SUMMARY

1. An acetone extract of the livers of rats dosed with *OO*-diethyl *O*-*p*-nitrophenyl thiophosphate (parathion) has been made, and by paper chromatography the existence of a labile derivative of *p*-nitrophenol which strongly inhibits cholinesterase *in vitro* has been detected.

2. This extract has been purified by chromatography on a cellulose powder column, and the identity of the inhibitor with diethyl *p*-nitrophenyl phosphate (paraoxon) has been suggested by a comparison of their migration on filter paper, their absorption spectra, their hydrolysis constants and their anticholinesterase activities.

I wish to express my thanks to Messrs Albright and Wilson for a supply of pure parathion, to Wellcome Research Laboratories for a regular supply of horse erythrocytes, and to Miss Sylvia Morrissey for assistance in the experimental work.

REFERENCES

Aldridge, W. N. (1951). *Biochem. J.* **49**, i.
Binkley, F. (1949). *J. biol. Chem.* **181**, 317.
Chamberlain, W. F. & Hoskins, W. M. (1951). *J. econ. Ent.* **44**, 177.
Diggle, W. M. & Gage, J. C. (1951*a*). *Biochem. J.* **49**, 491.
Diggle, W. M. & Gage, J. C. (1951*b*). *Nature, Lond.*, **168**, 998.
Gage, J. C. (1952). *Analyst*, **77**, 123.
Gage, J. C. & Payton, J. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 433.
Grob, D., Garlick, W. A. & Harvey, A. M. (1950). *Johns Hopk. Hosp. Bull.* **87**, 106.
Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **46**, 451.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Mounter, L. A. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 525.

The Assimilation of Amino-acids by Micro-organisms

16. CHANGES IN SODIUM AND POTASSIUM ACCOMPANYING THE ACCUMULATION OF GLUTAMIC ACID OR LYSINE BY BACTERIA AND YEAST

By R. DAVIES, JOAN P. FOLKES, E. F. GALE and LOIS C. BIGGER
Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, University of Cambridge

(*Received* 31 *October* 1952)

Many Gram-positive bacteria and yeasts are able to effect a high concentration of certain amino-acids in the free state within the cell (Gale, 1947; Taylor, 1947). In *Streptococcus faecalis* and *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) lysine enters the cell by diffusion, but the accumulation of glutamic acid only takes place if exergonic metabolism such as glucose fermentation is occurring (Gale, 1947; Najjar & Gale, 1950). In yeasts the accumulation of both lysine and glutamic acid occurs only during the metabolism of glucose (Taylor, 1949). Accumulation of free glutamic acid has been observed in a number of mammalian tissues (Krebs, Eggleston & Hems, 1949) and Terner, Eggleston & Krebs (1950) found that brain cells lose potassium ions when incubated with glucose whereas if glutamic acid is also present, this loss of potassium is prevented. Pulver & Verzár (1940) have shown that potassium leaks from yeast cells during incubation in water and that this leakage is prevented by the fermentation of glucose, but no information is available concerning the changes in sodium or potassium in bacterial or yeast cells during accumulation of amino-acids. Roberts, Roberts & Cowie (1949), Cowie, Roberts & Roberts (1949) and Roberts & Roberts (1950) have shown that sodium and potassium diffuse readily into the 'water space' of *Escherichia coli* and that, in the presence of glucose, potassium enters into non-ionic combination with breakdown products of glucose and becomes stored within the cell in a non-diffusible form.

Christensen & Riggs (1952) and Christensen, Riggs, Fischer & Palatine (1952*a*, *b*) have found that mouse ascites carcinoma cells concentrate a number of amino-acids, including the D-isomers and 'unnatural' amino-acids such as $\alpha\gamma$-diaminobutyric acid. The accumulation of glycine is accompanied by a loss of potassium from the cells, a gain of sodium and osmotic swelling of the cells. Most of the amino-acids tested displace potassium from the cells, this displacement being almost complete in the case of diaminobutyric acid.

The present investigation was undertaken to determine what changes occur in the sodium and potassium content of bacterial and yeast cells during the accumulation within those cells of glutamic acid or lysine.

EXPERIMENTAL

Organisms. Three organisms have been used in the course of these studies: *Strep. faecalis* ST used by Gale (1947) for the initial investigations on glutamic acid and lysine accumulation; *Staph. aureus* Duncan used for earlier studies in this series (Gale, 1951; Gale & Folkes, 1953); *Saccharomyces fragilis* Jorgensen obtained from the Delft C.B.S. Collection and used by Bigger (1953) for investigations on amino-acid accumulation and metabolism in yeast.

Methods of cultivation. The bacteria were cultivated in the 'deficient medium' containing salts, glucose, Marmite and arginine, with the addition of pyridoxine, pantothenic acid and riboflavin for the growth of *Strep. faecalis* (Gale, 1945*a*, 1947). For *Staph. aureus* the medium was dispensed in Roux bottles lying on their sides and growth took place during incubation at 30° for 16 hr. For *Strep. faecalis* the medium was put up in 2 l. flasks filled as full as possible, and incubation again took place for 16 hr. at 30°, the flasks being cold when placed in the incubator after inoculation. The yeast was grown in a medium containing glucose, Difco yeast extract, ammonium salts and trace elements, as described by Taylor (1949), and growth took place in Roux bottles incubated at 25° for 18 hr.

Preparation of suspensions. In all cases cells were harvested and washed in distilled water on the centrifuge in the usual way, and made up into washed suspension of density approximately 20–30 mg. dry wt./ml. in distilled water. Suspension densities were determined turbidimetrically on a Hilger absorptiometer calibrated against the organisms used. In the yeast experiments, the suspension density was determined in every case at the end of each experiment as it was found that, under some conditions, variations in the dry wt. of the cells occurred in the course of incubation. Such variations were not observed with suspensions of bacteria.

Estimation of glutamic acid and lysine. The free amino-acid content of cells was determined by use of the specific amino-acid decarboxylase preparations as previously described (Gale, 1945*b*, 1947; Taylor, 1947, 1949). The glutamic acid decarboxylase does not distinguish between glutamic acid and glutamine, consequently any glutamine within cells will be included as part of the 'glutamic acid' content.

Investigation of accumulation of glutamic acid and lysine. In general, the experimental conditions used for studying the accumulation of amino-acids within the cells were the same as those previously used in these investigations (Gale, 1947; Taylor, 1949). Modifications were, however, made in the composition of the buffered salt solution used during the incubation of cells with amino-acid solutions. Two basic solutions were used: a 'sodium salt solution' was prepared with the following quantities: $NaH_2PO_4.H_2O$, 3·0 g.; anhydrous Na_2HPO_4, 10·0 g.; NaCl, 3·0 g.; water, 1000 g.; a similar 'potassium salt solution' was prepared with the same quantities of K salts and, in each case, the pH was adjusted to 7·5 by the addition of NaOH or KOH respectively. Both salt solutions were diluted 1:3 for amino-acid accumulation experiments. A further salt solution was used in many experiments in which glucose fermentation occurred: 'minimal potassium solution' which consisted of the 'sodium salt solution' diluted 1:3 with KCl solution to give a final K concentration of 50 μg./ml. For the experiments with *Saccharomyces fragilis* it was necessary to work at pH 5·0 (Taylor, 1949), and for these experiments the salt solutions were adjusted approximately to pH 5·0 by the addition of HCl and then buffered by dilution 1:3 with the appropriate 0·1 M-citric acid-Na (or K) phosphate mixture. No significant change occurs in the pH during the accumulation of lysine by suspensions of *Strep. faecalis* or *Staph. aureus* and these experiments were therefore carried out in water with the addition of 'sodium salt solution' and 'potassium salt solution' to give final concentrations of 50 μg. Na and 50 μg. K/ml. These concentrations were checked by estimation in the flame photometer.

In all cases the cell suspensions were added to the incubation mixtures to give a final suspension density of 2·0 (bacteria) or 5·0 (yeast) mg. dry wt. of cells/ml. Amino-acids used were L-isomers. Incubations were stopped by rapid cooling to approx. 4° and the organisms harvested on the centrifuge. *Strep. faecalis* and *Saccharomyces fragilis* were washed once in distilled water but this was found inadvisable with *Staph. aureus* (see below). The harvested cells were made up into concentrated suspension (20–30 mg. dry wt./ml.) in water for estimation of amino-acids.

Flame photometer. Na and K were determined in a flame photometer designed and constructed by one of us (R.D.). The photometer consists of two photomultiplier tubes (931 A) arranged in a bridge circuit similar to that described by Heidel & Fassel (1951) and was designed for the estimation of Na and K within the range 0–3 μg./ml. It employs the internal-standard principle using Li as the reference element and has been developed from a single photocell instrument devised by Dr Holiday of the Medical Research Council Spectrographic Research Unit. The infrared line at 770 mμ. is used for estimating K and the 931 A phototube is made to respond to this wavelength by inserting an infrared image converter tube between the K filter and the phototube. For isolation of the K line the filter combination consisted of an Ilford 207 and two Ilford 503 filters. For isolation of the Na lines an interference filter (Barr and Stroud Ltd.) was used. Isolation of the Li 670 mμ. line was accomplished by means of two Ilford 608 filters and measurement depended on the insensitivity of the 931 A phototube to wavelengths greater than about 750 mμ.

Accuracy of the instrument. The standard deviations for pure aqueous solutions of NaCl and KCl did not exceed 0·03 μg./ml. for Na and 0·05 μg./ml. for K over the range 0–2·8 μg. Na or K/ml.

Interference by other ions. It has been reported that various ions and organic substances interfere with the estimation of Na and K in unbalanced flame photometers (Parkes, Johnson & Lykken, 1948; Domingo & Klyne, 1949). Berry, Chappell & Barnes (1946) have reported that their balanced flame photometer is comparatively insensitive to interference by other ionic and non-ionic substances, and this has been confirmed with the instrument used for these investigations. The following cations at concentrations of 280 μg./ml. have no significant effect on the estimation of 1·8 μg./ml. of Na; K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}: 280 μg./ml. of Na^+, NH_4^+, Mg^{2+}, Zn^{2+}, Mn^{2+} or Cu^{2+} have no effect on the estimation of 1·8 μg./ml. of K; but 280 μg. Fe^{2+}/ml. enhanced the K reading by 20% while 280 μg. Ca^{2+}/ml. depressed it by 35%. However, it is improbable that any of these cations would be present in the biological material in such high concentrations relative to Na or K, and it can be assumed that cations have not interfered with the estimations of Na or K in the work described in this paper. None of the following acids interfered with the estimation of Na when present in the sprayed solutions at concentrations of 0·1 N; HCl, HNO_3, H_2SO_4, acetic acid.

Phosphoric acid at 0·001 N did not interfere with the estimation of Na, but at 0·1 and 0·01 N there was considerable interference. The readings obtained with KCl solutions were unaffected by the presence of 0·1 N-H_2SO_4 or HNO_3, or 0·01 N-HCl; with 0·1 N-HCl and with 0·1 N- or 0·01 N-acetic acid the readings were 10% low. The presence of phosphoric acid resulted in severe depression of the readings when the concentration was 0·1 or 0·01 N and even at 0·001 N the readings were 14% low.

Preparation of samples for estimation of Na *and* K. Cell suspensions were ashed before metal estimation. A sample (0·5–1·0 ml.) of cell suspension containing 20·0 mg. dry wt. of cells/ml. was measured into platinum crucibles which were then maintained at dull-red heat in a muffle furnace for 2 hr.; after cooling, the crucibles were extracted three times with 1·0 ml. 0·1 N-HCl, the extracts combined and diluted to 400 ml. with distilled water for K estimations or to 100 ml. for Na estimations. Salt solutions and cell-free supernatant solutions were sprayed directly after appropriate dilution with distilled water. Li (as Li_2SO_4) was added as reference substance to all solutions, the final concentration being 12·0 μg. Li/ml. for Na estimations and 1·0 μg. Li/ml. for K estimations.

Units. Results are expressed as μmole metal/100 mg. dry wt. of organism. The approximate volume occupied by this weight of cells, after being packed on the centrifuge for 30 min. at 3000 *g*, is 0·35 ml. (*Strep. faecalis*), 0·40 ml. (*Staph. aureus*) or 0·70 ml. (*Saccharomyces fragilis*).

RESULTS

Preliminary work

Attempts were first made to estimate sodium and potassium contents of suspensions of organisms by spraying the cell suspensions through the flame without prior ashing. It was quickly obvious that sodium estimations could not be made accurately in this manner but consistent results were frequently obtained with potassium estimations. A series of estimations of potassium made on cell suspensions with and without ashing was then undertaken. Results were very variable. In some series agreement was obtained between ashed and intact cell samples to within 5%; on other occasions the intact cell samples gave potassium assays consistently 20% lower than those obtained with ashed preparations, and occasionally results were obtained which gave wide and inconsistent variations between the two preparations. Accordingly, spraying of cell suspensions was abandoned except for preliminary experiments designed to test conditions of sampling.

In the three organisms studied, the content of potassium was considerably greater than that of sodium and the question arose whether there was any alteration of the metal contents during preparation of suspensions or cooling and washing of cells after incubation. Accordingly, the effect was tested of standing the cells in water, with and without cooling, and in the various salt solutions in the absence of other substances. With *Strep. faecalis* and *Saccharomyces fragilis* no significant loss of potassium from the cells occurred on standing in water or sodium salt solutions for short periods up to 30 min. with or without cooling and it appeared that washing these cells after incubation in experimental solutions would introduce no serious error in the estimation of potassium content. However, there was a significant loss of potassium from *Staph. aureus* on standing in water or sodium salt solutions and it was concluded that washing of these cells was not permissible. When incubation mixtures include glucose, and fermentation is essential for amino-acid accumulation, it is necessary to buffer the mixture, and consequently some buffer salts are carried down with cell samples which have not been washed in water. Various methods of overcoming the difficulties in estimating metals under such conditions were attempted, and that finally adopted was to incubate the cells in sodium salt buffers when potassium estimations were made, and in potassium salt buffers for sodium estimations. It is shown below that the accumulation of glutamic acid by the cells tested is accompanied by an uptake of potassium ions and the absence of available potassium may result in a decreased ability to accumulate glutamic acid. Consequently it was decided to add small amounts of potassium to the sodium salt buffers so that both ions would be available but that the one estimated would be present in such small amounts in the salt solution that insignificant quantities would be carried down with the cells on harvesting. With bacteria there appear to be no significant changes in sodium content under the various conditions studied here (other than the accumulation of glutamic acid by *Staph. aureus* in the absence of available potassium) and the major changes studied relate to potassium, although it is realized that the use of 'minimal potassium solutions' does not present the cells with balanced media of the type usually employed in metabolic experiments.

Wide variations were experienced in the metal-ion content of cells harvested on different days, and in the quantitative nature of the responses encountered. For example, analyses of twenty-one batches of *Staph. aureus* cells gave an average potassium content of 64·9 μmoles/100 mg. dry wt. of cells, S.D. 0·85, range 52·8–83·0; the analyses for sodium content on eleven samples gave mean = 5·1 μmoles/100 mg. dry wt. of cells, S.D. 2·4, range 1·0–5·8. Consequently, all experiments were carried out on a comparative basis, and it has not been possible to draw exact quantitative conclusions from the data obtained. The growth media used contain sodium and potassium salts and attempts were made to obtain organisms with a small potassium content by preparing media with a

sodium salt solution instead of the usual salt mixture. However, all the media contain either Marmite or yeast extract and the organisms appear to utilize the potassium in such preparations so efficiently that it was not possible to decrease the cell potassium content by more than 15–18%. Consequently, the usual basal salt medium was used for the experiments described here.

Sodium and potassium changes during accumulation of glutamic acid by bacteria and yeast

Staphylococcus aureus. Fig. 1 shows the accumulation of glutamic acid and potassium within washed suspensions of *Staph. aureus* when the cells

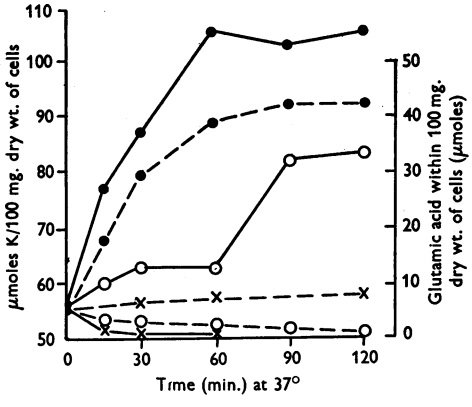

Fig. 1. Accumulation of K and glutamic acid within washed suspensions of *Staphylococcus aureus.* Conditions as for Table 2. Full lines = K; broken lines = glutamic acid. Incubation mixture contains ○, glucose only; ×, glutamic acid only; ●, glucose and glutamic acid.

are incubated in 'minimal potassium solution' with glucose and glutamic acid separately or together. When the cells are incubated in the presence of glutamic acid without glucose, there is little change in either potassium or glutamic acid content; when glucose but not glutamic acid is present in the external medium, there is no change in the internal concentration of glutamic acid but an accumulation of potassium which increases in rate as the incubation continues. The pH of the incubation mixture falls from 7·5 to approximately 7·0 during the first hour of incubation and this is followed by a more rapid fall to approximately 6·2–6·3 by the end of the second hour; it is probable that the accelerated uptake of potassium during the second hour is associated with the increase in acidity within the cell. When both glutamic acid and glucose are present in the incubation mixture, there is a rapid accumulation of both glutamic acid and potassium within the cells, the amount of potassium taken up being greater than that due to glucose alone. In twenty experiments the extra potassium uptake in the presence of glutamic acid, over and above that taking place in the presence of glucose, has given a mean value of 0·73 g. atom/mole glutamic acid accumulated with S.D. = 0·25. Table 1 shows the alteration of the potassium content of cells and external medium in a typical experiment under these conditions; within experimental error the amount of potassium withdrawn from the external medium can be accounted for by the increase in the cell content of potassium.

There is no significant change in the sodium content of cells incubated under the conditions described above. Fig. 2 shows that the accumulation of glutamic acid is slower if the cells are washed and incubated in a medium freed from potassium as far as possible. The cells carry down a certain amount of potassium from the growth medium but it can be seen that, if the cell suspension is incubated for some time in a sodium buffer before glutamic acid is added, then the absence of potassium has

Table 1. *Changes in potassium and glutamic acid during the accumulation of glutamic acid by washed suspensions of* Staphylococcus aureus

(*Staph. aureus* Duncan grown in deficient medium, harvested and made into washed suspension. Cells incubated at 37° at final suspension density = 2·0 mg. dry wt./ml. in 'minimal potassium solution' with 1% glucose and 2·7 µmoles sodium glutamate/ml. Changes in K and glutamic acid of cells and external medium expressed as µmoles/100 mg. dry wt. of cells. Initial K content: cells, 54·9; external medium 100 µmoles/100 mg. dry wt. of cells (≡50 ml. medium). Initial glutamic acid content of cells = 3·2 µmoles/100 mg. dry wt.)

Time of incubation (min.)		Incubation medium: Glucose — Glutamic acid (µmoles)	Glucose — Potassium (µmoles)	Glucose + Glutamic acid — Glutamic acid (µmoles)	Glucose + Glutamic acid — Potassium (µmoles)	Glutamic acid — Glutamic acid (µmoles)	Glutamic acid — Potassium (µmoles)
30	Internal content of cells	−2·0	+8·7	+26·4	+30·3	+1·8	−3·55
	External medium	+3·0	−11·8	−43·5	−32·2	−4·4	+2·1
	Balance	+1·0	−3·1	−17·1	−1·9	−2·6	−1·45
60	Internal content of cells	−3·2	+17·5	+36·4	+41·5	+3·6	−12·7
	External medium	+3·7	−17·5	−74·9	−43·8	−6·9	+10·3
	Balance	+0·5	0·0	−38·5	− 2·3	−3·3	−2·4

a marked effect in decreasing the rate of internal accumulation of glutamic acid. Potassium could not be replaced by calcium, ammonium or magnesium ions, but a final concentration of 30 μg. potassium/ml. appeared to give full stimulation.

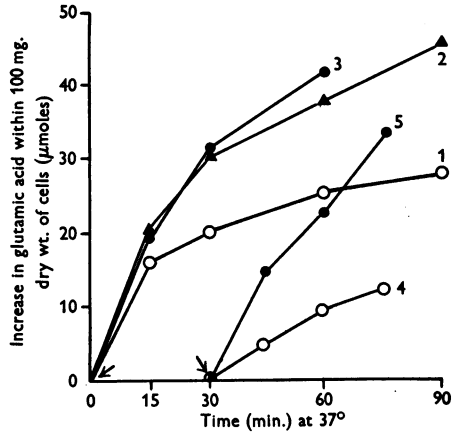

Fig. 2. Dependence of glutamic acid accumulation on K content of incubation mixture. Deficient *Staph. aureus* incubated at 37° in 'sodium salt solution' containing 1% (w/v) glucose and 2·7 μmoles sodium glutamate/ml. with the following additions: curves 1 and 4, no addition; curve 2, 30 μg.K^+/ml.; curves 3 and 5, 150 μg. K^+/ml. Sodium glutamate added at time 0 in curves 1, 2 and 3; at time 30 in curves 4 and 5.

Fig. 3 shows the changes occurring in the sodium and potassium contents of the cells when incubation is carried out in 'sodium salt solution'. When both glutamic acid and glucose are present, there is a small accumulation of potassium (probably derived from the growth medium) and a significant accumulation of sodium. The total (sodium + potassium) ion accumulated is approximately equivalent to the glutamic acid accumulated.

Streptococcus faecalis. Table 2 shows that the accumulation of potassium by *Strep. faecalis* during glucose fermentation is greater and more rapid than is the case with *Staph. aureus*. When glutamic acid is also present in the external medium, the resulting accumulation of glutamic acid within the cells is accompanied by an increase in the potassium

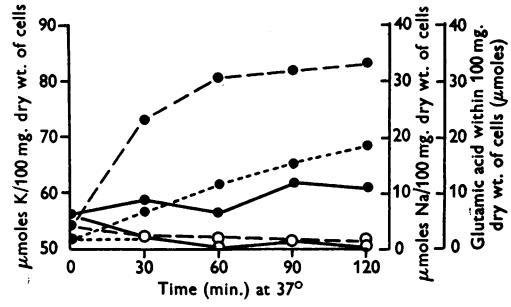

Fig. 3. Accumulation of Na and K in *Staph. aureus* suspensions incubated in 'sodium salt solution'. Deficient *Staph. aureus* incubated at 37° and suspension density 2·0 mg. dry wt. of cells/ml. in 'sodium salt solution' containing ○, 1% glucose; ●, 1% glucose + 2·7 μmoles sodium glutamate/ml.; – – –, glutamic acid; ——, K; · · · · ·, Na.

content of mean value 0·85 (range 0·70–1·05) g. atom/g. mole glutamic acid accumulated.

Saccharomyces fragilis. Table 3 shows that the metabolism of glucose by yeast suspensions is accompanied by an accumulation within the cells of potassium and sodium. If glutamic acid is added to the incubation medium, accumulation of the amino-

Table 2. *Accumulation of potassium and glutamic acid by washed suspensions of* Staphylococcus aureus *and* Streptococcus faecalis

(Cells incubated at final suspension density = 2·0 mg. dry wt./ml. in 'minimal potassium solution' at pH 7·5 with additions as below: glucose 1%; sodium glutamate or glutamine 2·7 μmoles/ml. Content of K or glutamic acid expressed as μmoles/100 mg. dry wt. of cells.)

	Incubation of *Staph. aureus* for 1 hr. at 37°				Incubation of *Strep. faecalis* for 30 min. at 37°			
	Potassium				Potassium			
Additions to incubation mixture	Content (μmoles)	Change (μmoles)	Net change due to amino-acid (μmoles)	Glutamic acid accumulated (μmoles)	Content (μmoles)	Change (μmoles)	Net change due to amino-acid (μmoles)	Glutamic acid accumulated (μmoles)
Initial	48·4	—	—	—	72·1	—	—	—
No additions	42·5	−5·9	—	0	90·5	+18·4	—	0
Glutamic acid	43·7	−4·7	+1·2	4·1	90·5	+18·4	0	0·7
Glutamine	44·2	−4·2	+1·7	4·0	90·5	+18·4	0	0·6
Glucose	56·1	+7·7	—	0	115·5	+43·4	—	0·0
Glucose + glutamic acid	78·9	+30·5	+22·8	39·5	121·0	+48·9	+5·5	6·8
Glucose + glutamine	70·5	+22·1	+14·4	29·0	114·0	+41·9	−1·5	9·5

Table 3. *Changes in sodium and potassium content of* Saccharomyces fragilis *during the accumulation of glutamic acid or lysine*

(Cells incubated for 1 hr. at 25° in 'minimal potassium solution' at pH 5·0 with 2% (w/v) glucose and 5 μmoles glutamic acid, glutamine or lysine/ml. as below. Final suspension density = 5·0 mg. dry wt. of cells/ml. Content of Na, K, lysine and glutamic acid expressed as μmoles/100 mg. dry wt. of cells. Initial content of amino-acids given in brackets.)

		Potassium			Sodium		
Incubation mixture (additions to 'minimum potassium solution')	Accumulation of glutamic acid (μmoles)	Content (μmoles)	Change (μmoles)	Net change due to amino-acid (μmoles)	Content (μmoles)	Change (μmoles)	Net change due to amino-acid (μmoles)
Initial cells	(8·9)	37·0	—	—	15·3	—	—
No addition	0·0	37·0	0	—	17·2	—	—
Glutamic acid	1·63	32·8	−4·2	−4·2	18·0	+0·8	+0·8
Glutamine	1·33	43·0	+6·0	+6·0	17·7	+0·5	+0·5
Glucose	0·0	43·0	+6·0	—	18·3	+1·1	—
Glucose + glutamic acid	44·9	60·5	+23·5	+17·5	31·5	+14·2	+13·1
Glucose + glutamine	34·8	31·8	−5·2	−11·2	18·0	+0·7	−0·4
	Accumulation of lysine (μmoles)						
Initial cells	(3·6)	30·0	—	—	24·4	—	—
No addition	0·0	29·8	—	—	26·5	—	—
Lysine	0·4	33·4	+3·6	+3·6	28·8	+2·3	+2·3
Glucose	0·0	37·5	+7·7	—	36·8	+10·3	—
Glucose + lysine	38·1	15·5	−14·3	−22·0	25·3	−1·2	−11·5

acid takes place within the cells, and this is accompanied by an increase in the base content of the cells. In general, it was found that increasing the potassium content of the external medium resulted in a higher proportion of the accumulated base consisting of potassium, but results were not consistent. It would appear that the uptake of glutamic acid is accompanied by an uptake of sodium and potassium, the latter ion being preferentially accumulated.

Effect of replacing glutamic acid by glutamine in the external medium

Table 2 shows the effect of replacing glutamic acid in the external medium by glutamine. McIlwain, Roper & Hughes (1948) have shown that the internal glutamic acid of *Strep. faecalis* contains a small proportion of glutamine and that the accumulation of glutamine increases if glutamine is supplied externally instead of glutamic acid; even in the latter condition the amount of glutamine within the cells represents less than 16% of the total 'glutamic acid' which would be estimated by the glutamic acid decarboxylase method. McIlwain *et al.* (1948) observed that the final concentration of glutamine plus glutamic acid which was reached in the cells when glutamine was supplied, was frequently greater than when ammonium glutamate was supplied. A similar finding was made in the present investigations, but Table 2 shows that whereas the accumulation is accompanied by an uptake of potassium when glutamic acid is the external source, there is no potassium uptake corresponding to the increase in internal 'glutamic acid' when glutamine forms the external source. A similar situation with regard to potassium uptake is found with *Saccharomyces fragilis* (Table 3).

With *Staph. aureus* there is no obvious difference in the amount of potassium accumulation accompanying glutamic acid accumulation whether the external source is glutamic acid or glutamine (Table 2). The internal accumulation of glutamic acid is smaller with this organism if the external source is glutamine than if it is an equivalent concentration of glutamic acid.

Sodium and potassium changes during the accumulation of lysine by bacteria and yeast

Staphylococcus aureus and *Streptococcus faecalis*. Table 4 shows the changes in the sodium and potassium contents of washed cells of these organisms when incubated in water containing lysine and traces of sodium and potassium ions. The changes are small and somewhat erratic but there would appear to be a loss of sodium and a gain of potassium in both organisms during the accumulation of lysine, although the changes in potassium content for *Staph. aureus* are scarcely significant, and the fluctuations in the sodium values are too great to allow any firm conclusions to be drawn therefrom.

Saccharomyces fragilis. Lysine does not accumulate within yeast cells unless glucose is being metabolized (Taylor, 1949). Table 3 shows that the accumulation of lysine which occurs in *Saccharo-*

Table 4. *Changes in sodium and potassium content of* Staphylococcus aureus *and* Streptococcus faecalis *during accumulation of lysine*

(Cells harvested from deficient medium and incubated at 25° in Na and K solution containing 2·2 μmoles Na and 1·3 μmoles K/ml. at pH = 7·5. Suspension density = 2·0 mg. dry wt. of cells/ml. Concentration of lysine in external medium = 10 μmoles/ml. Changes expressed in each case as difference between values obtained for cells incubated with and without lysine and given in μmoles/100 mg. dry wt. of organism. (*a*) and (*b*) represent different batches of cells in each case.)

Organism	Incubation time (min.)	Lysine		Sodium		Potassium	
		Content (μmoles)	Change (μmoles)	Content (μmoles)	Change (μmoles)	Content (μmoles)	Change (μmoles)
Staph. aureus (*a*)	0	2·8	—	9·8	—	64·5	—
	15	8·1	+5·3	8·0	−1·8	64·5	0·0
	30	13·6	+10·8	6·9	−2·9	68·5	+4·0
(*b*)	0	4·5	—	14·8	—	71·1	—
	15	10·9	+6·4	13·7	−1·1	71·6	+0·5
	30	16·1	+11·6	10·9	−3·9	75·2	+4·1
Strep. faecalis (*a*)	0	1·3	—	5·8	—	62·0	—
	15	2·4	+1·1	4·7	−1·1	65·8	+3·8
	30	6·0	+4·7	6·2	+0·4	69·7	+7·7
(*b*)	0	2·8	—	12·8	—	66·1	—
	15	6·9	+4·1	11·7	−1·1	73·1	+7·0
	30	10·0	+7·2	4·8	−8·0	85·3	+19·2

myces fragilis incubated in the presence of glucose and lysine, is accompanied by a loss of both sodium and potassium from the cells.

DISCUSSION

The accumulation of amino-acids by mouse ascites carcinoma cells appears to involve the displacement of potassium from the cells (Christensen *et al.* 1952*a*, *b*). The accumulation of lysine by the yeast cells studied here may involve a similar process since it is accompanied by migration of both potassium and sodium out of the cells. Such displacement has not been observed during the accumulation of glutamic acid which, in the microbial cells studied, is accompanied by a gain in cell potassium. It seems probable that the loss or gain of basic ions by the cell will be largely controlled by the acidic or basic nature of the amino-acid which is accumulated.

The uptake of glutamine points to an interesting difference between the organisms studied in that it is accompanied by an uptake of potassium in *Staph. aureus* but not in *Strep. faecalis* or *Saccharomyces fragilis*. McIlwain (1946) has shown that both streptococci and yeasts decompose glutamine during metabolism of glucose, whereas *Staph. aureus* is unable to do so. It is possible that the liberation of ammonia from glutamine in organisms able to accomplish its decomposition dispenses with the need for an uptake of potassium. However, other differences between the processes resulting in an accumulation of glutamic acid in *Staph. aureus* and *Strep. faecalis* have been reported (Gale, 1951); thus sodium azide and 2:4-dinitrophenol inhibit accumulation in *Staph. aureus* but increase and accelerate it in *Strep. faecalis*. Terner *et al.* (1950) found that glutamine was not effective as replacement for glutamic acid in preventing the loss of potassium from brain cells.

SUMMARY

1. *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*), *Streptococcus faecalis* and *Saccharomyces fragilis* accumulate free glutamic acid within the cells when these are incubated with glucose and glutamic acid. The accumulation is accompanied by an uptake of potassium ions amounting to not more than 1 g. atom K/mole glutamic acid accumulated. Some uptake of sodium accompanies the accumulation in *Saccharomyces fragilis*.

2. Potassium stimulates the accumulation of glutamic acid in *Staphylococcus aureus*; in the absence of available potassium the accumulation of glutamic acid is restricted and is accompanied by an uptake of sodium.

3. The accumulation of lysine by *Streptococcus faecalis* is accompanied by a gain of potassium within the cells; the changes in sodium content of this organism and of sodium and potassium content in *Staphylococcus aureus* during lysine accumulation are small and of doubtful significance.

4. The accumulation of lysine by *Saccharomyces fragilis* will not take place in the absence of glucose, and is accompanied by a loss of sodium and potassium from the cells.

The authors wish to thank Mr W. Leonard for assistance with some of the estimations, and Dr Holiday for his advice concerning the construction of the flame photometer.

REFERENCES

Berry, J. W., Chappell, D. G. & Barnes, R. B. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 19.
Bigger, L. C. (1953). (In preparation.)
Christensen, H. N. & Riggs, T. R. (1952). *J. biol. Chem.* **194**, 57.
Christensen, H. N., Riggs. T. R., Fischer, H. & Palatine, I. M. (1952*a*). *J. biol. Chem.* **198**, 1.
Christensen, H. N., Riggs, T. R., Fischer, H. & Palatine, I. M. (1952*b*). *J. biol. Chem.* **198**, 17.
Cowie, D. B., Roberts, R. B. & Roberts, I. Z. (1949). *J. cell. comp. Physiol.* **34**, 243.
Domingo, W. R. & Klyne, W. (1949). *Biochem. J.* **45**, 400.
Gale, E. F. (1945*a*). *Brit. J. exp. Path.* **26**, 225.
Gale, E. F. (1945*b*). *Biochem. J.* **39**, 46.
Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
Gale, E. F. (1951). *Biochem. J.* **48**, 286.
Gale, E. F. & Folkes, J. P. (1953). *Biochem. J.* **53**, 483, 493.
Heidel, R. H. & Fassel, V. A. (1951). *Anal. Chem.* **23**, 784.
Krebs, H. A., Eggleston, L. V. & Hems, R. (1949). *Biochem. J.* **44**, 159.
McIlwain, H. (1946). *J. Path. Bact.* **58** (2), 322.
McIlwain, H., Roper, J. A. & Hughes, D. E. (1948). *Biochem. J.* **42**, 492.
Najjar, V. A. & Gale, E. F. (1950). *Biochem. J.* **46**, 91.
Parkes, T. D., Johnson, H. O. & Lykken, L. (1948). *Analyt. Chem.* **20**, 822.
Pulver, R. & Verzár, F. (1940). *Nature, Lond.*, **145**, 823.
Roberts, R. B. & Roberts, I. Z. (1950). *J. cell. comp. Physiol.* **36**, 15.
Roberts, R. B., Roberts, I. Z. & Cowie, D. B. (1949). *J. cell. comp. Physiol.* **34**, 259.
Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 86.
Taylor, E. S. (1949). *J. gen. Microbiol.* **3**, 211.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.

A New Method for Preparing Flavin-adenine Dinucleotide

By L. G. WHITBY
*Biochemical Laboratory, University of Cambridge**

(*Received* 17 *October* 1952)

Flavin-adenine dinucleotide (FAD) was first prepared by Warburg & Christian (1938*a*, *b*), and their method has since been used by many workers, some of whom recorded the purity of their products (e.g. Klein & Kohn, 1940; Hellerman, Lindsay & Bovarnick, 1946; Bessey, Lowry & Love, 1949; Schrecker & Kornberg, 1950); only Hellerman *et al.* (1946) claimed that they had prepared pure FAD. Burton (1951) prepared FAD by chromatography on alumina, and Sanadi & Littlefield (1951) by chromatography on Florisil, but in neither case was a pure product obtained.

This paper describes a convenient and simplified method of isolating FAD, making use of chromatography on powdered cellulose; by this method, products containing 90% FAD have been obtained. The absorption spectra of FAD and related compounds are discussed.

EXPERIMENTAL

Spectrophotometric measurements. These were made in quartz cells, 1 cm. thickness, in a Beckman model DU spectrophotometer, which had been allotted the number B9 in a collaborative test (Gridgeman, 1951). The performance of the instrument was satisfactory, except at 313 mμ. where $K_2Cr_2O_7$, the standard substance used in the test, has a trough in its spectrum; at 313 mμ., B9 differed from the mean value in its estimate of the strength of solution C by 5%. The molecular extinction coefficients (ϵ) of riboflavin (Table 2) were confirmed, using another Beckman (B13 of Gridgeman, 1951) and a Hilger-Watts Uvispek spectrophotometer (number unavailable).

For measuring the absorption spectra, readings were made every 2·5 mμ. in the region of the maxima, and every 5 mμ. elsewhere. The slit widths at 450, 375 and 260 mμ. were 0·04, 0·1 and 1·3 mm., respectively; using the standard curve supplied by the Beckman manufacturers, these slit widths corresponded to band widths of 0·8, 0·9 and 3·4 mμ., respectively. The wavelength scale of the instrument had recently been checked, and the results in the collaborative test were satisfactory (Gridgeman, 1951). Measurements were made in 0·1 M-phosphate buffer, pH 7·0, except where stated to the contrary.

The content of FAD was estimated by the light absorption at 450 mμ., using $\epsilon = 11{\cdot}3 \times 10^3$ l. mole^{-1} cm.$^{-1}$ (Warburg & Christian, 1938*b*; present work). Impurities which absorbed light at 450 mμ. were detected by the addition of $Na_2S_2O_4$: the ratios in Table 1, stage *A*, have been corrected for the presence of 4% of an impurity which absorbed light at 450 mμ.; this impurity was removed by the later stages of the purification.

Preparation of FAD. Baker's yeast (28 lb.; United Yeast Co., Cambridge) was extracted by the method of Warburg & Christian (1938*b*) as far as the precipitation of the crude Ag salt of FAD. The precipitate was suspended in 140 ml. water and dissolved by adding excess saturated KCl; a few drops of 2N-HNO_3 were added to precipitate AgCl, the

* Present address: King's College, Cambridge.