AMINO ACID METABOLISM BY PLEUROPNEUMONIALIKE ORGANISMS

I. General Catabolism[1]

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Received for publication March 24, 1955

Earlier metabolic studies of the pleuropneumonialike organisms had failed to demonstrate significant activity beyond the dehydrogenation of monohydric alcohols (Lecce and Morton, 1954) and the conversion of adenine to hypoxanthine (Somerson, 1954). However, their ability to multiply readily in artificial media indicated that they should be able to catabolize some of the substances present in such media. The development of a synthetic medium capable of supporting the growth of some strains (Smith, *to be published*) has permitted a more rational approach to the study of the catabolic processes of these microorganisms. This paper presents the results of an investigation of amino acid metabolism by resting cells of certain human strains.

MATERIALS AND METHODS

Resting cells of two strains, 07 and 39, were grown and harvested as follows: An agar block containing colonies of a 3-day growth was placed in each of a series of tubes containing 10 ml of heart infusion broth (Morton *et al.*, 1951) supplemented with 1 per cent Bacto PPLO serum fraction. Following 3 days' incubation at 37 C, each 10-ml culture was inoculated into 100 ml of medium. After 40 hours' incubation, each 100-ml culture was used to inoculate 1500 ml of broth in a 2-liter flask containing thallium acetate (1:2,000) (Morton and Lecce, 1953). The cells were harvested after 40 hours' incubation at 37 C by first concentrating in the Sharples centrifuge at 15,000 rpm. The concentrate was then centrifuged in a Servall angle centrifuge at 12,000 rpm and the sedimented cells washed once with 15 M phosphate buffer, pH 8.0. Cellular nitrogen was measured turbidimetrically (Lecce and Morton, 1954) and the washed cells resuspended in 15 M phosphate buffer of appropriate pH so as to give a concentration of 2 mg nitrogen per ml. Yields of 1 to 1.5 mg cellular nitrogen per L were regularly obtained. For the transamination experiments, cell-free extracts were employed to eliminate the possibility of impermeability of whole cells to α-ketoacids. These extracts were prepared by subjecting suspensions of whole washed cells containing 2 mg cellular nitrogen per ml in phosphate buffer to sonic vibrations in a 9-Kc Raytheon magneto-constriction oscillator for a period of 10 min. Cellular debris was removed by centrifugation at 10,000 rpm at 0 C for 10 minutes in a Spinco ultracentrifuge, model L.

The amino acids, α-ketoacids and pyridoxal phosphate employed in this study were obtained from Nutritional Biochemicals Corporation.

Experiments designed to detect the breakdown of amino acids were carried out as follows:

The complete reaction mixture, containing approximately 50 μM amino acid, 10^{-5} M pyridoxal phosphate and resting cells equivalent to one mg nitrogen in pH 8.0 phosphate buffer was contained in a final volume of 1.0 ml. One set of the mixture was incubated in test tubes in a 37 C water bath under aerobic conditions. Another set was run in Thunberg tubes under anaerobic conditions. Anaerobiosis was achieved by alternately evacuating and filling each tube with nitrogen four times and then incubating in an atmosphere of nitrogen. At the end of the reaction time, (3 hours unless otherwise specified), one ml of 10 per cent trichloracetic acid and 3 ml of distilled water were added to each reaction mixture. The clear supernatant overlying the precipitated protein was employed for paper chromatographic analysis. In each experiment a substrate control for each amino acid and cell controls containing no substrate were run in the same manner.

[1] This work was supported in part by a contract (NONR 551(04)) between the Office of Naval Research and the University of Pennsylvania.

Experiments for detection of possible transamination were run in the same way except that 30 μM of α-ketoacid were added to the reaction mixture and an additional α-ketoacid control was run. Varying cellular concentrations, reaction times and pH values were employed. All transamination reactions were run anaerobically.

Quantitative estimation of the amino acids was accomplished by paper chromatographic methods. Known amounts of amino acids were spotted in duplicate or triplicate in a narrow line on 1½-inch wide strips of Whatman no. 1 filter paper. These strips were allowed to develop overnight by ascending chromatography employing a water-saturated phenol solvent. After air-drying, the paper strips were saturated by spraying with 0.25 per cent ninhydrin in water-saturated butanol and then heated at 100 C for 5 minutes. The ninhydrin positive areas were cut out, clarified with mineral oil containing 2 per cent α-bromnaphthalene, and the density of each mm area determined in a photoelectric densitometer (Bender and Hobein, München 15, Lindwurmstrasse 71, Germany). Density was plotted against the length of the area in mm and the area underlying the curve determined in cm^2 by the use of a K & E compensating polar planimeter. The area plotted against the concentration in μM resulted in a standard curve. Most amino acids gave standard curves with an error of +0.05 μM or less. Exceptions to this were tryptophan and lysine. The range of levels of amino acids which could be detected and accurately fitted to Beer's law varied greatly with different amino acids. Reproducibility, however, was easily obtainable if exact conditions were maintained. Standard curves for proline and hydroxyproline were prepared in a similar manner, except that the developing solvent consisted of water-saturated *n*-butanol plus 12 per cent glacial acetic acid, and the color developer was 0.2 per cent isatin in *n*-butanol containing 4 per cent acetic acid (Acher, *et al.*, 1950). No satisfactory standard curve was obtained for hydroxyproline.

Unmetabolized and converted amino acids were then quantitatively determined by the above procedure, by chromatographing different amounts of the reaction mixture and substrate controls and determining the concentration in μM from the standard curves. Enough replicate determinations were made to achieve reliable results.

α-Ketoglutarate was determined by the method of Cavallini *et al.* (1949).

RESULTS

Synthetic media commonly employed for the cultivation of lactobacilli failed to support the growth of stock strains of human pleuropneumonialike organisms even with the addition of serum or the lipoprotein growth factor (Smith *et al.*, 1954). However, growth of some strains, including Campo, occurred upon substitution of the basal medium with the TC medium no. 199 (Difco) which conforms with the formula of Morgan *et al.* (1950). Due to the altered nature of the Campo strain (Smith *et al.*, 1954) it was considered unsatisfactory for metabolic studies. Another group of stock strains, which includes strains 07 and 39, was found to grow well on the TC medium no. 199 with added lipoprotein factor and Bacto-peptone. It was subsequently found that the ash of this peptone contained the growth requirement. Other strains such as 48 and 60 failed to grow even with added peptone. Thus, strains 07 and 39 were selected for metabolic studies.

Modification of this synthetic medium by omission of the fat-soluble vitamins and glucose did not alter its growth supporting properties even though no energy sources other than amino acids were available for the pleuropneumonialike organisms. Two criteria were used in attempting to elucidate the possible metabolic functions of these amino acids, viz.: (1) their degradation as indicated by their disappearance and (2) their conversion to α-keto acids by transamination.

Catabolism of amino acids. The amino acids contained in the synthetic medium in quantity and therefore employed as substrates for resting cells of pleuropneumonialike organisms, in particular strain 39, were DL-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-glutamine, glycine, L-histidine, L-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, L-methionine, DL-phenylalanine, L-proline, DL-serine, L-threonine, DL-tryptophan, L-tyrosine and DL-valine.

Table 1 lists those amino acids which were catabolized and the extent of their breakdown in 3 hours. The disappearance of tyrosine and tryptophan in an aerobic atmosphere was in-

TABLE 1

Catabolism of amino acids by resting cells of a pleuropneumonialike organism (strain 39)

Amino Acid	Gaseous Environment	μM Amino Acid		
		Initial	Utilized	Remaining converted compound
L-Arginine	Aerobic	42.0	42.0	29.9*
	Anaerobic	42.0	42.0	45.0
L-Aspartic acid	Aerobic	36.3	5.0	
	Anaerobic	36.3	17.5	
L-Glutamic acid	Aerobic	59.8	38.3	
	Anaerobic	59.8	20.2	
L-Glutamine	Aerobic	38.8	38.8	27.2*
	Anaerobic	38.8	38.8	26.3
L-Histidine	Aerobic	72.5	11.3	
	Anaerobic	72.5	0	
L-Leucine	Aerobic	42.5	10.3	
	Anaerobic	42.5	0	
L-Threonine	Aerobic	72.5	13.7	
	Anaerobic	72.5	0	
DL-Tryptophan	Aerobic	53.8	5.3	
	Anaerobic	53.8	13.9	
L-Tyrosine	Aerobic	43.8	1.9	
	Anaerobic	43.8	10.5	

* Arginine → citrulline; glutamine → glutamic acid.

Each tube contained: 0.2 ml amino acid; 0.1 ml 10^{-4} M pyridoxal PO_4; 0.2 ml pH 8.0 15 M phosphate buffer; 1.0 mg cellular nitrogen in 0.5 ml phosphate buffer.

Reaction time: 3 hr.

significant, while no loss of histidine, leucine and threonine occurred in an anaerobic atmosphere. Strain 39 was found to utilize aspartic acid at a slower rate than strain 07. There was little difference in aspartic acid utilization by the latter strain under aerobic and anaerobic conditions. Arginine disappeared completely in 3 hours. The rate of arginine utilization is shown in figure 1b together with the concomitant rate of appearance of citrulline. Although arginine was converted to citrulline as shown by identical Rf values and the additive effect of the reaction product on citrulline in paper chromatographs, citrulline was not recovered in amounts equivalent to the arginine utilized. No other ninhydrin positive areas appeared on the chromatographs of the arginine reaction mixture. Glutamine, in a manner similar to arginine, disappeared completely in 3 hours. Figure 1a shows the rate of glutamine breakdown and glutamic acid appearance. Quantitative recovery of glutamine as glutamic acid was not possible since the latter compound is further metabolized. Figure 2 shows the rate of glutamic acid disappearance. This rate is much slower than the rates for arginine and glutamine disappearance. Further analysis of the reaction mixture in which glutamic acid was the substrate has revealed a substance which gives a color reaction with isatin and increases in quantity with

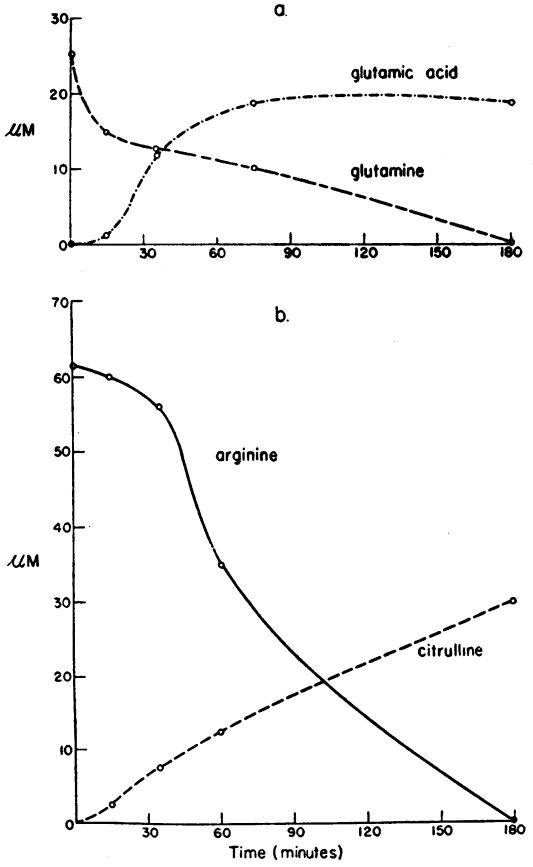

Figure 1. Rates of breakdown of amino acids by the human pleuropneumonialike organism strain 39. a: Rates of disappearance of glutamine and appearance of glutamic acid. b: Rates of disappearance of arginine and appearance of citrulline.

increased reaction time. No α-ketoglutaric acid could be demonstrated in the reaction mixture nor was this α-keto acid utilized by resting cells. The isatin-positive compound was found not to be identical with proline. Furthermore, α-pyrrolidone carboxylic acid, which has been suggested as an intermediate between glutamic acid and proline (Braunstein, 1947), did not give a color reaction with isatin.

Transamination attempts. Initial experiments designed to demonstrate transamination reactions were carried out with the Campo strain. In a few experiments employing 2 mg cellular nitrogen per ml, a pH of 8.4 and a reaction time of 3 hours, insignificant transamination between aspartic acid, leucine or valine and α-ketoglutaric acid occurred. The QTN varied from 0.7 to 2.0. Obviously these values are extremely low when compared to values obtained for bacteria. No transamination occurred between glutamic acid, glutamine, or asparagine and pyruvic acid. Variation of the reaction time from 30 minutes to 3 hours, of the cellular nitrogen from 0.4 to 2.5 mg per ml and of the pH from 7.8 to 8.4, the addition of varying levels of pyridoxal phosphate or the use of whole cells or cell-free extracts did not result in any increase in the rate of transamination. These studies with the Campo strain were terminated when this strain was found to be associated with diphtheroids in liquid culture.

Further attempts to demonstrate significant transamination were carried out with strains 07 and 39. Resting cells and cell-free extracts prepared from 26, 40 and 60-hour-old cultures failed to transaminate between glutamine or aspartic acid and α-ketoglutaric acid. No demonstrable activity resulted when pyridoxal phosphate was added or when the cellular nitrogen was varied from 1.5 to 2.3 mg per ml, the pH from 8.0 to 8.5 and the reaction time from 2 to 3 hours. In the reaction mixture containing glutamine and α-ketoglutarate, glutamic acid was formed but not in excess of the glutamine-cell control.

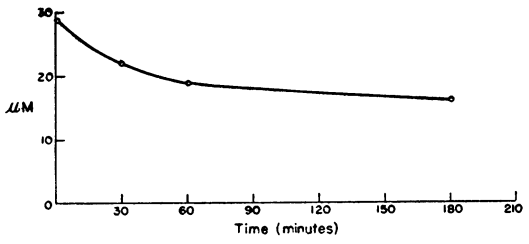

Figure 2. Rate of breakdown of glutamic acid by the human pleuropneumonialike organism strain 39.

DISCUSSION

The presence of a very active diphosphopyridine nucleotide linked alcohol dehydrogenase in all strains of pleuropneumonialike organisms tested to date poses the question of the origin of monohydric alcohols in their metabolism. Neither hexose sugars nor intermediates of the Embden-Meyerhof and Krebs cycles are metabolized to any appreciable extent. Ribose, reported to cause reduction of triphenyltetrazolium in the presence of resting cells (Lecce and Morton, 1954), is present in the synthetic medium in nucleic acids totaling only 10 mg per liter. This concentration seems much too small to be an adequate source of energy. On the other hand the plentiful supply of amino acids in the media made plausible the conjecture that these compounds may give rise to monohydric alcohols. Although studies have not progressed far enough to indicate such a metabolic route, it was found that deamidation was a significant function of these microorganisms. Parallel metabolic and nutritional studies are being continued in an effort to demonstrate key metabolic pathways.

Transamination studies have not resulted in significant findings, although this may have been due to our failure to use the correct α-keto acids. Certainly the limited number of amino acids required for the growth of these organisms suggests that they can synthesize the other amino acids that are found in virtually all living cells.

SUMMARY

Eighteen amino acids and glutamine were employed as substrates with resting cells of certain strains of pleuropneumonialike organisms. Slow anaerobic breakdown of tyrosine and tryptophan and aerobic breakdown of histidine, leucine and threonine occurred. Aspartic and glutamic acids were metabolized at a more rapid rate. Conversion of glutamine and arginine to glutamic acid and citrulline respectively was complete in 3 hours. Attempts to demonstrate transaminases were either unsuccessful or inconclusive.

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