substrate, metabolized its carbohydrate reserves very slowly. Carbohydrate analysis suggested that a slight fall in glycogen content, of the same order as the CO_2 output, occurred after 2 hr. (Table 1, Exp. 1). In a parallel experiment, in which $1.25 \times 10^{-3}\text{M}$ - NaN_3 was added, the analysis at 2 hr. was the same as in its absence. Azide, therefore, did not promote endogenous fermentation (cf. Reiner & Spiegelman, 1947).

finding has already been discussed (Trevelyan & Harrison, 1952). Application of the complete analytical fractionation (Table 1, Exp. 2) showed that in these experiments it is justifiable to treat the change in yeast carbohydrate occurring during glucose fermentation as mainly due to synthesis of glycogen. However, it cannot be assumed without further study that the same happens, for example, during the fermentation of higher concentrations of sugar: indeed, the mere fact of the occurrence in yeast of other forms of carbohydrate shows that their synthesis must take place. The sum of the separate carbohydrate fractions was less than the directly measured total carbohydrate, and the discrepancy increased after fermentation. The significance of this is not yet clear.

The values obtained for mannan by direct colorimetric comparison with glucose standards have to be multiplied by 1.82 to correct for the lower chromogenic value of mannose. However, because of the relatively small change in this fraction under our experimental conditions, changes in the total carbohydrate figure may be taken as a measure of variation of the hexose of the yeast.

By-products of glucose fermentation

In overall balance experiments, the discrepancy between the recorded CO_2 output and that calculated from the glucose fermented, which we shall term the glucose defect, was due to side reactions of which carbohydrate synthesis was only one. No more than 40% of the glucose defect was accounted for as synthesized cell carbohydrate (Table 1, items 11 and 12). The remainder was only ascribable in part to glycerol formation (Fig. 1). In one experiment 2 mg. of glucose were completely fermented by 10 mg. of yeast, and lactic acid was

Table 1. *Yeast carbohydrate (a) before addition of substrate, and (b) after complete fermentation of 10 mg. of pressed yeast (about 2.5 mg. dry weight) in 2.00 ml. of 0.067M- KH_2PO_4 at 30°*

(All analytical values expressed as μg . glucose.)

Exp. no.	...	...	...	...	1	2	3	4	5	6
Substrate (glucose) (μg .)	...	...	...	...	0	2000	2000	2000	2000	2000
NaN_3 ...	...	...	...	...	0	0	0	0	$5.0 \times 10^{-5}\text{M}$	$2.5 \times 10^{-3}\text{M}$
					(a)	(b)	(a)	(b)	(a)	(b)
(1) Total carbohydrate					849	830	899	1198	871	1053
(2) Extracellular					13	5	5	51	8	20
(3) TCA extract (trehalose)					243	233	238	246	226	255
(4) Total polysaccharide					577	523	604	786	518	665
(5) KOH extract					—	—	—	—	—	—
(6) Glycogen					217	175	211	381	—	—
(7) Mannan					195	190	217	229	167	170
(8) Glucan					165	158	176	176	183	202
(9) Sum of fractions (2)–(4).					833	761	847	1083	879	1053
Fermentation time (min.)					—	120	—	95	—	100
(10) CO_2 (as hexose)					—	16	—	1427	—	1495
(11) Substrate not converted to CO_2					—	—	—	573	—	505
(glucose defect)					—	—	—	—	—	208
(12) Increase in total carbohydrate					—	-19	—	+299	—	+279
									—	+43
										-340

Glycogen synthesis during glucose fermentation

When 2 mg. of glucose were completely fermented by 10 mg. of yeast the total carbohydrate increased, due largely to an increase in the polysaccharide fractions (Table 1, Exps. 2–4). Only part of this material was extracted from the yeast cell by KOH treatment: the significance of this

determined in the medium by the method of Barker & Summerson (1941): only 37 μg . of lactic acid were produced. It is probable that several other compounds are formed, each in relatively small amount, as was found for oxidative metabolism of yeast by Pickett & Clifton (1943). The contribution of carbohydrate synthesis to the glucose defect may vary from yeast to yeast: but it is clearly not justifiable

to interpret manometric findings in terms of carbohydrate synthesis alone unless actual analysis is first used to establish the properties of the particular organism studied.

Fig. 1. Fermentation of 2 mg. of glucose by 10 mg. of yeast in 1 ml. of 0.067M-KH₂PO₄ at 30° (Procedure A, Exp. 7).

Fermentability of glycogen in presence of substrate

In Exp. 7 polysaccharide (glycogen) was formed up to 45 min. in amount proportional to the CO₂ output (Fig. 1). After 45 min. the fermentation rate fell away owing to exhaustion of substrate. Glycogen actually decomposed, but the rate of destruction decreased rapidly as the external glucose level fell. This suggested that yeast can ferment its endogenous carbohydrate as long as external substrate is present. This interpretation is supported by a consideration of the results of Reiner, Gest & Kamen (1949), who showed that external glucose accelerated the turnover of cell products which had been synthesized by yeast from radioactive glucose. A kinetic interpretation of glycogen formation during fermentation is clearly indicated, i.e. the reaction

is fully reversible as long as external substrate is present, the direction of the overall reaction depending upon the concentration of the various components.

Formation of intermediates during fermentation

The curves for CO₂, polysaccharide and glycerol formation (Exp. 7, Fig. 1) are of similar form, and the amount of each of these products at any stage of the fermentation is roughly proportional. If other by-products, as yet unidentified, behave similarly, the implication is that the CO₂ output at any stage of the fermentation should be a constant fraction of the glucose uptake. In the case studied, this fraction increased from 0.54 after 20 min. to 0.75 at 75 min. This was probably due to the formation within the yeast cell of intermediates of the Meyerhof cycle in concentrations which were, after a rapid initial formation, in equilibrium with the external glucose concentration and consequently decreased to zero as the substrate was removed by the fermentation (cf. Fales & Baumberger, 1948). A similar explanation may be adduced for the peculiar variation in carbohydrate extracted by TCA (Fig. 1). Even at ice-bath temperature carbohydrate resistant to hypobromite oxidation was formed; this material could scarcely have been trehalose, and presumably repre-

sented such intermediates as fructose phosphates and di-hydroxyacetone phosphate. The terminal increase in TCA-extractable carbohydrate, however, was probably due to genuine trehalose formation.

Percentage conversion of glucose to CO₂

In agreement with Fales & Baumberger (1948) we have found that CO₂ production does not entirely cease when external glucose has been exhausted. The percentage conversion of glucose to CO₂, calculated from manometric data, thus depends somewhat upon the time after disappearance of external substrate at which the experiment is stopped. If

Fig. 2. Rate of fermentation of 2 mg. of glucose by 10 mg. of yeast in 2 ml. of 0.067M-potassium hydrogen phthalate, pH 4.5 at 30° (Exp. 8).

the velocity of gas production is plotted against the total CO₂ evolved, as in Fig. 2, it is found that, as the external glucose concentration falls, the curve approximates to a straight line. We define the 'CO₂ output' (x_{∞}) as the intercept on the 'CO₂ volume' axis obtained by extrapolating the straight line portion of this curve.

Action of azide on cell carbohydrate

When fermentation of glucose was carried out in the presence of 2.5×10^{-3} M-NaNaN₃, the CO₂ evolved was equivalent to the complete conversion of added glucose to CO₂ and ethanol. Carbohydrate analysis (Table 1, Exp. 6), however, showed that part of the yield was due to the metabolism of cell carbohydrate, trehalose in particular, though polysaccharide (probably glycogen) also decreased. Fig. 3 shows that the equivalence of glucose uptake, CO₂ output, and ethanol production applied approximately throughout the fermentation. Trehalose and polysaccharide of the yeast decreased (Fig. 3), and the rate of disappearance of these compounds fell as the external substrate was removed, in accordance with the finding that azide, in the absence of substrate, did not promote endogenous metabolism. Glycerol formation, though decreased as compared with the control, was still appreciable. The decomposition of trehalose in a similar fermentation was confirmed by chromatographic methods previously described (Trevelyan & Harrison, 1952). Yeast was incubated for 90 min. at 30°: (a) anaerobically

without substrate, (b) aerobically with glucose, (c) anaerobically with glucose, and (d) anaerobically with glucose and $2.5 \times 10^{-5} \text{ M-NaN}_3$, after which the cells were washed with water and extracted with 10% (w/v) trichloroacetic acid.

Fig. 3. Fermentation of 2 mg. of glucose by 10 mg. of yeast in 1 ml. of $0.067 \text{ M-KH}_2\text{PO}_4$ and $2.5 \times 10^{-5} \text{ M-NaN}_3$ at 30° (Procedure B, Exp. 9).

Fig. 4. Filter-paper chromatogram of sugars extracted from yeast after incubation at 30° for 90 min. G, glucose, $R_F = 0.48$; 1, after anaerobic metabolism of glucose in the presence of $2.5 \times 10^{-5} \text{ M-NaN}_3$, showing some unfermented glucose; 2, after anaerobic metabolism of glucose; 3, after aerobic metabolism of glucose; 4, after anaerobic incubation with water; T, trehalose, $R_F = 0.28$. Sugar spots detected by alkaline silver reagent after developing with collidine.

The extracts were examined chromatographically with the results shown in Fig. 4, where glucose and trehalose were used as markers. The amount of trehalose was of the same order in tests (a), (b) and (c), but in the presence of azide (d) the amount was much smaller, indicating breakdown of trehalose during fermentation of the glucose.

Fig. 3 suggests that in the last 20 min. of the fermentation a conversion of polysaccharide to trehalose occurred. In

Exp. 9 (Fig. 3), throughout the fermentation the extra-cellular carbohydrate was almost entirely glucose, for after hypobromite oxidation the following values for non-glucose carbohydrate were obtained in successive 20 min. periods: 23, 5, 2, 5, 9, 23 $\mu\text{g.}$ (as glucose)/ml. The initial glucose concentration was $2000 \mu\text{g./ml.}$ Thus the disappearance of trehalose promoted by $2.5 \times 10^{-5} \text{ M-NaN}_3$ was not due to a permeability change allowing the diffusion of trehalose into the medium, and its subsequent hydrolysis and re-absorption.

Effect of different concentrations of azide on carbohydrate synthesis

With a concentration of NaN_3 of $5 \times 10^{-5} \text{ M}$, the trehalose and polysaccharide content of yeast was almost unchanged by the complete fermentation of 2 mg. of glucose (Table 1, Exp. 5). Table 2 shows that lower concentrations of azide allowed synthesis of cell carbohydrate, whereas higher concentrations brought about a net decomposition. Over the range $0-15 \times 10^{-5} \text{ M-NaN}_3$, increase in CO_2 output was largely due to the effect on carbohydrate synthesis; the plot of glucose deficit against increase in cell carbohydrate was approximately linear, with a slope of 1.1. Stated in more conventional terms, this means that in higher concentrations of azide part of the CO_2 originated from the fermentation of preformed cell carbohydrate.

Table 2. Effect of NaN_3 on the fermentation of 2 mg. of glucose by 10 mg. of yeast

NaN_3 concentration ($\text{M} \times 10^{-5}$)	Glucose deficit, i.e. glucose not converted to CO_2 ($\mu\text{g.}$)	Increase in total yeast carbohydrate expressed as glucose ($\mu\text{g.}$)	Initial rate of CO_2 production ($\mu\text{l./min.}$)
Exp. 10. Medium $0.067 \text{ M-KH}_2\text{PO}_4$ (pH 4.5)			
0	528	223	8.00
1.0	456	189	8.33
2.5	296	38	9.16
5.0	232	-2	9.01
7.5	212	-49	8.58
15.0	164	-72	8.33
Exp. 11. Medium $0.067 \text{ M-potassium hydrogen phthalate}$ (pH 4.5)			
0	600	259	6.83
1.0	356	23	8.83
2.5	188	-78	7.92

Omission of phosphate from the medium greatly increased the effect of a given concentration of azide (Table 2, Exp. 11). Such an effect would seem to be contrary to the known effect of azide in preventing phosphate uptake during fermentation (Spiegelman, Kamen & Sussman, 1948): however, the addition of azide together with substrate, as used in our experiment, may explain this, as we have observed that azide added to a yeast suspension before glucose had a more pronounced inhibitory effect.

Effect of azide on fermentation rate

Azide concentrations below $2.5 \times 10^{-5} \text{ M}$. As the conversion of glucose to CO_2 is raised from 73% in the absence of azide to 86% in the presence of $2.5 \times 10^{-5} \text{ M-NaN}_3$, the rate of CO_2 production is increased in exact proportion to the increase of CO_2 output. Because of a decrease in carbohydrate synthesis the rate of glucose uptake is not significantly affected.

Azide concentrations between $2.5 \times 10^{-5} \text{ M}$ and $2.5 \times 10^{-3} \text{ M}$. The rate of glucose consumption is decreased, the inhibitory action of azide on this rate being greater at the beginning of the fermentation than near the end. This has the effect of decreasing the slope of the lower part of the curve obtained by plotting CO_2 production against time, with the result that the curve becomes nearly linear over a greater part of the fermentation period at higher azide concentrations.

Fig. 5. Effect of azide on (a) carbohydrate synthesis, (b) initial rate of CO_2 output, and (c) final volume of CO_2 (x_∞), during the fermentation of 2 mg. of glucose by 10 mg. of yeast in 1 ml. of $0.067 \text{ M-KH}_2\text{PO}_4$ at 30° (Exp. 12). $\bullet$ — $\bullet$, Carbohydrate synthesis when the CO_2 evolved $= x_\infty$; $\triangle$ — $\triangle$, initial velocity of CO_2 output calculated between 6 and 30 min.; $\circ$ — $\circ$, final CO_2 evolved, x_∞ , expressed as a percentage of glucose used.

When (a) the amount of carbohydrate synthesized, (b) the initial rate of fermentation, as CO_2 output per min., and (c) the final CO_2 volume (x_∞), as a percentage of the glucose used, are plotted against the logarithm of the azide concentration, sigmoid curves are obtained (Fig. 5). The curves for fermentation in phosphate-free medium (not shown) are similar, but displaced towards the region of low azide concentration; the slope of the linear part of the curves is approximately the same as when phosphate is present.

Effect of azide on phosphate metabolism

Spiegelman *et al.* (1948) have stated that in their experiments the inorganic phosphate of yeast decreased progressively as fermentation proceeded, and that this effect was almost entirely repressed by $2.5 \times 10^{-3} \text{ M-NaN}_3$. They interpreted this as showing that 'azide can effectively prevent the flow of phosphate from the inorganic to the organic fraction which accompanies the fermentation of glucose'. However, Mirski & Wertheimer (1939) showed that in glucose fermentation the internal inorganic phosphate dropped rapidly on addition of glucose, the lowest value being recorded after 1–2 min., and that as fermentation progressed the inorganic phosphate recovered almost to the initial level. We have suggested above, that the intermediates of the Meyerhof cycle are extremely labile and rapidly reach equilibrium with the external sugar concentration. Thus, if the latter is decreased by centrifugation of the yeast followed by washing with water, the internal inorganic phosphate of the yeast cell will increase (cf. Dische, 1940). As it is not entirely clear how the measurements of Spiegelman *et al.* were arrived at, or whether precautions were taken to

prevent decomposition of labile phosphate during analysis, we have studied the effect of $2.5 \times 10^{-3} \text{ M-NaN}_3$ on the inorganic phosphate of yeast during fermentation of glucose (2 mg./ml.) by yeast (10 mg./ml.) suspended in 0.017 M -potassium succinate buffer (pH 4.5). Fermentation was

Fig. 6. Orthophosphate content of fermentation mixtures after fermenting 2 mg. of glucose with 10 mg. of yeast in 1 ml. of potassium succinate buffer (pH 4.5) at 30° with and without the addition of NaN_3 (Procedure B, Exp. 13). Different time scales used for normal and azide experiments because of different fermentation rates. $\bullet$ — $\bullet$, Normal; $\circ$ — $\circ$, with $2.5 \times 10^{-3} \text{ M-NaN}_3$.

stopped by adding TCA to a final concentration of about 5% (w/v), and after extraction at ice-bath temperature the inorganic phosphate, in TCA solution, was determined by the method of Berenblum & Chain (1938). The fermentation was carried out at 30° in centrifuge tubes using procedure B. Results are shown in Fig. 6. The variations in inorganic phosphate level during azide-free fermentation followed the course described by Mirski & Wertheimer (1939). With $2.5 \times 10^{-3} \text{ M-NaN}_3$, however, the inorganic phosphate of the suspension actually increased. Now, although the fermentation rate in the presence of azide was much less than that of the control (initial rate 34% of the control), some formation of phosphorylated intermediates must have occurred for fermentation to proceed at all. Hence the rise in inorganic phosphate must have been due to the preponderance of decomposition of a pre-existing phosphorus compound of the yeast other than orthophosphate, over a simultaneous formation of labile phosphorylated intermediates. Hence, the interpretation of the effect of azide on the inorganic phosphate of yeast, as due to an interference with fermentative phosphorylation, is not justified.

DISCUSSION

Synthesis of cell carbohydrate in yeast fermentation

Under our experimental conditions, the synthesis of cell carbohydrate is largely accounted for as glycogen formation. There is no reason to suppose that the

synthesis of glycogen in yeast requires any other mechanism than that shown to operate in the separate isolated enzyme systems (see, for example, Colowick & Sutherland, 1942). Kiessling (1939), using yeast enzyme, showed that equilibrium was reached in the reaction

at a definite value of the ratio [glucose-1-phosphate]/[orthophosphate]. Later workers, using muscle and plant phosphorylases (cf. Cori, Cori & Green, 1943), established the dependence of this ratio on pH: in particular, the ratio is about 0.2 at pH 6.4, which may be taken as approximately the internal pH of the yeast cell (Conway & O'Malley, 1946). Thus a consideration of *in vitro* findings leads to the conclusion that glycogen synthesis in yeast would occur when the ratio [glucose-1-phosphate]/[orthophosphate] inside the cell is greater than 0.2.

It is interesting to note that Sussman, Spiegelman & Reiner (1947) have reported manometric experiments which they interpret as showing that carbohydrate synthesis is repressed when the pH of the medium is increased from 4.5 to 8.5, and suggest a direct effect upon the internal phosphorylase equilibrium as being responsible. As it seems unlikely that the overall internal pH of the cell would be much affected by the external pH (cf. the treatment of phosphate uptake by Hevesy, 1947), such explanations should be accepted with caution.

Nickerson (1949) has suggested that in living yeast metaphosphate is formed concurrently with glycogen, and that the insolubility of the former drives the phosphorylase reaction to the side of glycogen synthesis. Table 2 (Exp. 13) shows that in the absence of external phosphate 259 $\mu\text{g.}$ of cellular carbohydrate were formed after fermentation of 2 mg. of glucose. Formation of an equivalent amount of metaphosphate would bind about 60 $\mu\text{g.}$ of phosphorus, i.e. twenty times the orthophosphate phosphorus present in the yeast before fermentation. In the absence of further evidence, it would seem that Nickerson's mechanism is implausible. Similarly, quantitative considerations rule out the treatment of glycogen synthesis as an important dephosphorylating mechanism (Brockmann & Stier, 1947), for the small amount of glycogen formed would remove only about 6% of the adenosinetriphosphate bonds generated by fermentation.

We consider, therefore, that glycogen synthesis during fermentation is due to (a) the production of ester phosphate, and (b) the decrease of intracellular orthophosphate to a low level. As glucose becomes exhausted, the orthophosphate rises: thus eventually a point should be reached when the conditions favour the breakdown of glycogen. To some extent this has been shown to occur towards the end of fermentation, but the rate of breakdown rapidly

falls to a negligible value when substrate has disappeared from the medium. This point is clearly connected with the virtual non-fermentability of cell carbohydrate in the absence of external substrate. It is likely that a kinetic explanation is required, connected with the findings of Dische (1940) that the 'labile pyrophosphate' (presumably adenosinetriphosphate), formed rapidly on the addition of glucose to yeast suspensions, was capable of spontaneous destruction by enzymes at speeds approaching that of fermentation, with the result that, as the concentration of glucose decreased during fermentation, the amount of the phosphorylated fraction fell away. The enzyme concerned in the breakdown of 'labile pyrophosphate' was presumably adenosinetriphosphatase (Meyerhof, 1945).

Indirect evidence of the action of adenosinetriphosphatase during fermentation is provided by the heat produced during fermentation (Winzler & Baumberger, 1938) which may be due to the wastage of high-energy phosphate bonds rather than their use in synthesis. If the breakdown of polysaccharide into hexosemonophosphate is slow relative to the action of adenosinetriphosphatase, the resulting level of adenosinetriphosphate in the cell may become so low as to limit the overall speed of the endogenous fermentation. Alternatively, the level of other important phosphorylated compounds may fall, such as that of the coenzyme of phosphoglucumutase (i.e. glucose-1:6-diphosphate).

Action of azide on glycogen synthesis

It seems certain that a given level of azide will have widely different inhibitory powers on fermentation by different yeasts, and this should be borne in mind when comparing the results reported here with those of other investigators. Azide interferes with several aspects of the anaerobic metabolism of yeast; e.g. adaptive enzyme formation (Spiegelman, 1947), carbohydrate assimilation (Reiner & Spiegelman, 1947), and exchange of internal and external phosphate (Spiegelman *et al.* 1948). These last authors consider that the fundamental action of azide is in reducing the supply of energy-rich phosphate normally generated during fermentation. An analogy with the action of arsenate on the triose phosphate dehydrogenase system (Warburg & Christian, 1939) has been assumed, although *in vitro* experiments designed to show such an effect were negative. Reiner (1948), however, suggested that the action of azide differed from that of arsenate, and was unlikely to be due simply to the suppression of high-energy phosphate generation.

We have suggested that synthesis of glycogen by living yeast will occur when the ratio [glucose-1-phosphate]/[orthophosphate] inside the cell exceeds a certain critical value, and that fermentation of

intracellular carbohydrate takes place (as long as external substrate is present) when the ratio falls below the equilibrium ratio. In normal fermentation the critical ratio appears to be attained at a very low level of external glucose concentration, and consequently in the complete fermentation of milligram amounts of glucose under the conditions of our experiments the overall change in the level of internal cell carbohydrate is not greatly affected by the usage of the polysaccharides of the cell. It is probable that the effect of azide on carbohydrate synthesis may be explained on the same basis, i.e. as primarily due to (a) a fall in glucose-1-phosphate concentration, or (b) a rise in orthophosphate concentration.

(a) *Diminution in glucose-1-phosphate.* Since low concentrations of azide reduce glycogen formation without alteration in the rate of glucose uptake, a general lowering of high-energy phosphate cannot initially occur, for the rate of the hexokinase reaction with a given amount of enzyme depends on the concentration of glucose at the enzyme surface, which must have been unaltered, and the concentration of adenosinetriphosphate, which therefore also was presumably unaffected. However, a specific lowering of the concentration of glucose-1-phosphate may occur, analogous to the action of arsenate on the phosphorylase of *Pseudomonas saccharophila* (Doudoroff, Barker & Hassid, 1947) and of potato (Katz, Hassid & Doudoroff, 1948). Here arsenate competes with phosphate and brings about the breakdown of sucrose and starch respectively to free glucose via an unstable glucose-1-arsenate.

(b) *Rise in orthophosphate.* We have already considered reasons why the rise in orthophosphate induced by 2.5×10^{-3} M-sodium azide during fermentation in phosphate-free medium suggests the breakdown of a phosphate compound other than the intermediates of the Meyerhof cycle. This may be metaphosphate, since Wiame (1949) states that the synthesis of metaphosphate, which requires phosphate, potassium ions, and substrate, is suppressed by concentrations of azide which do not interfere with fermentation.

Either mechanism would explain the competition between azide and (internal) inorganic phosphate which is suggested by the influence of addition of extracellular phosphate in our experiments. Such a competition probably accounts for the finding that the inhibitory action of azide on fermentation rate is greater in the initial stages of fermentation, since

the orthophosphate content of the cell rises as fermentation proceeds. The effect of higher concentrations of azide on rate of fermentation would be explainable as due to a drain of high-energy phosphate which could clearly occur by either of mechanisms (a) or (b). Mechanism (b) would also explain the potentiation by azide of the inhibitory action of fluoride on fermentation (Spiegelman *et al.* 1948), since the inhibition of enolase by fluoride depends on the concentration of orthophosphate (Warburg & Christian, 1942). As both mechanisms are outside the main reaction chain of the fermentation cycle, the failure of Spiegelman *et al.* (1948) to demonstrate azide inhibition in cell-free extracts or *in vitro* enzyme systems is explicable.

The breakdown of trehalose induced by higher concentrations of azide suggests that trehalose, like glycogen, participates inside the yeast cell in an equilibrium with some intermediate of the Meyerhof cycle, possibly glucose-1-phosphate.

SUMMARY

1. The changes in total carbohydrate, trehalose, glycogen, mannan and glucan of baker's yeast occurring during glucose fermentation have been determined.

2. Under the experimental conditions employed cellular carbohydrate increased during fermentation; this was mainly due to synthesis of glycogen.

3. About 70 % of the glucose metabolized was converted to carbon dioxide and ethanol. Less than half of the remainder was accounted for as synthesized cell carbohydrate.

4. Sodium azide inhibited carbohydrate synthesis. The magnitude of the effect depended upon the concentration of azide: synthesis occurred below 2.5×10^{-5} M, whereas greater concentrations promoted breakdown of glycogen and trehalose. With 2.5×10^{-3} M-sodium azide, carbon dioxide and ethanol formed were equivalent to 100 % conversion of fermented glucose, but partly originated by fermentation of preformed cell carbohydrate.

5. The mechanism of glycogen synthesis has been discussed in terms of the known properties of the enzymic reactions of fermentation.

6. The action of azide upon glycogen synthesis during fermentation has been discussed and possible mechanisms of action considered.

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The Preparation and Properties of Aucubin, Asperuloside and Some Related Glycosides

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Some of the many types of glycosides which have been described have been thought to be specific products of narrow taxonomic groups of plants. However, more and more of them are proving to occur widely in the plant kingdom. The work described in this paper shows that aucubin, asperuloside and related glycosides, which are probably derived from substituted furan aglycones, constitute one such widely occurring type.

Aucubin was first isolated by Bourquelot & Hérissé (1905) from the leaves of *Aucuba japonica* (family Cornaceae). It is a monohydrate of a monoglucoside of an unstable aglucone, which these workers were unable to isolate or identify. Asperuloside was first isolated by Hérissé (1925*a*, *b*, 1926*a*, *b*, 1927, 1933) from several members of the family Rubiaceae. He showed that it, too, is a monoglucoside of an unstable aglucone, which, like aucubin, loses 1 mol. of water when heated to 105–110°.

Karrer & Schmid (1946) suggested for aucubin the substituted furan structure (I) which explains the properties described by Bourquelot & Hérissé (1905) and Bergmann & Michalis (1927). This

glucoside has now been isolated from plants belonging to the following families: Cornaceae (Bourquelot & Hérissé, 1905), Garryaceae (Hérissé & Lebas, 1910), Plantaginaceae (Bourdier, 1907), Orobanchaceae (Bridel, 1929), Globulariaceae (Zellner, 1934), Scrophulariaceae (Braecke, 1923*a*), and Eucommiaceae (Plouvier, 1944).