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Biosynthesis of Amino Acids in *Clostridium pasteurianum*

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1. *Clostridium pasteurianum* was grown on a synthetic medium with the following carbon sources: (*a*) ^{14}C-labelled glucose, alone or with unlabelled aspartate or glutamate, or (*b*) unlabelled glucose plus ^{14}C-labelled aspartate, glutamate, threonine, serine or glycine. The incorporation of ^{14}C into the amino acids of the cell protein was examined. 2. In both series of experiments carbon from exogenous glutamate was incorporated into proline and arginine; carbon from aspartate was incorporated into glutamate, proline, arginine, lysine, methionine, threonine, isoleucine, glycine and serine. Incorporations from the other exogenous amino acids indicated the metabolic sequence: aspartate → threonine → glycine ⇌ serine. 3. The following activities were demonstrated in cell-free extracts of the organism: (*a*) the formation of aspartate by carboxylation of phosphoenolpyruvate or pyruvate, followed by transamination; (*b*) the individual reactions of the tricarboxylic acid route to 2-oxoglutarate from oxaloacetate; glutamate dehydrogenase was not detected; (*c*) the conversion of aspartate into threonine via homoserine; (*d*) the conversion of threonine into glycine by a constitutive threonine aldolase; (*e*) serine transaminase, phosphoserine transaminase, glycerate dehydrogenase and phosphoglycerate dehydrogenase. This last activity was abnormally high. 4. The combined evidence indicates that in *C. pasteurianum* the biosynthetic role of aspartate and glutamate is generally similar to that in aerobic and facultatively aerobic organisms, but that glycine is synthesized from glucose via aspartate and threonine.

Pathways that can account for the biosynthesis of all the common amino acids from glycolytic intermediates are already firmly established as a result of tracer, mutant and enzymic studies. In general, however, investigations have been concentrated on a few aerobic or facultatively aerobic organisms, notably yeasts, *Neurospora crassa* and *Escherichia coli*. Comparatively little information is available on biosynthesis in strict anaerobes, such as the clostridia.

Clostridium pasteurianum ferments sugars with the formation of acetic acid, butyric acid, hydrogen, carbon dioxide and possibly ethanol and butanol (Prévot, 1957). It grows vigorously on media containing carbohydrate, mineral salts, biotin and *p*-aminobenzoic acid, with molecular nitrogen or ammonia as its principal nitrogen source. It thus synthesizes all of the common amino acids and appeared to be a suitable anaerobe for biosynthetic studies. This paper reports tracer and enzymic studies with this organism; part of the work has already been briefly reported (Dainty & Peel, 1966).

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MATERIALS AND METHODS

Maintenance and growth of bacteria. Clostridium pasteurianum strain W5 (N.C.I.B. 9486; A.T.C.C. 6013) was maintained by monthly subculture in tubes of potato broth medium (Jensen & Spencer, 1947). Tubes were heated at 100°C for 10 min to remove dissolved oxygen and were then cooled rapidly, immediately before inoculation with about 5% (v/v) of a previous culture. Incubation was at 30°C until vigorous growth ensued (1–3 days).

For all other purposes synthetic media containing glucose, salts and vitamins were used. The medium of Carnahan & Castle (1958) (referred to as 'carbonate medium') was used in preliminary isotope experiments; in other experiments the modified version of Lovenberg, Buchanan & Rabinowitz (1963) was used, in which potassium phosphate replaces $CaCO_3$ as the buffering agent (referred to as 'phosphate medium'). Growth was erratic immediately after transfer from potato broth to the synthetic medium and the organism was therefore subcultured daily for at least 14 days on synthetic medium before experimental cultures were grown. Tubes of synthetic medium were inoculated in the same way as the potato broth cultures and were then fitted with a pyrogallol plug and closed with a rubber bung as described by Somerville & Peel (1967). For the tracer experiments, boiling tubes were used in a similar fashion to accommodate the required culture volume of 20 ml. Where required,

^{14}C-labelled glucose and supplements of amino acids, pyrimidines or purines, in some cases containing ^{14}C, were autoclaved separately and added immediately before inoculation.

Larger cultures (1.5l) for enzymic work were grown in 3l conical flasks with provision for flushing with a filtered stream of gas, excess of gas being exhausted through a Bunsen valve. The medium was flushed with H_2+CO_2 (95:5) for 10min before inoculation. With gas still passing, the rubber bung was then raised slightly out of the neck of the flask, the inoculum added, the bung replaced and flushing continued for a further 5min before incubation at 30°C.

Peptostreptococcus elsdenii (N.C.I.B. 8927) was maintained and 1 litre batches were grown as described by Somerville & Peel (1967).

Veillonella alcalescens (*Veillonella gazogenes*; *Micrococcus lactilyticus*) was an oral strain isolated by Dr J. N. Ladd at the University of Sheffield. It was maintained and grown in the same way as *P. elsdenii*.

Harvesting of bacteria. All three organisms were harvested when the evolution of gas ceased, as judged visually, after 16–24h of growth. The cells were centrifuged off (4000*g* for 15min at 5°C) and the cell paste was washed thrice by resuspending in a volume of buffer equal to that of the original culture and centrifuging as before. The buffer was 50mM-potassium phosphate (KH_2PO_4–KOH), pH7.0 (all potassium phosphate buffers consisted of the appropriate KH_2PO_4–KOH mixture), containing 0.004% (v/v) of thioglycollic acid, except for the cell-free experiments with *C. pasteurianum* in which pyruvate was used as substrate, when 50mM-tris–HCl buffer, pH7.4, containing 0.004% of thioglycollic acid was used. The washed cells were finally suspended in the same buffer to give approx. 100mg dry wt./ml.

Preparation of cell-free extracts. Ultrasonic extracts were prepared by treating freshly prepared bacterial suspensions (approx. 100mg dry wt. of cells/ml of buffer) in an MSE 60W ultrasonic disintegrator, operating between 18000 and 20000 Hz for 1.5min. The beaker containing the bacterial suspension was surrounded by an ice–water mixture throughout the procedure. After centrifuging (22000*g* for 15min at 5°C) the supernatant was stored in a stoppered tube at 0°C and used on the same day.

Extracts of dried cells of *C. pasteurianum* were prepared by extracting the dried cells (Dainty, 1970) with 50mM-potassium phosphate buffer, pH7.0, containing 0.004% of thioglycollic acid (10ml/g of dried cells) under vacuum in a Thunberg tube. The suspension was incubated at 30°C for 1h with occasional shaking, centrifuged (20000*g* for 15min at 5°C) and the supernatant was stored as above. For one experiment, designed to detect glutamate dehydrogenase activity, this procedure was modified by using cells dried rapidly at 40°C under vacuum in a rotary evaporator immediately after harvesting (Carnahan, Mortenson, Mower & Castle, 1960).

Dialysed extracts. Before use, all dialysis tubing was soaked in 0.1mM-EDTA for at least 10min and thoroughly rinsed with deionized water. Extracts were dialysed at 0–5°C, with magnetic stirring, against dilute buffer as indicated.

Anaerobic dialysis was carried out in a Buchner flask closed with a bung. Dissolved gases were removed from the buffer by evacuation and thioglycollic acid was added to give a final concentration of 0.001% (v/v). The dialysis sac containing the enzyme preparation was then placed in the buffer and the flask was evacuated and refilled with N_2 purified as described by Somerville (1968).

Isolation of amino acids from the cell protein of labelled cultures. The bacteria were grown as described above with the appropriate supplements and ^{14}C-labelled substrates. A sample (0.5ml) was removed immediately after inoculation for determination of the specific radioactivity of the substrate. After growth, the cells were harvested as described above except that thioglycollic acid was omitted from the buffer. The cells were then fractionated by the method of Roberts, Cowie, Abelson, Bolton & Britten (1955) by using 10ml portions of the extraction reagents and carrying out the whole procedure in a 25ml polypropylene centrifuge tube. The final residue, the 'principal protein fraction', was suspended in 3ml of 6M-HCl and transferred to a Pyrex tube, which was then sealed in a flame and heated at 110°C for 18h. The contents of the tube were transferred to a 25ml round-bottomed flask and evaporated to dryness at 50°C under a stream of air. The residue was redissolved in 3ml of water and the solution was again evaporated to dryness to remove HCl. This step was repeated thrice more. Finally, the residue was dissolved in 0.5ml of water and stored at −20°C until the individual amino acids were separated by the techniques given below.

Paper chromatography and ionophoresis. Unless otherwise stated all chromatography and ionophoresis was carried out on Whatman 3MM paper. All chromatograms were developed in the descending direction at 22°C. The paper was allowed to equilibrate with the solvent vapours overnight before chromatography. Ionophoresis was carried out either in a horizontal-plate apparatus (Locarte Co., London S.W.7, U.K.) or in an apparatus similar to that of Ryle, Sanger, Smith & Kitai (1955).

The protein hydrolysate from the preliminary tracer experiments was separated on Whatman no. 4 paper with the two-dimensional chromatographic system of phenol–water followed by butan-1-ol–propionic acid as described by Benson *et al.* (1950). The protein hydrolysates from the experiment of Table 1 were separated with a two-dimensional system of ionophoresis at pH1.8 in formic acid (88%, w/v)–acetic acid–water (25:87:888, by vol.), followed by chromatography with butan-2-ol–aq. 3% (w/v) NH_3 (5:2, v/v) (Rothman & Higa, 1962). In the experiments of Table 2 the same method was used except that the butan-1-ol–butyl acetate–acetic acid–water (19:1:5:25, by vol.) solvent of Richmond & Hartley (1959) was used for the chromatographic step; the top layer was used as the mobile phase.

Radioactive areas on the resulting papers were located by radioautography as described by Knight (1962) and Somerville & Peel (1967). The individual amino acids were eluted with water in a closed cabinet (Dent, 1947) and the eluates (0.5ml) were dried under vacuum over conc. H_2SO_4. Each amino acid was then purified further by the following paper methods: for lysine, arginine and histidine, chromatography in ethanol–butanol–water–diethylamine (10:10:5:2, by vol.) (Hardy, Holland & Nayler, 1955); for alanine, glycine, valine, methionine, phenylalanine and tyrosine, chromatography in methanol–butanol–water (2:2:1, by vol.) (Hardy *et al.* 1955); for threonine,

proline and serine, chromatography in butanol–acetone–water (2:2:1, by vol.) (Hardy *et al.* 1955); for isoleucine and leucine, chromatography in 2-methylpropan-1-ol–ethyl methyl ketone–water (7:5:3, by vol.) (Kemble & Macpherson, 1954); for glutamate and aspartate, ionophoresis at pH 3.6 in the pyridine–acetic acid–water (1:10:89, by vol.) system of Dixon, Kauffman & Neurath (1958). The amino acids were located by radioautography and eluted as before. Radioactivities and amino acid contents were determined on portions of the eluates.

For examination of the products of enzymic experiments the reaction was stopped by adding trichloroacetic acid to a final concentration of 5%, the precipitate was removed by centrifuging, and about one-fiftieth of the supernatant used for separations on paper. When aspartate, glutamate and dicarboxylic acids were involved, the ionophoresis system at pH 3.6 mentioned above was used. For threonine with glycine the ionophoresis system at pH 1.8 mentioned above was used. For threonine with homoserine a two-dimensional treatment was given in which electrophoresis at pH 1.8 was followed by chromatography with propan-1-ol–water–diethylamine (85:15:4, by vol.) (Redfield, 1953).

Amino acids were located by spraying with or dipping the paper into 0.1% (w/v) ninhydrin in acetone and then heating for 2 min at 100°C (Toennies & Kolb, 1951). Where more permanent records were required the chromatograms were subsequently sprayed with 0.5 M-$NiCl_2$ (Kawerau & Wieland, 1951). Organic acids were detected by spraying the paper with 0.04% (w/v) Bromocresol Green in ethanol to which a few drops of aq. NH_3 soln. were added just before use (Bryant & Overell, 1953).

Measurement of radioactivity. The radioactivity of the various cell fractions, radioactive substrates and eluted amino acids were measured at infinite thinness after plating on disposable stainless-steel planchets. One drop of 0.2% (v/v) Teepol (Shell Chemical Co. Ltd., London S.E.1, U.K.) was pipetted on to the planchets to promote even spreading of the sample, which was then evaporated to dryness under an infrared lamp. The radioactivity of the dried samples was measured in a Packard model 20A windowless gas-flow counter with a Baird–Atomic 1035 scaler. The gas phase was methane (Dept of Public Health Engineering, Mogden Works, Isleworth, Middx., U.K.).

Enzymic tests. Several enzymes were demonstrated qualitatively by incubating ultrasonic extracts as described below and testing for the appropriate products by ionophoretic or chromatographic means. Unless otherwise indicated, no product was detected when any of the substrates was omitted.

For the carboxylation of phosphoenolpyruvate, a total volume of 0.9 ml contained 110 mM-tris–HCl buffer, pH 7.4, 5 μmol of phosphoenolpyruvate, 5 μmol of $MgCl_2$, 10 μmol of L-glutamate, 20 μmol of $NaH^{14}CO_3$ (10^6 c.p.m.) and ultrasonic extract dialysed anaerobically overnight against 50 mM-tris–HCl buffer, pH 7.4, and containing 4.2 mg of protein. Incubation was under N_2 at 30°C for 1 h.

For the carboxylation of pyruvate, a total volume of 2 ml contained 100 mM-tris–HCl buffer, pH 7.4, 10 μmol of pyruvate, 10 μmol of $MgCl_2$, 10 μmol of L-glutamate, 10 μmol of NADH and 20 μmol of $NaH^{14}CO_3$ (10^6 c.p.m.). Other details were as for the carboxylation of phosphoenolpyruvate.

For transamination between glutamate and oxaloacetate, a total volume of 1 ml contained 50 mM-potassium phosphate buffer, pH 7.0, 5 μmol of oxaloacetate, 5 μmol of L-glutamate and ultrasonic extract containing 8.0 mg of protein. Incubation was in air at 30°C for 30 min. For the reverse reaction 5 μmol of 2-oxoglutarate plus 5 μmol of L-aspartate were used as substrates.

For the conversion of homoserine into threonine a total volume of 1.5 ml contained 50 mM-phosphate buffer, pH 7.0, 5 μmol of L-homoserine, 5 μmol of $MgCl_2$, 1 μmol of pyridoxal phosphate and ultrasonic extract containing 5.2 mg of protein. Incubation was under N_2 at 30°C for 1 h.

For transamination between glutamate and hydroxypyruvate or phosphohydroxypyruvate, a total volume of 1 ml contained 33 mM-tris–HCl buffer, pH 7.4, 6 μmol of L-glutamate, 5 μmol of hydroxypyruvate or 2.5 μmol of phosphohydroxypyruvate, and ultrasonic extract containing 5.7 mg of protein. Incubation was in air at 30°C for 30 min.

For examination of the products of the threonine aldolase reaction a total volume of 3.0 ml contained 83 mM-potassium phosphate buffer, pH 7.0, 20 μmol of L-threonine and ultrasonic extract containing 3.4 mg of protein. Incubation was in the outer compartment of a Conway microdiffusion unit at 30°C for 30 min and the centre well contained 1 ml of 100 mM-semicarbazide hydrochloride to trap acetaldehyde.

Quantitative tests for enzymic activities were done by incubating ultrasonic extracts as described below. Reactions leading to the reduction or oxidation of nicotinamide nucleotides were followed by measuring the change in E_{340} with a Unicam SP.800 recording spectrophotometer. Where indicated measurements were carried out anaerobically under purified N_2 in cuvettes fitted with a side arm as described by Somerville (1968). Unless otherwise mentioned, progress curves were linear, or nearly so, with time over the test period and the initial rates were proportional to the amount of extract protein over the range investigated. Quantitative results given in the text and in Table 3 have been corrected by subtraction of the rates in the absence of substrate. The latter were usually negligible and never more than 25% of the rate with the complete system.

For the formation of oxaloacetate from aspartate by transamination, a total volume of 3 ml contained 75 mM-potassium phosphate buffer, pH 7.0, 10 μmol of L-aspartate, 10 μmol of 2-oxoglutarate, 0.3 μmol of NADH, 0.05 ml of malate dehydrogenase containing 18 units of activity and ultrasonic extract containing 5.6 mg of protein. Measurements were made anaerobically at 30°C over the first 5 min.

For the synthesis of citrate from oxaloacetate, a volume of 1.5 ml contained 33 mM-tris–HCl buffer, pH 7.6, 6.7 mM-$MgCl_2$, 10 mM-Na_2S, 10 μmol of potassium oxaloacetate, 10 μmol of potassium acetate, 10 μmol of ATP and crude or dialysed ultrasonic extract containing 8.7 mg of protein. After incubation for 20 min at 30°C, 0.15 ml of 50% trichloroacetic acid was added and citrate was estimated. The dialysed extract was prepared from the crude ultrasonic extract by dialysing anaerobically against 0.05 M-tris–HCl buffer, pH 7.6, for 6 h at 0–5°C.

For the oxidation of tricarboxylic acids, a total volume of 3 ml contained 83 mM-potassium phosphate buffer

pH 7.0, 3.3 mM-$MgCl_2$, 0.3 μmol of $NADP^+$, 1 μmol of L-isocitrate, citrate or *cis*-aconitate and ultrasonic extract containing 0.72 mg of protein (experiments with isocitrate) or 3.6 mg of protein (experiments with citrate or *cis*-aconitate). Measurements were made anaerobically at 30°C over the first 3 min. The oxo acid product of the reaction with isocitrate was investigated after 60 min by treating the mixture with 2.4-dinitrophenylhydrazine (Friedemann, 1957) and examining the absorption spectrum. A broad maximum at 380 nm was observed similar to that of the derivative from authentic 2-oxoglutarate and distinct from that from glyoxylate, which has a maximum at 465 nm.

For aspartate kinase assays, a total volume of 1.5 ml contained 60 mM-potassium phosphate buffer, pH 7.0, 13 mM-$MgCl_2$, 0.27 M-hydroxylamine 50 μmol of L-aspartate, 20 μmol of ATP and dialysed ultrasonic extract containing 2.1 mg of protein. The dialysed extract was prepared from crude ultrasonic extract by dialysing for 3 h against 0.05 M-phosphate buffer pH 7.0. Incubation was for 12 min at 30°C after which time hydroxamate was estimated (Black & Wright, 1955*a*).

For aspartate semialdehyde dehydrogenase assays, a total volume of 3 ml contained 80 mM-potassium phosphate buffer, pH 7.3, 7 mM-$MgCl_2$, 50 μmol of L-aspartate, 20 μmol of ATP, 0.4 μmol of NADPH and ultrasonic extract dialysed as for the aspartate kinase tests and containing 5.9 mg of protein. Measurements were made anaerobically at 30°C over the first 3 min.

For homoserine dehydrogenase assays, a total volume of 3 ml contained 83 mM-tris–HCl buffer, pH 8.8, 10 μmol of L-homoserine, 0.5 μmol of NAD^+ and ultrasonic extract containing 3.6 mg of protein. Measurements were made anaerobically at 30°C.

For threonine aldolase assays, a total volume of 3 ml contained 83 mM-potassium phosphate buffer, pH 7.0, 20 μmol of L-threonine, 0.3 μmol of NADH, 10 units of alcohol dehydrogenase and ultrasonic extract containing 2.7 mg of protein. Measurements were made anaerobically at 30°C.

For glycerate dehydrogenase and 3-phosphoglycerate dehydrogenase assays, a total volume of 3 ml contained 83 mM-potassium phosphate buffer, pH 7.0, 10 μmol of $MgCl_2$, 0.4 μmol of NADH or 0.35 μmol of NADPH, and either 1.5 μmol of 3-phosphohydroxypyruvate plus ultrasonic extract containing 0.1 mg of protein or 10 μmol of hydroxypyruvate plus ultrasonic extract containing 3.6 mg of protein. Measurements were made anaerobically at 30°C over the first 3 min.

For alcohol dehydrogenase assays, a total volume of 3 ml contained 77 mM-phosphate buffer pH 7.0, 40 μmol of acetaldehyde, 0.6 μmol of NADH or NADPH and ultrasonic extract containing 6 mg of protein. Measurements were made anaerobically at 30°C over the first 8 min.

Miscellaneous methods. Amino acids were determined by the ninhydrin method of Yemm & Cocking (1955). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard. Inorganic phosphate was determined by a phosphomolybdate method, with ascorbic acid as the reductant (Chen, Toribara & Warner, 1956). Citrate was determined by the pentabromoacetone method of Taylor (1953). Glucose was determined with a blood-sugar test kit based on glucose oxidase [Boehringer Corp. (London) Ltd., London W.5, U.K.]. Except where indicated otherwise, buffer solutions were prepared as described by Gomori (1955).

Chemical reagents. Except where indicated otherwise, all reagents were A.R. grade. Ninhydrin, glyceric acid (calcium salt), isocitric acid (trisodium salt) and all amino acids except homoserine were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Homoserine and aspartyl-β-hydroxamate were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Malate dehydrogenase, alcohol dehydrogenase, NAD^+, $NADP^+$, NADH, NADPH, ATP, ADP, oxaloacetic acid, malic acid, CoA and 3-phosphoglyceric acid were from the Boehringer Corp. (London) Ltd. Pyridoxal phosphate, *cis*-aconitic acid and the tricyclohexylammonium salt of the dimethylketal of phosphohydroxypyruvate were obtained from Calbiochem A.-G., Lucerne, Switzerland. Free phosphohydroxypyruvate was prepared from this derivative as described by Ballou (1960). Glutaconic acid was obtained from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Tris (laboratory reagent; BDH Chemicals Ltd., Poole, Dorset, U.K.) was recrystallized from methanol before use.

All radioactive compounds were obtained from The Radioachemical Centre, Amersham, Bucks., U.K. Before use [U-^{14}C]glucose was purified by chromatography in the butanol–propionic acid–water system of Benson *et al.* (1950) and radioactive amino acids were purified by electrophoresis at pH 1.8 (Rothman & Higa, 1962).

RESULTS AND DISCUSSION

Effect of exogenous amino acids on the incorporation of glucose carbon into the amino acids of the cell protein. Exploratory tracer experiments, conducted in a fashion similar to those described in full below, showed that when *C. pasteurianum* was grown in 'carbonate medium' containing [U-^{14}C]glucose as the sole carbon source, 2.5% of the isotope was incorporated into the cells and 1.1% into the cell protein. When the same medium was supplemented with a mixture of 18 amino acids and five purine and pyrimidine bases the specific radioactivity of the protein fraction was decreased from 862 to 235 c.p.m./μg-equiv. of α-amino N. Examination of the protein hydrolysates by two-dimensional separation and radioautography showed that growth in the supplemented medium eliminated or considerably decreased the radioactivity in all chromatographic areas; only those areas corresponding to alanine, aspartate, glutamate, serine and threonine still retained appreciable radioactivity. The organism is thus able to utilize exogenous amino acids and hence it appeared that the competition inhibition method (Roberts *et al.* 1955) might yield useful information about amino acid biosynthesis.

In all subsequent experiments 'phosphate medium' was used to avoid manipulative difficulties when calcium carbonate was present in the medium.

Table 1. *Effect of exogenous glutamate and aspartate on the incorporation of* ^{14}C *from* [U-^{14}C]*glucose into amino acids of the cell protein of* C. pasteurianum

Cultures were grown in 20ml of 'phosphate medium' with 0.75% of [U-^{14}C]glucose supplemented where indicated with 0.025% of glutamic acid or aspartic acid. From each culture one-fifth of the total protein hydrolysate was taken for analysis; the results have been adjusted to refer to the whole culture. 'Proportion recovered' is the ratio of 'Radioactivity recovered' in each amino acid to the radioactivity present in the initial glucose. Other details are given in the Materials and Methods section.

	Unsupplemented culture		Culture with glutamate			Culture with aspartate		
Amino acid	$10^{-3}\times$ Radioactivity recovered (c.p.m.)	$10^{5}\times$ Proportion recovered (A)	$10^{-3}\times$ Radioactivity recovered (c.p.m.)	$10^{5}\times$ Proportion recovered (B)	$\frac{B}{A}\times 100$	$10^{-3}\times$ Radioactivity recovered (c.p.m.)	$10^{5}\times$ Proportion recovered (C)	$\frac{C}{A}\times 100$
Initial glucose ...	34200	—	39400	—	—	41200	—	—
Aspartate	41.1	120	51.4	130	108	18.4	45	38
Threonine	20.4	60	24.5	62	103	8.0	19	32
Methionine	9.8	29	8.7	22	76	4.9	12	41
Lysine	44.9	131	50.7	129	99	33.4	81	62
Isoleucine	44.3	129	49.4	125	97	44.3	107	83
Glycine	14.4	42	20.7	53	126	6.3	15	36
Serine	24.1	71	27.7	70	99	16.3	40	56
Glutamate	57.8	169	24.5	62	37	43.3	105	62
Proline	14.7	43	7.3	19	44	14.3	35	81
Arginine	22.2	65	15.5	39	60	23.0	56	86
Alanine	34.5	101	37.3	95	94	38.7	94	93
Histidine	5.4	16	7.2	18	112	6.0	15	94
Tyrosine	33.6	98	34.3	87	89	36.3	88	90
Leucine	51.1	149	51.4	131	88	53.6	130	87
Phenylalanine	32.6	95	37.4	95	100	37.7	91	96
Valine	30.4	89	33.8	86	97	34.8	84	94

A glucose concentration of 0.75% was selected as the most suitable for assimilation experiments as this gave close-to-maximum growth with complete utilization of the glucose. Tests with aspartate and glutamate as supplements showed that 0.025% was sufficient to ensure that the amino acid was not exhausted before growth ceased. In these tests the culture supernatant was examined by paper electrophoresis at pH 3.6 and it was observed incidentally that basic and neutral amino acids appear in the medium during growth even on the unsupplemented medium. The identity of these amino acids has not been investigated further; the excretion of soluble nitrogenous compounds into the medium by *C. pasteurianum* fixing $^{15}N_2$ was reported earlier by Rosenblum & Wilson (1950).

The effect of exogenous aspartate or glutamate on the incorporation of ^{14}C from [U-^{14}C]glucose into the individual amino acids of the cell protein was next examined. Reliable specific radioactivities could not be obtained in this experiment because the particular batch of chromatographic paper used gave unexpectedly high blanks in the amino acid determination. Incorporations were therefore compared by expressing the radioactivity recovered in each amino acid as a proportion of the total initially added as [U-^{14}C]glucose (Table 1). The values so obtained for the two supplemented cultures (B and C) are also given as a percentage of those for the unsupplemented culture (A). Under these circumstances the conclusions drawn involve the assumption that the relative recoveries of the amino acids during their isolation from each of the three cultures were the same. The similar values of A, B and C for alanine, histidine, tyrosine, leucine and phenylalanine, none of which is believed to have any direct metabolic connexion with aspartate and glutamate, indicate that this assumption is justified.

The addition of exogenous glutamate to the medium caused a marked decrease in the radioactivity recovered in glutamate, proline and to a smaller extent arginine, indicating that glutamate is utilized for the formation of both proline and arginine. The presence of exogenous aspartate caused analogous effects, which suggested that aspartate is a precursor of threonine, methionine, lysine, glycine, serine and glutamate. The smaller decreases in the radioactivity recovered in some amino acids, e.g. methionine from the culture with glutamate and isoleucine, arginine and proline from

the culture with aspartate, were considered suggestive but too small to permit firm conclusions to be drawn.

Assimilation of ^{14}C-labelled amino acids. To obtain more evidence for the metabolic connexions suggested by the above experiments, the incorporation of carbon from [U-^{14}C]aspartate and [U-^{14}C]-glutamate was examined. Experiments were also done with [U-^{14}C]threonine, [2-^{14}C]glycine and [U-^{14}C]serine in an attempt to clarify their interrelation. With glutamate as ^{14}C source, the specific radioactivity of the glutamate isolated from the protein hydrolysate was close to that of the original exogenous glutamate (Table 2). When the other amino acids were used as ^{14}C sources, however, their specific radioactivities after isolation from protein were considerably less than the initial values, presumably owing to dilution with carbon from glucose. For comparative purposes therefore Table 2 also gives the specific radioactivities of the various isolated amino acids as a percentage of that of the ^{14}C source after isolation from protein. Accordingly, in the considerations of carbon flow that follow, any quantitative mention of amino acids as precursors relates to the amino acid within the cell rather than to the exogenous material supplied in the medium.

High relative specific radioactivities confirmed that glutamate gives rise to a large proportion of the carbon of proline and arginine and that aspartate gives rise to a large proportion of the carbon of threonine, methionine, lysine, glycine, serine and, to a smaller extent, isoleucine. In addition, there was a high incorporation of aspartate carbon into glutamate and pronounced incorporation into arginine and proline. These experiments with labelled exogenous amino acids give a much more sensitive detection of minor metabolic connexions than did the experiments with labelled glucose. They confirmed most of the interrelations suggested by the latter experiments and revealed others not previously detected, such as the appearance of small amounts of aspartate carbon in histidine.

The relationships observed between glutamate, aspartate and the other amino acids are in the main comparable with those observed in *E. coli* (Roberts *et al.* 1955). The incorporation of aspartate carbon into glycine and serine is, however, much higher in *C. pasteurianum* than in *E. coli*, indicative of a metabolic route not well developed in the latter organism. Where the isolated amino acid contains fewer carbon atoms than the exogenous source of ^{14}C, the maximum possible relative specific radioactivity will be less than 100%. In this instance, since glycine contains only two carbon atoms, whereas the radioactivity of the exogenous [U-^{14}C]-aspartate was distributed through all four carbon atoms, the observed relative specific radioactivity of glycine (50%) shows that all of the glycine carbon is derived from aspartate. Similarly, in the experiment with exogenous [U-^{14}C]threonine, all of the glycine carbon came from threonine. In this experiment there was also a high incorporation of threonine carbon into isoleucine and a marked incorporation into serine, but no radioactivity was detected in aspartate, indicating that threonine is a probable intermediate in the flow of carbon from aspartate to isoleucine, glycine and serine. There was a high incorporation of radioactivity from exogenous glycine into serine, but very little or no radioactivity passed into aspartate, isoleucine or threonine. Radioactivity from exogenous serine did not pass into isoleucine or threonine and very little passed into aspartate, but there was a marked incorporation into glycine. These observations suggest the following metabolic relations between these amino acids and glucose:

$$\text{Glucose} \rightarrow \text{aspartate} \rightarrow \text{threonine} \begin{cases} \nearrow \text{glycine} \rightleftharpoons \text{serine} \\ \searrow \text{isoleucine} \end{cases}$$

Enzymic experiments on aspartate formation. Conventional pathways for the formation of aspartate from glucose involve the carboxylation of a C_3 intermediate such as pyruvate or phosphoenolpyruvate. Ultrasonic extracts of *C. pasteurianum* were therefore incubated with [^{14}C]-bicarbonate, glutamate and either pyruvate or phosphoenolpyruvate under the conditions indicated in the Materials and Methods section and the radioactive products were examined by ionophoresis and radioautography. With pyruvate as the C_3 substrate, four radioactive products were detected with the respective properties of aspartate, malate, oxaloacetate and a neutral amino acid, possibly alanine; the presumptive aspartate was not formed when glutamate was omitted from the incubation. Similar results were obtained with respect to aspartate when phosphoenolpyruvate served as the C_3 substrate. In this case aspartate was the only radioactive product detected with the complete incubation system; oxaloacetate and an unidentified radioactive substance were detected when glutamate was omitted.

Transamination between glutamate and oxaloacetate and the reverse reaction were demonstrated by incubating the appropriate substrates with ultrasonic extract and identifying the amino acid product. The activity in the direction of glutamate formation was also measured by coupling the reaction with malic dehydrogenase (Table 3). The organism is thus able to form aspartate from carbon dioxide plus pyruvate or phosphoenolpyruvate, probably via oxaloacetate with glutamate as the amino donor. The mechanism of carboxylation has not been investigated in detail.

Table 2. *Incorporation of* ^{14}C *into amino acids of the cell protein from exogenous* [^{14}C]*-glutamate, -aspartate, -threonine, -glycine and -serine*

Cultures were grown in 20 ml of 'phosphate medium' with 0.75% of glucose and 0.025% of L-[U-^{14}C]glutamate, L-[U-^{14}C]aspartate, L-[U-^{14}C]threonine, [2-^{14}C]glycine or L-[U-^{14}C]serine. The following portions of the protein hydrolysate were taken for separation: one-thirtieth for the cultures with glutamate and glycine, one-twenty-fifth for aspartate and threonine and one-fiftieth for serine. The results have been adjusted to refer to the whole culture. Other details are given in the Materials and Methods section. 'Relative specific radioactivity' is the specific radioactivity as a percentage of that of the isotope source after isolation from the cell protein in the same experiment.

Amino acid	10^{-3} × Radioactivity recovered (c.p.m.)	Amino acid (μmol)	10^{-3} × Specific radioactivity (c.p.m./μmol)	Relative specific radioactivity
Expt. 1, with glutamate				
Initial exogenous glutamate	13100	34.0	386	—
Protein hydrolysate	890	—	—	—
Glutamate	456	1.2	380	(100)
Arginine	140	0.6	230	61
Proline	102	0.3	340	89
Threonine	22	0.6	36	9
Expt. 2, with aspartate				
Initial exogenous aspartate	9250	37.6	246	—
Protein hydrolysate	1330	—	—	—
Aspartate	438	3.8	116	(100)
Threonine	563	0.5	110	95
Methionine	608	0.5	120	103
Lysine	250	2.5	100	86
Isoleucine	558	1.25	45	39
Glycine	116	2.0	58	50
Serine	69	1.75	39	34
Glutamate	151	1.25	120	103
Arginine	29	1.0	29	25
Proline	50	1.0	50	43
Histidine	8	0.5	16	14
Alanine	10	2.25	4	4
Expt. 3, with threonine				
Initial exogenous threonine	8000	42.0	191	—
Protein hydrolysate	230	—	—	—
Threonine	57	0.75	76	(100)
Glycine	76	1.75	43	57
Serine	24	1.5	16	21
Isoleucine	66	1.25	52	68
Glutamate	8	1.0	7	9
Alanine	2	2.5	4	5
Expt. 4, with glycine				
Initial exogenous glycine	28400	66.6	436	—
Protein hydrolysate	1320	—	—	—
Glycine	642	1.8	360	(100)
Serine	324	1.5	220	61
Alanine	75	1.8	42	12
Glutamate	24	1.5	16	4
Aspartate	15	3.6	4	1
Histidine	7	0.6	12	3
Expt. 5, with serine				
Initial exogenous serine	10000	47.6	210	—
Protein hydrolysate	490	—	—	—
Serine	171	1.5	114	(100)
Glycine	94	2.5	37	32
Glutamate	21	1.0	21	18
Alanine	48	3.5	14	12
Lysine	25	2.5	10	9
Aspartate	19	2.0	9	8
Methionine	4	0.5	7	6
Histidine	9	1.0	9	8
Arginine	5	1.0	5	4
Valine	12	2.0	6	5

Table 3. *Enzymic activities determined quantitatively in cell-free extracts of* C. pasteurianum

The nicotinamide coenzymes used in the individual dehydrogenase assays are indicated in parentheses. In the test for aconitase, *cis*-aconitate was used as substrate for the reduction of $NADP^+$ under conditions where the isocitrate dehydrogenase was not limiting. The assays for threonine aldolase and aspartate aminotransferase involved the oxidation of NADH by coupled reactions. Other details are given in the Materials and Methods section.

Enzyme	Specific activity (μmol/h per mg of protein)
Citrate synthase	0.2
Isocitrate dehydrogenase	4.7 ($NADP^+$)
Aconitase	2.1
Aspartate kinase	2.4
Aspartate semialdehyde dehydrogenase	1.9 (NADPH)
Homoserine dehydrogenase	0.8 (NAD^+)
Threonine aldolase	1.9
Alcohol dehydrogenase	0.4 (NADH), 0.6 (NADPH)
3-Phosphoglycerate dehydrogenase	208 (NADH), 73 (NADPH)
Glycerate dehydrogenase	0.5 (NADH), 1.3 (NADPH)
Aspartate aminotransferase	0.2

Enzymic experiments on glutamate formation. The formation of glutamate from oxaloacetate by the reactions of the tricarboxylic acid cycle as far as 2-oxoglutarate, followed by amination, is a well-established biosynthetic route in aerobic species (e.g. Umbarger & Davis, 1962) and strong evidence for the same route in anaerobes has recently accumulated (Somerville & Peel, 1967; Somerville, 1968; Gottschalk & Barker, 1967; Gottschalk, 1968). The present tracer experiments are consistent with this route in *C. pasteurianum* and Gottschalk & Barker (1967) showed that cell-free extracts of this organism incorporate isotope from [1-^{14}C]acetyl phosphate into C-5 of 2-oxoglutarate. We have tested for individual reactions of the tricarboxylic acid route in *C. pasteurianum* under the conditions described in the Materials and Methods section. Ultrasonic extracts catalysed the formation of citrate from oxaloacetate and with dialysed extracts a stimulation was obtained with CoA. ATP and acetate were not essential for citrate formation, presumably because the extracts were able to form pyruvate and acetyl-CoA from the oxaloacetate. Isocitrate dehydrogenase (EC 1.1.1.42) was demonstrated by observing the reduction of NADP and the formation of 2-oxoglutarate; no activity was observed with NAD. The presence of aconitase (EC 4.2.1.3) was inferred from the ability of the extracts to reduce NADP in the presence of aconitate or citrate. The observed rates of these reactions are given in Table 3.

Several unsuccessful attempts were made to demonstrate glutamate dehydrogenase (EC 1.4.1.2) in extracts by testing spectrophotometrically for the oxidation of NADH or NADPH in the presence of 2-oxoglutarate and ammonia. Tests were made with ultrasonic extracts, extracts of vacuum-dried cells and extracts of cells crushed in a bacterial press (Hughes, 1951), in buffers over the range pH 5–9.5. No activity was ever detected, although in parallel tests with *P. elsdenii* the reaction with NADH could be readily demonstrated. Several of the reaction mixtures were examined for glutamate after incubation, but none was found. The reverse reaction could not be demonstrated spectrophotometrically with or without a keto fixative present.

The possibility that *C. pasteurianum* might contain a glutamate dehydrogenase using some reductant other than NADH or NADPH was also explored. In the saccharolytic clostridia, the electrons released in the anaerobic oxidation of pyruvate are passed to ferredoxin, at a lower potential than the NAD^+–NADH couple, and the possibility exists that such organisms may possess a glutamate dehydrogenase capable of using ferredoxin or some other low potential carrier as electron donor, rather than NADH or NADPH. Tests were therefore made in which ultrasonic extracts were incubated anaerobically with pyruvate, 2-oxoglutarate, ammonia and CoA and the products examined for glutamate. In some tests ferredoxin was added to the system and in one such experiment a ninhydrin spot corresponding to glutamate was detected. Attempts to repeat the experiment, however, were unsuccessful.

Tests for the formation of glutamate when extracts were incubated with ammonia and glutaconate were also negative. Glutaconate would be the precursor of glutamate in an alternative (but hypothetical) pathway of glutamate synthesis via vinylacetyl-CoA or crotonyl-CoA (Somerville & Peel, 1967).

Tracer experiments with $^{15}N_2$ and $^{15}NH_3$ with the same strain of *C. pasteurianum* as in the present work indicated that glutamate is the first amino acid into which inorganic nitrogen is incorporated (Wilson & Burris, 1953). This renders unlikely the possibility that glutamate is formed from 2-oxoglutarate by transamination from aspartate, even though this reaction was observed with cell extracts. Tests with cells grown in medium supplemented with glutamate showed a marked decrease in the rate of citrate synthesis (0.12–0.17 μmol/h per mg of extract protein from cells grown without glutamate, 0.02–0.09 μmol/h per mg of extract protein from cells grown with glutamate). On the other hand, the activity of isocitrate dehydrogenase (4.0–4.7 μmol/h per mg of extract protein) was not affected by growth with glutamate. These observations are in line with several other instances where

the end product of a specific biosynthetic sequence (cf. glutamate in this case) represses or inhibits the enzyme catalysing the first reaction beyond the branch point (cf. oxaloacetate in this case) at which the specific portion of the biosynthetic route begins (see, e.g., Fry, 1964).

Although Gottschalk & Barker (1967) demonstrated the formation of [^{14}C]citrate from acetyl-CoA plus [^{14}C]oxaloacetate and of [^{14}C]glutamate from [^{14}C]acetyl phosphate plus oxaloacetate by extracts from *C. pasteurianum*, the glutamate was isolated after treatment of the reaction products with added glutamate dehydrogenase and the presence of this enzyme in the extracts was not reported. On balance, therefore, the available evidence indicates that in *C. pasteurianum* 2-oxoglutarate is formed by the tricarboxylic acid route, but that the subsequent amination to give glutamate may involve some system other than a conventional glutamate dehydrogenase.

Enzymic experiments on threonine formation. The results of the experiments with radioactive compounds indicate that threonine is formed from aspartate. The following reaction sequence is already known in other organisms (Umbarger & Davis, 1962) and could account for this conversion:

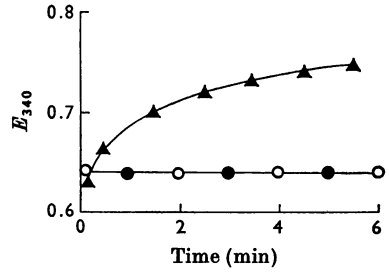
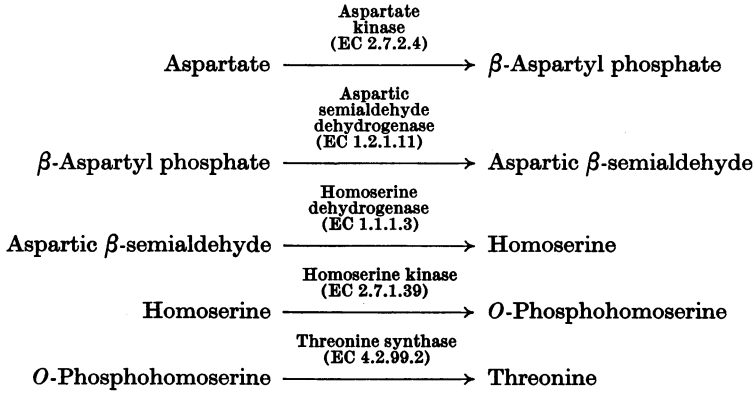


Fig. 1. Homoserine dehydrogenase in ultrasonic extracts of *C. pasteurianum*. Experimental details are given in the Materials and Methods section. ▲, Complete system; ○, without homoserine; ●, $NADP^+$ replacing NAD^+.



Evidence for this pathway was therefore sought in *C. pasteurianum*.

Aspartate kinase was demonstrated by measuring the formation of hydroxamate when an extract was incubated with aspartate, ATP and hydroxylamine as trapping agent (Black & Wright, 1955*a*) (Table 3). Since β-aspartyl phosphate was not readily available, the aspartic semialdehyde dehydrogenase was demonstrated by substituting NADPH for hydroxylamine in the aspartate kinase assay and following its oxidation spectrophotometrically (Table 3). Both reactions were completely dependent on ATP and marked stimulations were observed with Mg^{2+}, in keeping with the Mg^{2+} requirement of the aspartate kinase of yeast (Black & Wright, 1955*a*). NADH did not replace NADPH in the dehydrogenase reaction.

Homoserine dehydrogenase was not tested for in the biosynthetic direction since aspartic β-semialdehyde was not available; instead the reverse reaction was demonstrated. Extracts readily reduced NAD^+ in the presence of homoserine, and $NADP^+$ would not substitute for NAD^+ (Fig. 1 and Table 3). Reduction of NAD^+ ceased after about 10min, at which time about 15% of the NAD^+ was reduced; this is consistent with observations that the equilibrium of the reaction in other micro-organisms favours homoserine formation (Patte, Le Bras, Loviny & Cohen, 1963; Black & Wright, 1955*b*).

No attempt was made to demonstrate homoserine kinase and threonine synthase separately but evidence for their combined action was obtained by incubating extracts with homoserine ATP, Mg^{2+} and pyridoxal phosphate and identifying threonine in the products. The necessary enzymes for converting aspartate into threonine via homoserine are therefore present in *C. pasteurianum*.

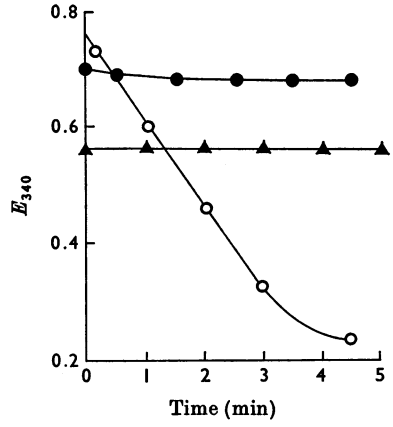


Fig. 2. Threonine aldolase in ultrasonic extracts of *C. pasteurianum*. Experimental details are given in the Materials and Methods section ○, Complete system; ●, without threonine; ▲, without extract.

Formation of glycine from threonine. The formation of glycine from threonine indicated by the tracer experiments could be accounted for by the action of threonine aldolase (EC 4.1.2.5), already known in animal tissues (Lin & Greenberg, 1954) and catalysing the reaction

$$CH_3 \cdot CH(OH) \cdot CH(NH_2) \cdot CO_2H \rightarrow CH_3 \cdot CHO + CH_2(NH_2) \cdot CO_2H$$

Extracts of *C. pasteurianum* were shown to contain this enzyme by using alcohol dehydrogenase (EC 1.1.1.1) to remove the acetaldehyde and following the oxidation of NADH (Fig. 2 and Table 3). The extracts contained sufficient alcohol dehydrogenase for this purpose but the rate of coenzyme oxidation with threonine varied with the preparation; higher and more consistent rates were obtained when an excess of alcohol dehydrogenase was added to the assay system. The extracts catalysed the oxidation of both NADH and NADPH by acetaldehyde (Table 3) and the threonine aldolase activity could be demonstrated with either coenzyme.

Preliminary identification of the products was obtained by carrying out the reaction in the outer chamber of a Conway microdiffusion unit with semicarbazide in the centre well. The absorption spectrum of the contents of the centre well after incubation showed an absorption peak at 224nm, indicating the formation of a volatile carbonyl compound. Glycine was tentatively identified as a reaction product by ionophoretic separation on paper of a sample of the deproteinized reaction mixture. These observations demonstrate the presence of threonine aldolase in cell extracts. A more rigorous identification of the products and results on the stoicheiometry of the reaction obtained with purified preparations from *C. pasteurianum* are described in the following paper (Dainty, 1970), together with fuller details of the assay procedure. These purified preparations were also shown to catalyse the reverse reaction. The tracer experiments, however, in which no incorporation of radioactivity from glycine (or serine) into threonine was observed, show that the reverse reaction is not significant *in vivo*.

The effect of adding glycine, serine and threonine to the growth medium on the threonine aldolase activity of cell extracts was examined. The activities observed were in the same range as with extracts from cells in unsupplemented medium (1.3–1.9 μmol/h per mg of protein).

These results show that threonine aldolase is present in *C. pasteurianum*, irrespective of the presence of threonine or glycine during growth, and could account for the formation of glycine from glucose via threonine. A second possible pathway of conversion of threonine into glycine would involve aminoacetoacetate as an intermediate:

Reaction 1

$$CH_3 \cdot CH(OH) \cdot CH(NH_2) \cdot CO_2H + NAD^+ \rightarrow CH_3 \cdot CO \cdot CH(NH)_2 \cdot CO_2H + NADH$$

Aminoacetoacetate

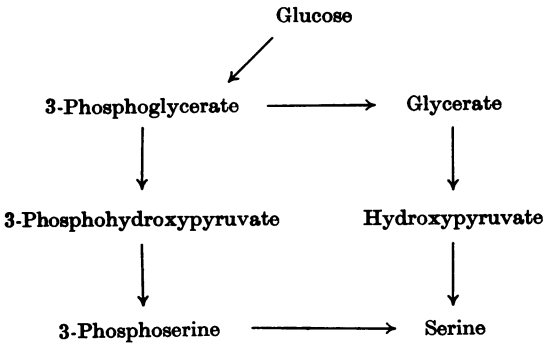
Reaction 2

$$CH_3 \cdot CO \cdot CH(NH_2) \cdot CO_2H + CoA \cdot SH \rightarrow CH_3 \cdot CO \cdot S \cdot CoA + CH_2(NH_2) \cdot CO_2H$$

Reaction 1 has been demonstrated in *Staphylococcus aureus* (Green & Elliott, 1964) and in *Rhodopseudomonas spheroides* (Neuberger & Tait, 1962), and both reactions have been demonstrated in an *Arthrobacter* species (McGilvray & Morris, 1969). Extracts of *C. pasteurianum* have been tested for both enzymes of this pathway by the methods of McGilvray & Morris (1969), which depend upon the detection of aminoacetone. Neither enzyme was detected under conditions where an activity of 2% of that of the threonine aldolase would have been apparent.

Enzymic experiments on serine synthesis. The tracer experiments showed that *C. pasteurianum* can interconvert glycine and serine but that glycine is not an obligatory intermediate in the formation of serine from glucose. Serine may be formed from glycolytic intermediates by the pathway known to occur in *S. typhimurium* (Umbarger, Umbarger & Siu, 1963; Pizer, 1963) and *P. elsdenii* (Somerville, 1965, 1968) and involving phosphohydroxypyruvate and phosphoserine as intermediates. Alternatively dephosphorylation may occur at an earlier stage or possibly via 2-phosphoglycerate (cf. Fallon & Byrne,

1965) followed by analogous reactions of non-phosphorylated intermediates.



Results from the present investigations are insufficient to determine whether either of these pathways functions in serine synthesis in *C. pasteurianum*, but the following results may be noted.

(1) In several tests with crude extracts no release of inorganic phosphate from phosphoserine was detected, although the same activity was readily detected in parallel experiments with extracts of *P. elsdenii* and *Veillonella alcalescens*.

(2) The formation of phosphoserine and serine by transamination from glutamate and phosphohydroxypyruvate or hydroxypyruvate was demonstrated qualitatively at pH 7.4 with extracts of *C. pasteurianum*.

(3) Glycerate dehydrogenase (EC 1.1.1.29) and 3-phosphoglycerate dehydrogenase could not be demonstrated in the biosynthetic direction, but crude extracts of *C. pasteurianum* catalysed the re-oxidation of both NADH and NADPH by either phosphohydroxypyruvate or hydroxypyruvate. Whereas, however, the activities with hydroxypyruvate were of similar magnitude to those observed with the other biosynthetic enzymes (Table 3), those with phosphohydroxypyruvate were about 100-fold greater. This high activity was observed with several preparations, although the first attempts to demonstrate the enzyme had been unsuccessful (Dainty & Peel, 1966). In view of this unexpectedly high activity its enzymic nature was checked by (*a*) heating at 100°C for 5 min, when the activity was lost, and (*b*) precipitating by the addition of ammonium sulphate (4 ml of saturated ammonium sulphate/ml of extract); the precipitated material had a specific activity (181 μmol of NADH oxidized/h per mg of protein) close to that of the original extract. The possibility that the oxidation of NADH is due to some other substrate, present as an impurity in the phosphohydroxypyruvate, is remote; in one experiment when the amount of phosphohydroxypyruvate was decreased to 1.5 μmol, 0.2 μmol of NADH was still reduced and the amount of active impurity required to account for this would be 13% on a molar basis.

The activity of this enzyme is vastly in excess of what would be needed to account for serine biosynthesis. This suggests that the amount of this enzyme is not subject to control or that it has some other important function that is not yet apparent.

General considerations. The present investigation shows that aspartate and glutamate are important intermediates in the biosynthesis of amino acids in *C. pasteurianum* grown with glucose as carbon source. Thus the tracer evidence indicates that glutamate acts as a precursor of arginine and proline and that aspartate acts as a precursor of threonine, methionine, isoleucine and lysine. The enzymic experiments show that aspartate may be formed by the carboxylation of pyruvate or phosphoenolpyruvate followed by transamination with glutamate as amino donor; with cells growing on glucose the carboxylation of phosphoenolpyruvate is perhaps the more probable route. The enzymic and tracer evidence in general supports the view that glutamate is formed from oxaloacetate by the tricarboxylic acid route; the failure to obtain a convincing amination of 2-oxoglutarate with cell-free extracts is the main gap in the evidence. The enzymic experiments also indicate that the conversion of aspartate into threonine is by the conventional route. In broad outline therefore the pathways forming and utilizing aspartate and glutamate in *C. pasteurianum* appear to be similar to those established previously in aerobic and facultatively aerobic organisms.

The most striking finding with *C. pasteurianum* is the role of threonine in the formation of glycine. The tracer experiments show that when exogenous aspartate or threonine is present it provides all the carbon used for glycine formation. When exogenous serine is present only about half the glycine carbon is derived from this substrate. The simplest interpretation of this observation is that half the number of glycine molecules are derived from serine, presumably by the action of serine hydroxymethyltransferase (Rabinowitz, 1960), whereas the remainder of the glycine is formed by some other means, probably via threonine. Together with the fact that threonine aldolase activity is found in cells grown on glucose, with or without supplements of threonine, serine and glycine, these observations strongly suggest that in this organism the sole route of glycine formation from glucose is by way of threonine. Tracer experiments suggest that in *E. coli* this is a minor pathway (Roberts *et al.* 1955) or may operate only when exogenous aspartate or threonine is present. Tests have shown that threonine aldolase is present in other strict anaerobes (Dainty, 1970) and it is possible that this pathway may be widespread among this physiological group of organisms.

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