reaction. It is suggested that 5-fluoronicotinic acid inhibits several steps in the synthesis of cozymase possibly by being metabolized along the synthetic route.

Thanks are due to Prof. H. A. Krebs, F.R.S., for his advice and criticism, and to Mr M. Clegg and Mr N. Blumson for technical assistance. The halogen-substituted nicotinic acids were gifts from Dr K. Rohrman, Eli Lilly Corp., U.S.A. This work was aided by a grant from the Rockefeller Foundation.

REFERENCES

Albert, A. (1952). *Selective Toxicity*. London: Methuen and Co. Ltd.
Barton-Wright, E. C. (1946). *Practical Methods for the Microbiological Assay of the B Complex and Essential Amino Acids*. London: Ashe Laboratories Ltd.
Ellinger, P. & Abdel-Kader, M. M. (1949). *Biochem. J.* **44**, 627.
Fildes, P. (1940). *Lancet*, **1**, 955.
Halvorson, H. O. & Spiegelman, S. (1952). *J. Bact.* **64**, 207.
Hawkins, G. F. & Roe, A. (1949). *J. org. Chem.* **14**, 328.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Hughes, D. E. (1951). *Photoelectric Spectr. Gr. Bull.* **5**, 121.
Hughes, D. E. (1952*a*). *Biochem. J.* **51**, xxii.
Hughes, D. E. (1952*b*). *Biochim. biophys. Acta*, **9**, 226.
Hughes, D. E. (1953). *6th Congr. int. Microbiol.* (*Abstr.*), p. 235.
Hughes, D. E. & Williamson, D. H. (1952*a*). *Biochem. J.* **51**, 330.
Hughes, D. E. & Williamson, D. H. (1952*b*). *Biochem. J.* **51**, 45.
Hughes, D. E. & Williamson, D. H. (1953). *Biochem. J.* **55**, 851.
Jackson, F. L. & Copping, A. M. (1952). *J. gen. Microbiol.* **7**, 107.
Krebs, H. A. (1937). *Biochem. J.* **43**, 51.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Levitis, N., Robinson, J., Rosen, F., Huff, J. N. & Perlzweig, W. A. (1945). *J. biol. Chem.* **167**, 169.
Lowry, O. H. & Bessey, O. A. (1946). *J. biol. Chem.* **161**, 288.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **44**, 285.
Matthews, R. E. F. (1953). *Nature, Lond.*, **171**, 1065.
Minor, J. T., Hawkins, G. F., Vanderwerf, C. A. & Roe, A. (1949). *J. Amer. chem. Soc.* **71**, 1125.
Mitchell, H. K. & Niemann, C. (1947). *J. Amer. chem. Soc.* **69**, 1232.
Nossal, P. M. (1951). *Biochem. J.* **50**, 349.
Partridge, S. M. & Westall, R. G. (1948). *Biochem. J.* **42**, 238.
Peters, R. A. (1952). *Proc. Roy. Soc.* B, **139**, 143.
Racker, E. (1949). *J. biol. Chem.* **184**, 313.
Rydon, H. N. (1949). *Symp. Soc. exp. Biol.* **3**, 60.
Shive, W. & Roberts, E. C. (1946). *J. biol. Chem.* **162**, 463.
Stekol, J. A. & Weiss, K. (1950). *J. biol. Chem.* **185**, 585.
Umbreit, W. W. & Waddell, J. G. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 293.
Woods, D. D. (1940). *Brit. J. exp. Path.* **21**, 74.
Woolley, D. W. (1952). *A Study of Antimetabolites*. London: Chapman and Hall Ltd.
Zatman, L. J., Kaplan, N. O. & Colowick, S. P. (1953). *J. Amer. chem. Soc.* **75**, 3293.

The Regulation of Respiration during the Assimilation of Nitrogen in *Torulopsis utilis*

By E. W. YEMM and B. F. FOLKES
Department of Botany, University of Bristol

(*Received* 7 *December* 1953)

In micro-organisms and in higher plants there is strong evidence that cellular respiration and the assimilation of inorganic nitrogen are closely interrelated metabolic processes. Some of the mechanisms underlying their linkage have been discussed by Yemm (1949), Burris & Wilson (1952), and Syrett (1953*b*). In order to investigate these interrelations more fully, experiments have recently been carried out with food yeast (*Torulopsis utilis*). This yeast has an outstanding capacity for the assimilation of nitrogen and synthesis of protein. Roine (1947) and Virtanen, Csaky & Rautanen (1949) have shown that pure cultures can be readily grown on a culture medium containing sucrose and minerals including ammonium salts, and then brought into a physiological condition in which they will rapidly utilize either ammonia or nitrate, by aeration for a few hours in a sucrose–mineral salt solution lacking in nitrogen. Their results indicate that glutamine, asparagine and alanine are the chief primary products of assimilation when such yeast is treated with a nitrogen source in the absence of external carbohydrate, and this clearly suggests a close parallel with the data now available for roots and other tissues of higher plants (e.g. Willis, 1950, 1951).

The experiments described in this paper were designed to investigate the interrelations of cellular respiration, the assimilation of nitrogen, and the metabolism of carbohydrates. For this purpose, pure cultures of the yeast were supplied with ammonium salts under controlled conditions; the

products of assimilation were determined together with the rates of respiration and of carbohydrate breakdown in the cells.

MATERIAL AND METHODS

Preparation of yeast suspensions

Food yeasts. Cultures of *Torulopsis utilis* (B.P. 60) and of *Torulopsis utilis* var. *major* (N.C.T.C. 6593) from the National Collection of Type Cultures were maintained on nitrate–sucrose agar. For experimental purposes the yeasts were grown on the complete culture medium (soln. *A*) modified from Virtanen *et al.* (1949) and with the following composition: sucrose 50 g., $(NH_4)_2HPO_4$ 3 g., $(NH_4)_2SO_4$ 4 g., K_2SO_4 1·5 g., $MgSO_4.7H_2O$ 1 g., $CaCl_2$ 0·05 g., 10 ml. micronutrient solution (Winzler, Burk & du Vigneaud, 1944: containing H_3BO_4 1 mg., $ZnSO_4$ 1 mg., $MnCl_2$ 1 mg., $FeCl_3$ 0·5 mg., $CuSO_4.5H_2O$ 0·1 mg. and KI 0·1 mg.), distilled water to 1 l. and the pH adjusted to 6·0–6·5 with HCl. Portions (125 ml.) of this medium were sterilized in 2 l. cylindrical culture flasks (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944), inoculated with a loopful of the yeast culture, and gently shaken on a 'Microid' flask shaker (Griffin and Tatlock Ltd.) in a constant-temperature room at 25° for 15–20 hr. When the culture became visibly turbid, the cotton-wool plugs were removed and the rate of shaking was increased to improve aeration for the next 4–5 hr. The yeast was then centrifuged off at 1500 *g* for 15 min. Total fresh weight was 1–2 g./flask.

Baker's yeast. Commercial pressed yeast (Distillers Co. Ltd.) was washed three times at the centrifuge with 20 times its weight of solution *C* (see below) at each washing.

Nitrogen-deficient cultures of yeast. The yeast, prepared as above, was resuspended in the N-deficient solution *B* modified from Virtanen *et al.* (1949), containing sucrose 50 g., KH_2PO_4 3 g., $MgSO_4$, $7H_2O$ 1 g., $CaCl_2$ 0·05 g., 10 ml. micronutrient solution, distilled water to 1 l. and with the pH adjusted to 7·2–7·5 with NaOH. A volume equal to that of the original culture was used and the suspension was divided into 125 ml. portions and shaken as before for 12–16 hr. at 25°. During this period the fresh weight of the yeast was approximately doubled. The culture was then centrifuged off and washed once by centrifuging in the mineral salt solution *C* modified from Virtanen *et al.* (1949). This solution contained KH_2PO_4 3 g., $MgSO_4$, $7H_2O$ 1 g., $CaCl_2$ 0·05 g., 10 ml. micronutrient solution, distilled water to 1 l., with the pH adjusted to $6·00 \pm 0·05$. The washed culture was resuspended in solution *C* to give 2 g. fresh weight yeast/100 ml. suspension.

Experimental procedures

Measurement of respiration. O_2 uptake was measured manometrically at 25° by the direct method of Warburg (Dixon, 1943). The N-deficient yeast suspension (3 ml.) was pipetted into the main compartment of each flask, with 0·5 ml. of 0·06 M-$(NH_4)_2HPO_4$, or 0·06 M-Na_2HPO_4, or of appropriate mixtures of the two, in the side bulb. The phosphate solutions were adjusted to pH $6·00 \pm 0·05$ with M-H_3PO_4. In experiments involving the use of 2:4-dinitrophenol appropriate amounts of this were included in the 0·5 ml. total volume in the side bulb. CO_2 output (and thus R.Q.) was occasionally determined on replicate samples by the direct method (Dixon, 1943). Bound CO_2 was estimated by the addition of 0·5 ml. 3 N-HCl from the side bulb, but the correction was small, never amounting to more than 2·5% of the total CO_2 produced in respiration.

Cultures for analysis. When large quantities of yeast suspension were required for analysis, samples were treated in the cylindrical culture flasks on the shaking machine at 25°, while a parallel experiment was performed in manometers to yield respiratory data. Each culture flask contained 90 ml. yeast suspension, and 15 ml. of the appropriate mixture of 0·06 M-$(NH_4)_2HPO_4$ and 0·06 M-Na_2HPO_4 at pH 6·0 was added after a suitable interval. The flasks were sampled after various periods of treatment, the contents of one flask being taken on each occasion. The suspension was washed into a centrifuge bottle with 20–30 ml. distilled water and centrifuged for 15 min. at 1500 *g*. The clear supernatant was decanted and made to 150 ml.; 5 ml. were then removed for pH determination by means of a glass electrode. The remainder of the culture solution was acidified with 5 ml. 4 N-H_2SO_4 and stored in a refrigerator for subsequent analysis. The sedimented yeast was weighed in the bottle and then chilled in an ice bath in preparation for analysis.

Analytical methods

Dry weight. Samples (1 ml.) of yeast suspension were dried at 98° in small weighing bottles until a constant value was reached (4–8 hr.). A blank, consisting of 1 ml. of the suspending solution, was dried alongside and used to correct the estimations.

Extraction of soluble constituents. The yeast, separated by centrifuging as described above, was chilled in the ice bath for 5 min. and treated with 2 ml. cold absolute ethanol for 15 min. Cold 0·2 N-H_2SO_4 (20 ml.) was then pipetted in, and the mixture was left in the ice bath for 1 hr. with shaking every 5 min. The yeast was then centrifuged (10 min. at 1500 *g*) and the extract decanted and saved for analysis. The yeast was re-extracted with 20 ml. 0·2 N-H_2SO_4, centrifuged, the second extract discarded, and the residue suspended in distilled water, made to 20 ml. and saved for analysis.

In this extraction procedure it was assumed that all soluble components came freely into solution, and the proportion extracted was the same as the proportion of total extractant recovered from the yeast. As the yeast contained approx. 80% moisture and was extracted with 2 ml. ethanol and 20 ml. H_2SO_4, the volume of total extractant used in ml. was $2 + 20 + 0·8 \times$ (fresh wt. yeast in g.) + wt. solution held on centrifuge bottle (in g.), making a total of 23·8–24·2 ml. in most experiments. Estimations of total N and glutathione on serial extracts showed the errors caused by these assumptions to be less than 1% of the total amount extracted.

Ammonia. The free-ammonia content of culture solution, extracts, and of the yeast suspensions from the manometric experiments, were determined by the boric acid–HCl procedure of Conway (1950) in standard microdiffusion units. The solution under test (0·5 ml.) was treated with 0·5 ml. saturated KBO_2 and left to diffuse at 0–5° for 90–120 min. before titration.

Total nitrogen. Portions (2 ml.) of extracts or residues were heated with 0·5 g. catalyst (Yemm & Folkes, 1953) and 2 ml. conc. H_2SO_4 for 20 hr. When cool, the solution was diluted to 10 ml. for extract or 25 ml. for residue. Portions (1 ml.) were taken for ammonia estimation as above but were neutralized with 1 ml. 10 N-NaOH for extract or 1 ml. alkaline metaborate (equal parts of saturated KBO_2 and

7 N-KOH) for residue. Diffusion was allowed to proceed for 18–20 hr. at room temperature.

Glutamic acid and glutamine. The free amino acid and the amide were estimated by means of the specific glutamic decarboxylase and glutaminase preparation of *Clostridium welchii* (S.R. 12) by the method developed by Krebs (1948). To 2 ml. extract was added 1 ml. acetate–cetavlon (0·45 M acetate buffer pH 4·9 containing 0·15% cetyltrimethylammonium bromide) and 0·8 N-NaOH (to adjust pH to 4·8–5·0). Part of this mixture (3 ml.) was used for the estimation of glutamic acid using 12·5 mg. of a freeze-dried enzyme preparation in 0·5 ml. 0·15 M acetate buffer, pH 4·9. Glutamine is both deamidated and decarboxylated by the enzyme (Krebs, 1948) and the glutamine content was calculated from the free ammonia present at the end of the enzyme treatment. For this estimation, 0·5 ml. portions were taken for the Conway units and 0·5 ml. saturated KBO_2.

The above procedure could also be applied to the small volumes of yeast suspension used for the manometric measurement of respiration, but here a preliminary extraction was necessary. The suspension (2 ml.) was pipetted into a tube containing 0·5 ml. 5 N-HCl, and 1 ml. acetate–cetavlon was added. The solution was refrigerated overnight, adjusted to pH 4·8–5·0 with 7·5 N-NaOH, and 3 ml. were taken for enzymic treatment.

Asparagine. A sample of extract (2 ml.) was mixed with 2 ml. 4 N-H_2SO_4 in a small tube fitted with a capillary condenser. The solution was heated in a boiling water bath for 3 hr., made up to 5 ml. and then 1 ml. portions were taken for ammonia estimation using 1 ml. alkaline metaborate, diffusion being allowed to proceed for 18–20 hr. at room temperature. Ammonia N in excess of the free ammonia + glutamine amide N was taken as asparagine amide N.

Alanine was estimated in extracts by means of ninhydrin oxidation and colorimetric estimation of the acetaldehyde produced (Folkes, 1953). The extract (0·5 ml.) was first dried *in vacuo* to remove ethanol. No correction was made for other acids, as chromatography showed that these were present in small amounts only.

Glutathione was estimated colorimetrically on 2 ml. extract by the nitroprusside reaction of Grunert & Phillips (1951), using a Unicam Absorptiometer with 1 cm. cells and an Ilford no. 624 (green) filter. Total glutathione was measured after reduction on a stirred mercury cathode for 10 min. at a current density of 3 mA/sq.cm. (Dohan & Woodward, 1939).

Chromatography of amino acids. Qualitative investigation of the amino acids in the extracts was by partition chromatography on Whatman no. 1 paper using water-saturated phenol, containing 1% (v/v) acetic acid, as the ascending solvent phase. Extracts were de-acidified first with 1% $BaCO_3$ and papers were run for 5–6 hr., then dried and amino acids detected by spraying with 0·2% ninhydrin in aqueous butanol. Standards containing glutamic acid, aspartic acid and alanine, or glutamine and asparagine, were run on each paper.

Total carbohydrate was determined colorimetrically by the anthrone method developed by Trevelyan & Harrison (1952). Where yeast suspensions from manometric experiments were tested, 1 ml. of suspension was pipetted into 2 ml. 4 N-H_2SO_4 to kill the cells and then diluted to 25 ml. with water. In some cases 0·1 ml. suspension only was used with appropriately smaller amounts of acid and water. Yeast residues were diluted, 0·5–50 ml., before analysis. The volume of diluted carbohydrate taken for the reaction was always 1 ml. and the colour intensity was measured in a Unicam Absorptiometer using 1 cm. cells and an Ilford no. 607 (orange) filter. Calibration was against glucose, and all results are expressed on this basis. Mannose was found to give much less intense colour than glucose (approx. 57%, cf. Trevelyan & Harrison, 1952, who found 55%) and correction was made for this when comparing estimates of insoluble carbohydrates by the anthrone and copper reagents.

Soluble sugars. The micro modification by Willis & Yemm (1954) of the method of Somogyi (1945) was used for the estimation of soluble sugars by measurement of the reducing power of the extracts both before and after hydrolysis. 'Glucose' was estimated by direct measurement of reducing power of 1 ml. portions of an extract previously neutralized and diluted 2·5 times. 'Sucrose' was determined similarly, but the acid extracts were heated for 10 min. at 100° before neutralizing and diluting; a correction for reducing power due to 'glucose' was of course made. Trehalose was estimated from the reducing power of extracts hydrolysed with 2 N-H_2SO_4 at 100° for 3 hr.; 3 ml. of the diluted and hydrolysed extract remaining from the estimation of asparagine were neutralized and made to 3·5 ml., 1 ml. portions being taken for the Somogyi estimation.

Insoluble carbohydrates were fractionated by the methods of Trevelyan & Harrison (1952). The glycogen + mannan fraction, extracted from 5 ml. residue with KOH, and then H_2SO_4, and the mannan fraction, precipitated with Fehling's solution from a KOH extract of 5 ml. residue, were each hydrolysed for 2 hr. at 100° in the presence of 2 N-H_2SO_4, then neutralized and diluted to 375 ml. Portions (1 ml.) were then taken for estimations of reducing power as above.

Chromatographic investigation of carbohydrate fractions. From about 4·5 g. fresh weight of sugar-fed yeast, three fractions were separated by the methods described above. These fractions were soluble sugars, glycogen + mannan, and mannan. The polysaccharide fractions were hydrolysed for 3 hr. at 100° with 2 N-H_2SO_4 and neutralized with $BaCO_3$. All were investigated by descending partition chromatography on Whatman no. 1 paper using butanol:pyridine:water (10:3:3, v/v) or ethyl acetate:acetic acid:water (9:2:2, v/v) as solvents and 1% *p*-anisidine hydrochloride in aqueous butanol for detection.

Calculation of results. Both fresh weight and dry weight are likely to change during experimental treatment of the yeast and so do not provide reliable bases for the calculation of results. All results have therefore been calculated on the basis of 100 ml. yeast suspension before it was diluted by the addition of phosphate solutions. The suspension generally contained 2 g. fresh weight of yeast/100 ml., but an exception was in the sugar-feeding experiment (see Table 2) where the suspension was approximately 1% before feeding and increased during the experiment.

RESULTS

The effect of ammonium ions on the respiration of nitrogen-deficient yeast

Numerous experiments have shown that the addition of an ammonium salt, such as ammonium phosphate, to nitrogen-deficient yeast results in a

considerable and very rapid rise in its rate of cellular respiration. Some typical measurements of oxygen uptake are given in Fig. 1.

The respiratory rate of nitrogen-deficient yeast, freshly transferred from a sugar-rich medium, shows at first a gradual decline, but after several hours it becomes almost constant. The addition of sodium phosphate to such a yeast causes no significant change in respiratory rate; this is apparent from the small scatter of values for both untreated and sodium phosphate treated yeasts in Fig. 1. The addition of ammonium phosphate, on the other hand, results in a sharp rise in the rate of oxygen uptake to 3 or 4 times its previous value. This high rate is maintained for about half an hour and then falls, rapidly at first and subsequently more slowly, to a value about double that of the yeast without added ammonium. This steady, or comparatively steady, rate of respiration is reached about 3 hr. after the addition of the ammonium solution. It is apparent from the curves given in Fig. 1 that the effect upon respiration of adding ammonium ions is extremely reproducible and relatively independent of the time of this addition.

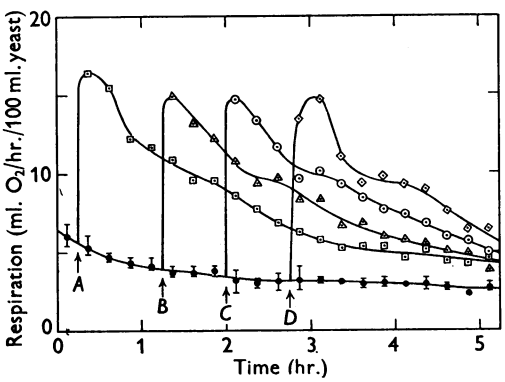

Fig. 1. The effect of ammonium ions on the rate of oxygen uptake of nitrogen-deficient yeast. ● Mean respiration rates, yeast untreated or with added sodium phosphate (range of values indicated). ⊡, △, ⊙, ◇ Respiration of individual samples treated at *A*, *B*, *C* and *D* respectively with a mixture of sodium and ammonium phosphates equivalent to 14 mg. N/100 ml. yeast suspension.

Data collected with *Torulopsis utilis* and other yeasts are summarized in Table 1, in which mean rates of respiration are given for a number of experiments. It can be seen that ammonium ions have a similar effect upon the respiration of all three yeasts tested, although some differences in the extent of response are apparent between baker's yeast and the two varieties of *Torulopsis utilis*. *T. utilis* B.P.60 was used for all the other experiments described in this paper.

The high degree of reproducibility in the experimental results is apparent from the data for *T. utilis* given in Table 1. The variation from experiment to experiment is small, and much of it is probably due to differences in the time of addition of ammonium phosphate. The two sets of results given for Expt. 9 illustrate this; the second series (*b*) was given by a sample treated with ammonia nitrogen 2 hr. after the first (*a*), and the differences between these two are nearly as large as any between pairs of experiments. Much of the variation in this and other experiments (see also Fig. 1) can be attributed to the differences between the rates of the nitrogen-deficient controls. These differences,

Table 1. *Respiration and the synthesis of glutamic acid in food yeasts and in baker's yeast*

In each experiment 3 ml. of 2% yeast suspension were treated at 25° in Warburg flasks with 0·5 ml. 0·06 M-Na_2HPO_4 (−N) or with 0·5 ml. of a mixture of equal parts of 0·06 M-$(NH_4)_2HPO_4$ and 0·06 M-Na_2HPO_4 (+N). O_2 uptake was measured for 2 hr. and then glutamic acid, free and as glutamine, was determined by means of glutamic decarboxylase.

Yeast	Expt. no.	Respiration (ml. O_2/hr./100 ml. suspn.) 1st hr. +N	1st hr. −N	2nd hr. +N	2nd hr. −N	Glutamic acid (mg. N/100 ml. suspn.) after 2 hr. +N	Glutamic acid (mg. N/100 ml. suspn.) after 2 hr. −N
Torulopsis utilis (BP60)	1	12·4	4·4	9·1	3·3	1·35	<0·02
	2	13·9	4·4	9·9	3·4	—	—
	3	14·1	4·9	9·7	3·5	1·44	0·02
	4	12·6	4·3	9·1	3·1	—	—
	5	13·3	5·0	9·3	3·5	—	—
	6	13·7	6·1	9·8	3·4	—	—
	7	14·0	4·6	9·4	3·4	—	—
	8	13·3	4·3	9·2	3·4	1·20	<0·02
	9*a*	13·9	5·1	9·6	4·0	—	—
	9*b*	12·5	3·3	8·8	2·9	—	—
	10*a*	12·6	4·2	9·6	3·1	1·28	<0·02
T. utilis var. *major* (N.C.T.C. 6593)	10*b*	11·2	3·9	8·3	3·1	1·23	<0·02
Baker's yeast	11	19·5	8·0	13·5	6·8	0·85	<0·02

although small, appear to influence in a direct additive manner the rate observed after nitrogen has been supplied.

The variation between replicate samples of the same experiment is small; in a typical experiment (Expt. 1) nine samples of untreated yeast showed a coefficient of variation in respiratory rate of ± 1·4 %, and eight samples after addition of ammonium ions gave a coefficient of variation of ± 0·8 %. This very small variation made extensive replication unnecessary and indicates that the results of different experiments can be compared with some confidence.

The high rate of oxygen uptake in ammonium-treated yeast is accompanied by a marked increase in the rate of carbon dioxide production, but the results shown in Fig. 2 indicate a change in R.Q. as compared with the control. At first the R.Q. of the control is about 1·05 and thereafter falls gradually to a value of about 0·90 after 3 hr. Treatment with ammonium ions leads to a sharp fall to about 0·85 and a subsequent slight rise to a value approaching that of the control.

The relation between the increase of respiration and the uptake of ammonium ions

When replicate samples of a nitrogen-deficient yeast suspension are treated with solutions containing various amounts of ammonium ions a close relationship is found between the increase of respiration and the amount of ammonium ions taken up by the yeast. The results of two similar experiments, shown together in Fig. 3, demonstrate this relationship. The rate of oxygen uptake after ammonium salts have been added is shown in detail for four levels of ammonia nitrogen in Fig. 3*a*, and in Fig. 3*b* total oxygen uptake over 3 hr., is plotted against the level of ammonium ion added to the yeast. From this it appears that there is a critical level of ammonium (approx. 10 mg. N/100 ml. under these conditions) below which variation in ammonium has a marked effect on respiration, and above which further increases of ammonium have no appreciable effect. Consideration

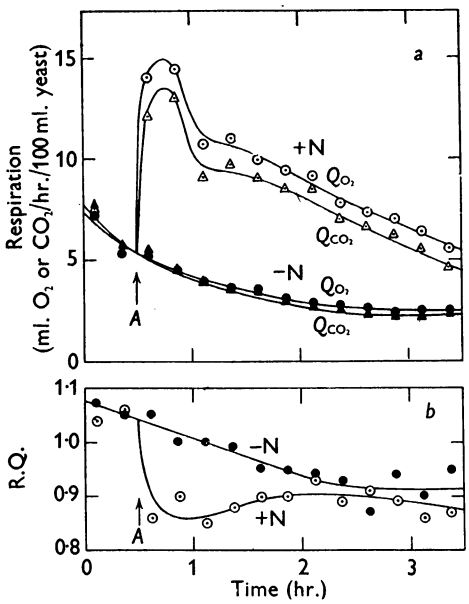

Fig. 2. Changes in respiratory activity of nitrogen-deficient yeast and of yeast treated at *A* with ammonium phosphate (14 mg. N/100 ml. yeast). *a*: rates of oxygen uptake and carbon dioxide output (each based on duplicate samples). *b*: changes in R.Q. (calculated from data of *a* above).

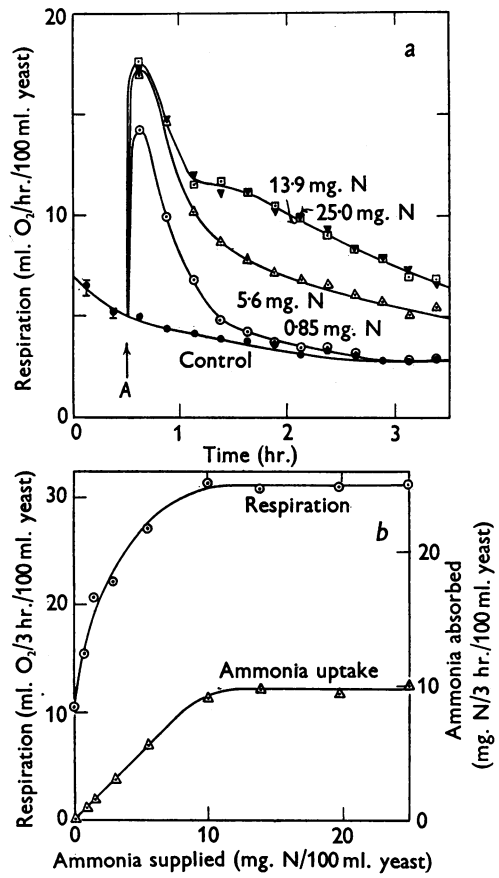

Fig. 3. The effect of varying level of ammonium N upon the respiration and ammonia uptake of nitrogen-deficient yeast. *a*: changes in respiration rate after adding (at *A*) mixtures of sodium and ammonium phosphates containing the stated amounts of N/100 ml. yeast suspension. The control was treated with sodium phosphate only. *b*: uptake of oxygen and ammonia N in 3 hr. after treatment with various levels of ammonium phosphate. The respiratory results are derived in part from those given in *a* (above). Ammonia uptake was estimated from the free ammonia remaining after 3 hr.

of the data on ammonium taken up by the yeast, also presented in Fig. 3*b*, shows that this critical level coincides with the level of ammonium which can be completely assimilated by the yeast during the period of the experiment. Below the critical level, all the ammonium is absorbed, while an increase in concentration above the level results in no further uptake of ammonium. The increase in respiration can therefore be related to the absorption of ammonium ions.

The curves of Fig. 3*a*, showing oxygen uptake at different levels of ammonium supply, support this interpretation. Little difference is apparent in the curves for 13·9 and 25·0 mg. N/100 ml. during the 3 hr. period, and in this range of concentration free ammonia is still available at the end of the experiment. At 5·6 mg. N/100 ml., the ammonium supply is adequate to maintain a high rate of oxygen uptake over the first half hour only, and the respiration rate subsequently falls to a lower level than that observed at 13·9 or 25·0 mg. N/100 ml. In this case all the ammonia nitrogen was exhausted by the end of the experiment. At the lowest ammonium level the highest rates of respiration are never reached, or at least not maintained over 15 min., and there is every indication that the ammonia nitrogen is rapidly utilized; the rate of oxygen uptake falls to that of the control after about 1·5 hr.

A level of about 14 mg. N/100 ml. yeast has been used in all experiments in which an adequate ammonium supply was required.

The effect of feeding with sugar upon the composition and metabolism of Torulopsis utilis

The physiological state of the yeast has a decisive effect on the metabolic changes which follow the addition of ammonia. When yeast is transferred from the complete nutrient solution *A* to one containing nutrient salts only (solution *C*), its respiration is not significantly affected by the presence or absence of ammonium salts. If, however, a brief treatment in the nitrogen-deficient, high-carbohydrate solution *B* is interposed, a typical increase in the rate of oxygen uptake is observed as already described. The changes which take place during the period of sugar feeding in solution *B* have been investigated by removing samples at 4-hourly intervals for analysis and measurement of +N and −N respiration. The results of one such experiment are given in Table 2. A small correction for sampling errors has been made by assuming that the total nitrogen of the yeast remained constant throughout the period of sugar feeding.

An important effect of sugar feeding upon the composition of the yeast is upon the partition of nitrogen between soluble and insoluble (mainly protein) fractions. During the first 4 hr. the level of soluble nitrogen falls to about half its original value and thereafter is maintained at this low value. A further result of sugar feeding is an increase both in dry weight and in carbohydrate content. The increase of dry weight cannot be attributed entirely to the accumulation of carbohydrates, and, while it may be partly due to the uptake of mineral ions, it is probable that formation of fat reserves also occurs. Some further evidence on this point will be given later, but it is well established that in some yeasts fat may constitute up to 12% of the fresh weight, and the amount is greatest under conditions of high carbohydrate, high phosphate, low nitrogen and high aeration (Smedley-Maclean & Hoffert, 1923).

Successive periods of sugar feeding lead to increased rates of oxygen uptake upon removal of the yeast to a sugar-deficient medium either with or

Table 2. *The effect of feeding with sugar on the composition and metabolism of yeast*

A 1% suspension of freshly grown *T. utilis* in soln. *B* was treated in the standard manner for the preparation of N-deficient yeast (see Material and Methods). At intervals, 25 ml. samples were taken, washed at the centrifuge and re-suspended in 25 ml. soln. *C*. Portions of this suspension were used for analyses of composition, and for metabolic studies with or without ammonia as described in Table 1. O_2 uptake was measured for 3 hr. and ammonia assimilated was estimated from the residual ammonia at the end of this period.

Duration of sugar feeding (hr.) ...	*0·3*	*4·3*	*8·3*	*12·3*	*16·3*
Composition (mg./100 ml. yeast suspn.)					
Fresh weight	1210	1550	1920	2130	2320
Dry weight	201	300	379	463	520
Carbohydrate (anthrone method, as glucose)	37	107	170	207	239
Dry matter other than carbohydrate (by difference)	164	193	209	256	281
Soluble nitrogen	2·1	1·1	1·0	1·0	1·0
Protein nitrogen	11·1	12·1	12·2	12·2	12·2
Metabolism (in 3 hr./100 ml. yeast suspn.)					
Respiration, −N (ml. O_2)	4·8	10·5	11·4	12·8	13·4
Respiration, +N (ml. O_2)	4·9	14·6	24·8	30·3	34·8
Difference in respiration between +N and −N (ml. O_2)	0·1	4·1	13·4	17·5	21·4
Ammonia assimilated, +N (mg. N)	0·1	3·2	7·3	9·0	10·3

without ammonia nitrogen. While the growth of the yeast probably accounts largely for the increased respiration in the absence of ammonium, the increase of respiration of the ammonia-treated yeast is much greater, and large differences in respiratory rate between the two treatments soon develop. These differences are closely correlated with an increase in the amount of ammonia nitrogen assimilated by the yeast. Thus the yeast, soon after removal from the nitrogen-rich solution *A*, shows little difference in rate of respiration between the +N and −N treatments and only negligible amounts of ammonia nitrogen are taken up, while after further sugar feeding both the difference in rates of oxygen uptake and the amount of ammonium assimilated show large increases. It is noteworthy that the greatest of these increases coincides with a period (0–8 hr.) in which changes in carbohydrate, total dry matter and nitrogenous constituents are greatest. The results clearly indicate that a high reserve of carbohydrate together with a low level of soluble nitrogen are two important factors influencing the assimilation of nitrogen and the associated changes in cellular respiration.

Changes in the composition of Torulopis utilis *during the assimilation of ammonium salts*

The changes of some of the nitrogenous constituents of this yeast during active assimilation of ammonia, under conditions similar to those used here, have already been described by Roine (1947) and by Virtanen *et al.* (1949). In the experiments described in this section the analyses were extended to include the soluble and insoluble carbohydrates of the yeast in order to give a more comprehensive account of the metabolic changes and their relation to cellular respiration.

Several experiments have given essentially similar results and those from one experiment are presented in Figs. 4 and 5. Samples were taken for analysis before ammonia treatment and 0·5, 1, 2 and 3 hr. after the addition of ammonium phosphate. A yeast deprived of nitrogen was also sampled after 3 hr. The respiratory behaviour determined from parallel samples, has been summarized in part in Table 1 (Expt. 3) and was very similar to that illustrated in Fig. 2.

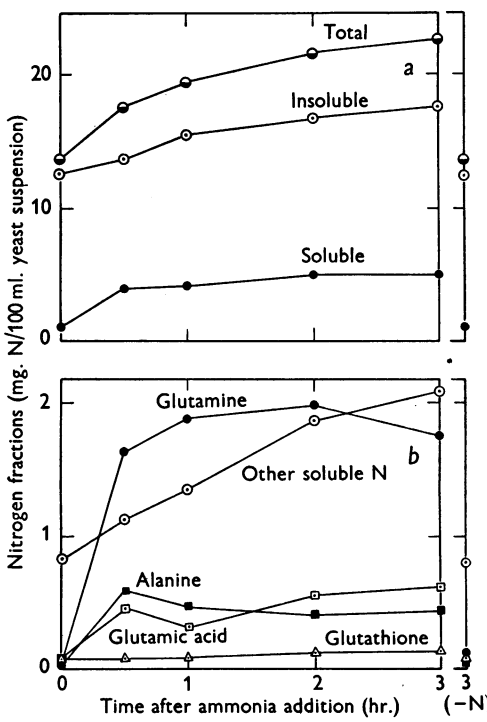

Fig. 4. Changes in nitrogenous fractions during the assimilation of ammonia N by nitrogen-deficient yeast. *a*: changes in total-nitrogen, insoluble- (protein) and soluble-nitrogen fractions. *b*: the major components of the soluble fraction. The corresponding levels in yeast treated with sodium phosphate (−N) for 3 hr. are indicated to the right of the figure.

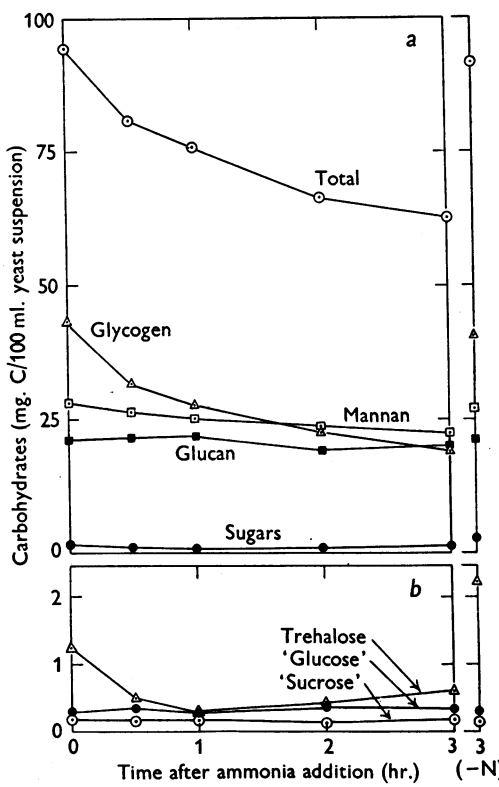

Fig. 5. Changes in carbohydrate fractions accompanying the changes in nitrogenous fractions shown in Fig. 4. *a*: the major carbohydrate fractions. *b*: the soluble sugars.

During the treatment with ammonium phosphate, nitrogen is assimilated by the yeast into a number of nitrogenous compounds. This is clear from Fig. 4. Both the insoluble and soluble nitrogen fractions show increases, although most of the increase in total nitrogen in the first half hour is attributable to the soluble fraction, whereas subsequently the insoluble fraction shows the main increase. As the soluble nitrogen has been corrected for free ammonia nitrogen, the increases in both total nitrogen and soluble nitrogen demonstrated here represent the incorporation of nitrogen into organic compounds and not merely the accumulation of ammonium ions within the cell. Indeed, as Roine (1947) has already reported, much of the increase in soluble nitrogen over the first 30 min. of assimilation is due to the synthesis of three compounds: glutamic acid, glutamine and alanine. In three similar experiments these compounds account for 90–100% of the increase in soluble nitrogen and 65–70% of the increase in total nitrogen over this first period, a period in which the rate of respiration is greatest. In later periods the levels of these compounds are, in general, maintained and further nitrogen assimilation is reflected mainly in increases in the insoluble protein nitrogen. This is especially marked in the period 0·5–1 hr. after addition of ammonia. Glutathione shows little change over the 3 hr. period of the experiment, and other nitrogenous compounds increase steadily throughout. The nature of these compounds was investigated both by examination for stable amide nitrogen and by chromatography. No amide other than glutamine could be detected in the former test so the traces of substances (R_F 0·48) appearing on the chromatogram may well have been glycine (R_F 0·49) and not asparagine (R_F 0·46). The only other coloured spot not accounted for by the analyses was one with R_F 0·70 (probably valine or methionine). In the −N yeast no appreciable change in any of the nitrogenous fractions was detectable over the period of the experiment.

The changes in the level of the carbohydrate fractions, given in Fig. 5, show that the assimilation of nitrogen is associated with high rates of carbohydrate breakdown. Glycogen is the main component disappearing, although mannan is also important. The composition of these two fractions was checked by chromatography of the hydrolysed fractions, and, as reported for baker's yeast by Trevelyan & Harrison (1952), they were found to yield predominantly (at least 70%) glucose and mannose respectively. The experimental error in the determination of glucan renders accurate assessment impossible, but it seems unlikely that it can make more than a minor contribution to the total carbohydrate disappearance, and the fact that this fraction is localized in the outer cell wall (Northcote & Horne, 1952) also suggests that it is not readily mobilized. Changes in the soluble sugars are very small and only trehalose is of importance, and that mainly during the first 30 min. period. Only trehalose, together with small amounts of glucose, could be detected chromatographically and the identity of the fraction reported as 'sucrose' is uncertain. More striking changes in the soluble sugars are apparent in the −N yeast where trehalose accumulates while the insoluble carbohydrates show only small decreases.

An interesting feature of these results is that, while the +N yeast exhibits a rate of respiration approximately three times that shown by the −N yeast, the losses in total carbohydrate over 3 hr. are 10 times as great in the +N yeast as in the −N. A balance sheet of the carbon changes drawn up in Table 3 throws some further light on this problem. In the absence of a full identification of the nitrogenous and other constituents of the yeast, such a balance sheet can only be approximate. Of the soluble fraction only the 'other soluble nitrogen' was in doubt and this was assumed to have a C/N ratio of 4/1. The insoluble fraction was considered to be a typical plant cytoplasmic protein (cf. Lindan & Work, 1951; Yemm & Folkes, 1953) and its carbon content was calculated on this basis. It is evident that the synthesis of amino acids and of proteins represents an important drain upon the total carbon available; roughly twice as much is used for this purpose as is lost as respiratory carbon dioxide. In the −N yeast slight protein breakdown occurred and this has been allowed for in the balance sheet.

The carbon derived from losses in carbohydrates is insufficient to account for that used in the synthesis of the nitrogenous compounds and for respiration. Carbon from some other source must

Table 3. *Carbon balance sheet for yeast treated for 3 hr. with sodium phosphate* (−N) *or with a mixture of sodium and ammonium phosphates* (+N)

Calculations are made from the experimental data given, in part, in Table 1 (Expt. 3), and in Figs. 4 and 5. For details see Results section.

	Changes during 3 hr. (mg. C/100 ml. yeast suspension)			
	−N		+N	
Products				
Respiratory CO_2		5·9		14·6
Syntheses				
Soluble amino acids	0·1		11·3	
Proteins	−0·6	−0·5	15·4	26·7
Total		5·4		41·3
Losses				
Carbohydrates		3·0		30·4
Other C compounds (to balance)		2·4		10·9
Total		5·4		41·3

also be available for metabolism and, as already suggested, fats may be important in this connexion. No direct determinations of fat content were made in these experiments, but an indirect estimate may be made from the R.Q. measurements after correcting for the changes in degree of oxidation involved in the synthesis of amino acids and proteins. Assuming an R.Q. of 0·7 for complete fat oxidation in the yeast (Daubney & Smedley-Maclean, 1927) it was calculated that over the 3 hr. period of the experiment, fat contributed about 0·4 mg. carbon in the −N yeast and 5·9 mg. carbon in the +N yeast. These estimates are lower than the estimates of 'other carbon compounds' made from the balance sheet: but it seems probable that fat contributes to the respiratory substrate under these conditions.

From the above it is apparent that the high rates of oxygen uptake accompanying nitrogen assimilation in low nitrogen yeasts are associated with the utilization of fats and carbohydrates, chiefly glycogen and mannan, and with the synthesis of soluble amino acids and, later, of proteins. Of the soluble compounds glutamic acid and glutamine are important and it is of interest that the accumulation of these compounds can be shown to occur in ammonium-treated samples not only of two strains of *Torulopsis utilis* but also of baker's yeast (see Table 1).

The uncoupling of nitrogen assimilation and respiration by means of 2:4-dinitrophenol

From these results it is evident that the assimilation of ammonia nitrogen, involving the synthesis of amino acids and proteins, is closely coupled with cellular respiration. Clifton (1946) has shown that several compounds, such as 2:4-dinitrophenol (DNP), strongly inhibit assimilation in micro-organisms under conditions in which respiration is much less affected. The observations of Massart & Heymans (1950*b*) with baker's yeast suggest a similar effect of dinitrophenols on the assimilation of nitrogen. After the work of Loomis & Lipmann (1948), it has been generally supposed that the differentiating effect of DNP on assimilation and respiration arises from an uncoupling of oxidation and phosphorylation, since the latter is now regarded as one of the chief ways in which respiratory energy is transferred to anabolic processes of the cell. It was of interest, therefore, to examine the effects of DNP on the metabolism of food yeast under some of the different conditions of nutrition which have been studied here.

For this purpose manometric experiments were performed in which various levels of DNP were added together with the sodium phosphate or sodium and ammonium phosphate mixtures. Measurements of oxygen uptake were continued for 3 hr. after the addition, and samples were then removed from the manometric flasks for estimates of carbohydrate loss, ammonia nitrogen assimilated, and of glutamic acid, free and as glutamine. Curves of oxygen uptake at three levels of DNP are given in Fig. 6 and the combined results of three similar experiments are shown together in Fig. 7.

It is apparent from the graphs in Fig. 6 that the addition of DNP to nitrogen-deficient yeast in the absence of added ammonium salts leads to very marked increases in respiration, of the same order as

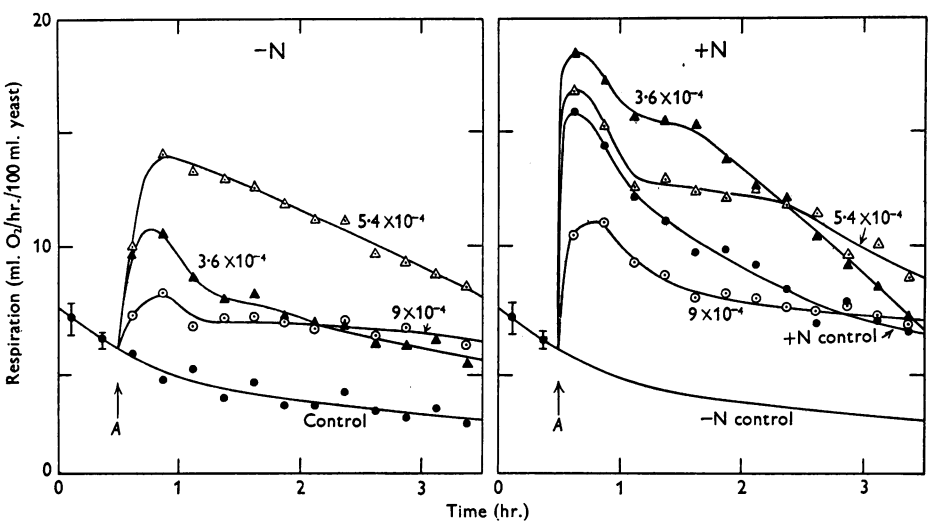

Fig. 6. Changes in respiration rate after adding (at *A*) DNP at the final molar concentrations shown, together with sodium phosphate (−N) or a mixture of sodium and ammonium phosphates (+N) to nitrogen-deficient yeast. The controls had no DNP addition.

those observed after addition of ammonia nitrogen in the absence of DNP, but somewhat different in form. These increases in the rate of oxygen uptake are greatest at the $5{\cdot}4 \times 10^{-4}$M level of DNP, and higher concentrations give progressively smaller increases: an inhibitory effect on oxygen uptake has been observed in other experiments at $1{\cdot}5 \times 10^{-3}$M and above.

When the data in Fig. 6 for samples in which DNP and ammonium were added together are considered, it is apparent that the separate effects of DNP and ammonia are not additive when the two are supplied together. Moreover, higher rates of oxygen uptake are observed with $3{\cdot}6 \times 10^{-4}$M DNP than with $5{\cdot}4 \times 10^{-4}$M, at least for the first 2 hr. after addition of DNP, the latter concentration giving somewhat higher rates subsequently. At 9×10^{-4}M the DNP is definitely inhibitory as compared with the +N control, although it still allows slightly higher rates of respiration than the same level of DNP gives in the −N series. The relationships underlying these data are obviously complex, but the chief features are more apparent from Fig. 7, where total changes over the 3 hr. after DNP addition are summarized.

The difference in sensitivity to DNP of the respiration of +N- and −N-treated yeasts can be seen from Fig. 7*a*. The +N yeast has a maximum oxygen uptake at 1–2×10^{-4}M DNP and a gradually decreasing rate at higher levels, whereas the optimum DNP level for the −N yeast is at about 5×10^{-4}M. On the other hand, far larger increases in respiration are observed in the −N yeast, although it never reaches the high levels of oxygen uptake observed in the +N series. The subsequent decline in rate of respiration in both +N and −N series is similar to that observed by Simon (1953*a*, *b*) for yeast with 3:5-dinitro-*o*-cresol in the presence and absence of external carbohydrate and suggested by him to be due to inhibition of electron-transfer systems in the yeast. The slight divergence in the two curves given in Fig. 7*a* is not necessarily in conflict with this hypothesis, as it may be due to slight differences in pH between the +N and −N cultures, leading to differences in the effective concentrations of DNP in the two series (Simon & Beevers, 1952). While all samples started at pH 6·0 and the pH of the −N series remained unchanged throughout the experiment, that of the +N series tended to become more acid in spite of the presence of phosphate buffer and pH's as low as 5·6 were recorded at the end of the experiment.

Definite evidence is given in Fig. 7*b* for an uncoupling of nitrogen assimilation and respiration. Low levels of DNP, while leading to somewhat increased rates of respiration, cause marked decreases in the rates of assimilation of nitrogen as evidenced both by total uptake of ammonia nitrogen and by the synthesis of glutamic acid. Nitrogen assimilation is almost completely inhibited at 10^{-3}M DNP, while at this level oxygen uptake is still 70% of that in the +N control.

The data for losses of carbohydrate during the 3 hr. of DNP treatment and presented in Fig. 7*c* are of interest in comparison with those of oxygen uptake given in Fig. 7*a*. Over the range of lower levels of DNP the effects are essentially similar, but at higher levels they differ in that, whereas the curve of oxygen uptake shows a pronounced maximum at 5×10^{-4}M DNP, the curve of carbohydrate loss continues to climb at this point and tends towards the steady level achieved at about the same DNP concentration by the +N series. Thus over a wide range of the higher DNP concentrations, carbo-

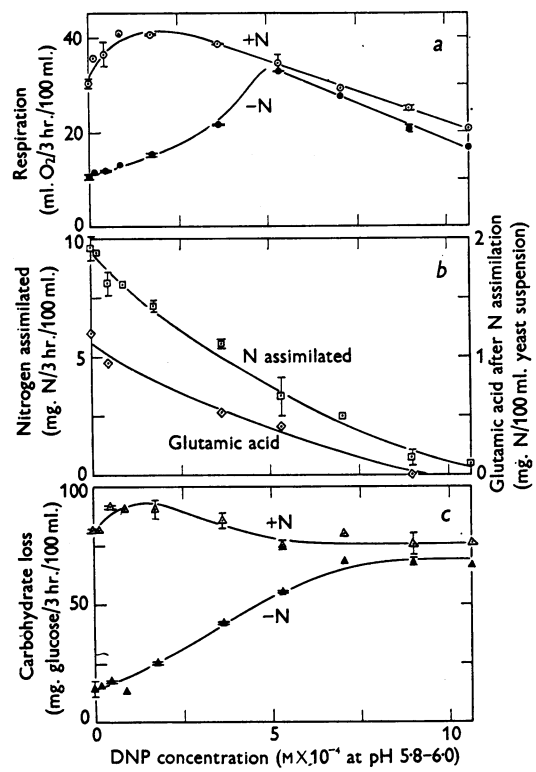

Fig. 7. The effect of DNP upon the metabolism of nitrogen-deficient yeast treated at the same time with sodium phosphate (−N) or with a mixture of sodium and ammonium phosphates (+N). All results represent total changes in 3 hr. after the addition of DNP and are from three similar experiments. Where results at any concentration of DNP are derived from more than one experiment the range of values is shown. *a*: total oxygen uptake/3 hr. in +N and −N yeast. These results are derived in part from those of Fig. 6. *b*: total ammonia N assimilated and increase of glutamic acid N in 3 hr. in the +N yeast. *c*: losses of total carbohydrate in +N and −N yeasts in 3 hr.

hydrate losses are being increased or maintained in both −N and +N conditions while oxygen uptake steadily decreases with increasing DNP. This implies an increasing component of fermentation; the ability of DNP to promote fermentation has been noted for yeast by Clifton (1946) and Simon (1953*a*, *b*) and for higher plants by Beevers (1953).

It is clear, therefore, that the process of assimilation of nitrogen responds in a similar manner to other assimilation processes (Clifton, 1946) to the action of DNP. The most important similarity is that an uncoupling of respiration and assimilation is apparent, both in the sense that assimilation is inhibited while oxygen uptake is little affected, and also that the high rates of respiration normally associated with the assimilation of ammonia nitrogen can be induced by DNP in the absence of ammonium.

DISCUSSION

The experiments described here have shown that, during the assimilation of ammonia nitrogen, there is at first a synthesis of amino acids and amides, largely identified as glutamic acid, glutamine and alanine. To this extent the results are in agreement with those of Roine (1947), who used somewhat different methods, and the three constituents given above account for 65–70% of the total nitrogen assimilated over the initial period of 30 min. Subsequently, the synthesis of proteins predominates, although the soluble nitrogenous fractions are maintained at a high level. Associated with these syntheses, high rates of cellular respiration are always observed, together with a breakdown of carbohydrates, mainly glycogen and mannan, in the yeast. There is some evidence that fats are also actively metabolized during the period of rapid assimilation of nitrogen. Many of the experiments indicate that these changes are coupled in the cellular mechanisms; for example, the increase in oxygen uptake can be closely correlated with the amount of nitrogen assimilated, which itself depends upon the level of available carbohydrate reserves. Moreover, uncoupling agents, such as DNP, are found to inhibit nitrogen assimilation while promoting oxygen uptake.

The important position of glutamic acid is apparent from the experimental results in which glutamine, together with free glutamic acid, are at first the chief products of the assimilation of nitrogen. Enzymic systems which catalyse the primary synthesis of both the free amino acid and the amide from ammonia nitrogen, have been separated from yeast by Adler, Günther & Everett (1938) and by Elliot (1951). In addition, it is well established that glutamic acid reacts in the synthesis of many amino acids by transamination and other reactions (Braunstein, 1947; Tatum, 1949). Transaminases leading to the synthesis of alanine and aspartic acid have been demonstrated in yeast by Roine (1947), and it is probable that alanine detected in the present experiments was formed in this way. Extensive synthesis of other amino acids is clearly indicated by the increase in protein in the later stages of assimilation, and recent theories (Fruton, 1950; Hanes, Hird & Isherwood, 1950) suggest that glutamine may be an important reactant in the formation of peptide bonds during this synthesis of protein. It is noteworthy that, despite the drain upon them by these reactions, both glutamic acid and glutamine are maintained at a high level within the cell throughout the period of active protein synthesis.

The highest rates of respiration are observed during the initial period of synthesis of amino acids and amides, and the relationship between this synthesis and the breakdown of carbohydrates is of primary importance. Elliot (1951) has shown that the synthesis of glutamine from glutamic acid and ammonia is enzymically coupled with the breakdown of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). Since, during glycolysis and respiration (the term 'glycolysis' is used throughout this discussion in a general sense to denote breakdown of sugars to C_3 compounds common to both fermentation and respiration), ATP is regenerated from ADP (Lipmann, 1941), this may constitute a direct energy link between cellular oxidations and the synthesis of the amide, and hence, by transfer reactions, of peptide bonds. The importance of phosphorylations as a link between respiration and assimilation, or other synthetic reactions, is widely accepted and has been discussed in relation to peptide synthesis by Lipmann (1949). In the present experiments further evidence of the linkage was obtained by the use of DNP. Although the exact mode of action of this inhibitor is uncertain, it is well established that it uncouples oxidation and phosphorylations in cell-free preparations. The selective inhibition of ammonia uptake by low concentrations of DNP is fully consistent with the importance of phosphorylations in the assimilation of nitrogen.

There are, however, two other ways in which the assimilation of nitrogen may interact with glycolysis and respiration. The synthesis of glutamic acid by the action of glutamic acid dehydrogenase is a reductive amination involving the oxidation of a phosphopyridine nucleotide. It is thus directly linked through the hydrogen-carrier systems with the oxidation of substrates in the cell. Hence the system synthesizing glutamic acid can be regarded as an accessory hydrogen-acceptor diverting electrons at a high level of oxidation–reduction energy and thus excluding oxidative phosphorylations normally associated with aerobic systems. As

the amino groups of many amino acids may be derived from glutamic acid, these reactions may achieve significant proportions during protein synthesis. For example, it can be calculated from the analytical data presented in Fig. 4 and Table 1, that, during a period of nitrogen assimilation lasting 3 hr., approximately one-sixth of the total electron transfer is utilized for amino acid synthesis rather than passed to atmospheric oxygen.

A third way in which the assimilation of nitrogen and the metabolism of carbohydrates are closely related is in the carbon skeletons of newly synthesized amino acids. Glutamic acid and alanine, the two amino acids most rapidly synthesized in the early stages of assimilation, are in all probability derived from α-oxoglutaric acid and pyruvic acid, both of which are well established intermediaries in the respiratory breakdown of carbohydrates. Krebs, Gurin & Eggleston (1952) have recently discussed the occurrence and significance of the tricarboxylic acid cycle in yeast and have emphasized its importance as a source of reactants for cellular syntheses. The present results strongly support this view; the carbon balance sheet drawn up in Table 3 shows that, of the increases in carbon consumption during nitrogen assimilation, 2–3 times as much is utilized for amino acid and protein syntheses as undergoes complete oxidation to carbon dioxide. A high proportion of the carbon diverted from oxidation in this way is derived from acids of the tricarboxylic acid cycle; for example, 26% can be accounted for by the glutamic acid and alanine accumulated in the soluble fraction alone.

The data presented here offer many similarities to those now available for higher plants and for other micro-organisms. Willis (1951) has shown with roots of barley that high rates of respiration and of carbohydrate breakdown are associated with the assimilation of ammonium and nitrate nitrogen, and that a primary synthesis of glutamine, together with smaller amounts of asparagine, occurs. Syrett (1953*a*, *b*) has obtained very similar results with *Chlorella vulgaris*. Although in the present experiments with yeast no appreciable amounts of asparagine were found, Virtanen *et al.* (1949) have given some evidence that this amide is formed in *Torulopsis utilis* when the latter is supplied with nitrate. Marked increases in oxygen uptake under some conditions have been observed on treatment with ammonia of other yeasts (Winzler *et al.* 1944; Runnström, Brandt & Marcuse, 1940; Massart & Heymans, 1950*a*) and some bacteria (Armstrong & Fisher, 1947; McLean & Fisher, 1947; Burris & Wilson, 1952). Increases of anaerobic glycolysis under similar conditions have also been recorded in yeast by Smythe (1939) and Winzler *et al.* (1944).

These observations of increased rates of carbohydrate breakdown and of oxygen uptake during the assimilation of nitrogen are relevant to the general problem of the regulation of cellular respiration. Much recent evidence converges to show that the rate of cell respiration is closely dependent on assimilation and other endergonic reactions. For example, high rates of respiration or glycolysis have been found to accompany not only nitrogen assimilation but also the assimilation of glucose in yeast (Simon, 1953*a*), the anabolic processes of young seedlings (Folkes, Willis & Yemm, 1952), the accumulation of salts by roots (Milthorpe & Robertson, 1948; Robertson, Wilkins & Weeks, 1951), and, in animals, muscular (Fenn, 1927) and nervous activity (McIlwain, 1950). Current theories (see, for example, Dixon, 1949) have laid great stress on phosphorylation as the underlying mechanism whereby catabolic processes are regulated to meet the energy requirements of synthesis and other functional activities of the cell. It is generally considered that the reversible reactions which make up the early stages of carbohydrate breakdown are controlled by the availability of free phosphate and/or suitable phosphate acceptors which may, in turn, depend upon their rates of formation from high energy phosphate compounds in endergonic reactions. These views have been put for nitrogen assimilation by Sussman & Spiegelman (1951) and by Syrett (1953*b*), and the increases in respiration and glycolysis observed in the present experiments with ammonia and with DNP can be interpreted along these lines: both DNP and ammonium may decrease the level of high-energy phosphate compounds, the one by uncoupling their formation and the other by providing phosphate-acceptor reactions in protein synthesis.

On such a theory, however, it is difficult to interpret completely the effects of DNP, both in the presence and absence of ammonia. There is some evidence (see Fig. 7) that both the uptake of oxygen and the breakdown of carbohydrate are inhibited by higher levels of DNP, although glycolysis is clearly less sensitive than respiration especially in the absence of ammonia. These inhibitory effects of DNP are particularly evident on the high metabolic rates observed during the assimilation of nitrogen and it is probably under these circumstances that other DNP-sensitive reactions become rate-limiting in carbohydrate breakdown and oxygen uptake. For example, Haas, Harrer & Hogness (1942) have shown that 10^{-3} M 2:4-dinitro-*o*-*cyclo*hexylphenol at pH 8·3 causes 70% inhibition of cytochrome reductase, and Simon (1953*c*) has suggested that this inhibition of flavoprotein enzymes, or of some other system, by dinitrophenols may be important during the high rates of respiration associated with glucose assimilation. A similar interpretation could apply to the present experiment. The action of DNP upon the glycolytic systems is more uncertain;

Bonner (1949) has suggested that in higher plants, a decrease in high-energy phosphate compounds may limit the initial phosphorylation of carbohydrates. But it is clear from our experiments that many other interactions are involved. The most substantial inhibition of carbohydrate breakdown by DNP occurs between $1{\cdot}5 \times 10^{-4}$M and 5×10^{-4}M in the presence of ammonia, and is associated with a marked decrease in the rate of nitrogen assimilation. Over the full range of DNP concentrations investigated in the presence of ammonia, there is a striking change in the fate of the products of glycolysis: in the absence of DNP, as already stated, about two-thirds of the glycolysis products are utilized for the synthesis of amino acids and proteins whereas, with 10^{-3}M DNP, only negligible amounts are utilized in this way, and at least two-thirds of the products of glycolysis appear to be diverted to fermentation reactions. While this implies an extensive switch in the fate of these products and in the enzymic mechanisms involved, there are several similarities between reductive amination and the dismutations associated with the formation of ethanol in fermentation. Both reactions involve a diversion of glycolysis products and of electrons from the normal aerobic channels of oxidative phosphorylation. In this regard the increased rates of glycolysis during the assimilation of nitrogen present a parallel with the Pasteur effect, in which low oxygen tension or inhibition of cellular oxidations leads to alcoholic fermentation and an increase in the rate of carbohydrate breakdown. However, in contrast to fermentation, the reductive synthesis of glutamic acid takes place under aerobic conditions and competes successfully with the electron-transfer systems leading to atmospheric oxygen. It seems probable that the occurrence of such powerful alternative electron-acceptor reactions will increase the proportion of oxidized to reduced phosphopyridine nucleotides and hence will promote the activity of triose phosphate dehydrogenase in glycolysis, whereas anaerobic fermentation will have an opposite effect. In addition, the assimilation of nitrogen generates high energy phosphate acceptors, leading to the synthesis of amide and peptide bonds. These multiple effects on glycolysis and cellular oxidations may well account for the observations that the rate of carbohydrate breakdown is higher during the assimilation of ammonia than it is in yeast treated with DNP alone, and that this rate decreases when nitrogen assimilation is itself inhibited by DNP.

There is little doubt that phosphorylation reactions constitute an important link between the breakdown of carbohydrate in respiration and the endergonic reactions of the cell. However, the present experiments strongly suggest that when the endergonic reactions involve extensive synthesis, other interactions may also be important in regulating cellular respiration and glycolysis. A large part of the products of glycolysis may be used for these syntheses and associated reduction reactions may alter the oxidation–reduction conditions within the cell. All of these components are closely integrated and are brought into action during nitrogen assimilation and protein synthesis. They may be largely responsible for the interdependence, commonly observed in higher plants and micro-organisms, between respiration and the metabolism of nitrogen.

SUMMARY

1. Nitrogen-deficient cultures of food yeast, *Torulopsis utilis*, suspended under aerobic conditions in a carbohydrate-free medium, have been shown to respond rapidly to the addition of ammonium phosphate by increasing their rate of oxygen uptake to 2 or 3 times the previous level. Their carbon dioxide output is also increased but the R.Q. falls from 1·05 to about 0·85.

2. This increase in respiration is accompanied by the assimilation of ammonia and a close quantitative relationship between these metabolic processes can be demonstrated.

3. Nitrogen deficiency can be induced by culture of the yeast in a high-carbohydrate, nitrogen-free solution, and its onset is associated with a marked increase in the level of stored carbohydrate and other materials within the yeast, and with an increase in the protein content at the expense of the soluble nitrogen compounds. This gives a greater capacity for nitrogen assimilation upon treatment with ammonia.

4. The assimilated nitrogen appears mainly as glutamine, alanine, glutamic acid and, later, as protein. These syntheses are accompanied by the breakdown of stored carbohydrates, mainly glycogen and mannan, and of fats.

5. 2:4-Dinitrophenol (DNP) has been found to inhibit the assimilation of nitrogen and to cause increased oxygen uptake and carbohydrate breakdown. At higher concentrations of DNP oxygen uptake also is inhibited and fermentation reactions predominate.

6. These results are discussed with special reference to the problem of the regulation of respiration and glycolysis within the cell. It is suggested that the assimilation of nitrogen may increase the rate of carbohydrate breakdown and of respiration in three ways: the synthesis of amide and peptide bonds may lower the level of high-energy phosphate compounds, the diversion of electrons for reductive amination reactions may alter the oxidation–reduction conditions within the cell, and the synthesis of amino acids and proteins may make large demands upon the organic acids produced in the course of glycolysis and respiration.

The authors wish to express their thanks to Dr A. J. Willis for his help with some of the analyses and in the preparation of the manuscript, and to Prof. M. Skene for helpful criticism.

REFERENCES

Armstrong, F. H. & Fisher, K. C. (1947). *J. gen. Physiol.* **30**, 279.

Adler, E., Günther, G. & Everett, J. E. (1938). *Hoppe-Seyl. Z.* **255**, 27.

Beevers, H. (1953). *Amer. J. Bot.* **40**, 91.

Bonner, J. (1949). *Amer. J. Bot.* **36**, 429.

Braunstein, A. E. (1947). *Advanc. Protein Chem.* **3**, 1.

Burris, R. H. & Wilson, P. W. (1952). *Biochem. J.* **51**, 90.

Clayton, J. C., Hems, B. A., Robinson, F. A., Andrews, R. D. & Hunwicke, R. F. (1944). *Biochem. J.* **38**, 452.

Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.

Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, 3rd ed. London: Crosby Lockwood.

Daubney, C. G. & Smedley-Maclean, I. (1927). *Biochem. J.* **21**, 373.

Dixon, M. (1943). *Manometric Methods*, 2nd ed. Cambridge University Press.

Dixon, M. (1949). *Multi-enzyme Systems*, Cambridge University Press.

Dohan, J. S. & Woodward, G. E. (1939). *J. biol. Chem.* **129**, 393.

Elliot, W. H. (1951). *Biochem. J.* **49**, 106.

Fenn, W. O. (1927). *Amer. J. Physiol.* **83**, 309.

Folkes, B. F. (1953). *Analyst*, **78**, 496.

Folkes, B. F., Willis, A. J. & Yemm, E. W. (1952). *New Phytol.* **51**, 317.

Fruton, J. S. (1950). *Yale J. Biol. Med.* **22**, 263.

Grunert, R. R. & Phillips, P. H. (1951). *Arch. Biochem.* **30**, 217.

Haas, E., Harrer, C. J. & Hogness, T. R. (1942). *J. biol. Chem.* **143**, 341.

Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.

Krebs, H. A. (1948). *Biochem. J.* **43**, 51.

Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.

Lindan, O. & Work, E. (1951). *Biochem. J.* **48**, 337.

Lipmann, F. (1941). *Advanc. Enzymol.* **1**, 99.

Lipmann, F. (1949). *Fed. Proc.* **8**, 597.

Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.

McIlwain, H. (1950). *Brit. med. Bull.* **6**, 301.

McLean, D. J. & Fisher, K. C. (1947). *J. Bact.* **54**, 599.

Massart, L. & Heymans, P. (1950*a*). *Bull. Soc. Chim. biol., Paris*, **32**, 879.

Massart, L. & Heymans, P. (1950*b*). *Bull. Soc. Chim. biol., Paris*, **32**, 888.

Milthorpe, J. & Robertson, R. N. (1948). *Aust. J. exp. Biol. med. Sci.* **26**, 189.

Northcote, D. H. & Horne, R. W. (1952). *Biochem. J.* **51**, 232.

Robertson, R. N., Wilkins, M. J. & Weeks, D. C. (1951). *Aust. J. sci. Res.* B**4**, 248.

Roine, P. (1947). *Ann. Acad. Sci. fenn.* Ser. A, **2**, *Chem.* no. 26.

Runnström, J., Brandt, K. & Marcuse, R. (1940). *Ark. Kemi Min. Geol.* B**14**, No. 8.

Simon, E. W. (1953*a*). *J. exp. Bot.* **4**, 377.

Simon, E. W. (1953*b*). *J. exp. Bot.* **4**, 393.

Simon, E. W. (1953*c*). *Biol. Rev.* **28**, 453.

Simon, E. W. & Beevers, H. (1952). *New Phytol.* **51**, 163.

Smedley-Maclean, I. & Hoffert, D. (1923). *Biochem. J.* **17**, 720.

Smythe, C. V. (1939). *Enzymologia*, **6**, 9.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.

Sussman, M. & Spiegelman, S. (1951). *Arch. Biochem.* **29**, 85.

Syrett, P. J. (1953*a*). *Ann. Bot.* **17**, 1.

Syrett, P. J. (1953*b*). *Ann. Bot.* **17**, 21.

Tatum, E. L. (1949). *Fed. Proc.* **8**, 511.

Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.

Virtanen, A. I., Csaky, T. Z. & Rautanen, N. (1949). *Biochim. biophys. Acta*, **3**, 208.

Willis, A. J. (1950). Nitrogen assimilation and respiration in barley. Ph.D. Thesis, University of Bristol.

Willis, A. J. (1951). *Biochem. J.* **49**, xxvii.

Willis, A. J. & Yemm, E. W. (1954). *New Phytol.* (in the Press).

Winzler, R. J., Burk, D. & du Vigneaud, V. (1944). *Arch. Biochem.* **5**, 25.

Yemm, E. W. (1949). *New Phytol.* **48**, 315.

Yemm, E. W. & Folkes, B. F. (1953). *Biochem. J.* **55**, 700.

The Estimation of Carbohydrates in Plant Extracts by Anthrone

By E. W. YEMM and A. J. WILLIS
Department of Botany, University of Bristol

(*Received* 4 *January* 1954)

The green colour produced when carbohydrates are heated with anthrone in acid solution was first used as a qualitative test by Dreywood (1946); since then anthrone has been extensively employed as a convenient and specific reagent for the colorimetric estimation of a variety of carbohydrates (see, for example, Viles & Silverman, 1949; Seifter, Dayton, Novic & Muntwyler, 1950; Trevelyan & Harrison, 1952). There has, however, been no systematic investigation of the reaction with the different hexose and pentose carbohydrates which may be encountered in biological material. Moreover, different conditions have been used for the reaction and the extent to which sugars may interfere with one another when present in mixtures is uncertain. Johanson (1953*a*) has, in fact, recently stated that