Free sulphhydryl groups are known to be a limiting factor in the metabolism of pyruvate; evidence for this was provided by Peters & Wakelin (1946) using maleate, and Peters, Sinclair & Thompson (1946) using arsenical vesicants.

Aneurin, one of the main factors concerned with the catabolism of pyruvate, was shown by Zima & Williams (1940) and Zima, Ritsert & Moll (1941) to be capable of undergoing reversible oxidation and reduction, $2\text{RSH} \rightleftharpoons \text{RSSR}$, both the reduced and oxidized forms having equal biological activity. The question arises, therefore, whether the catalytic activity of aneurin is due to oxidation of an addition compound formed between pyruvate and the reduced form of the vitamin, in a manner similar to

the coupled oxidation of pyruvate with glutathione described above.

SUMMARY

1. When glutathione or cysteine is oxidized either with copper ions or with the cytochrome c-cytochrome oxidase system, a secondary oxidation occurs when pyruvate is added.

2. The ratio of the extra carbon dioxide output to the extra oxygen uptake due to the added pyruvate is approximately 2, suggesting an oxidative decarboxylation to acetic acid and carbon dioxide.

3. It is suggested that this coupled oxidation of pyruvate with glutathione may throw light on the biological oxidation of pyruvate.

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The Effect of Amino-acids on the Growth of *Lactobacillus casei* A.T.C. 7469 in Pteroyl Glutamic Acid-Free Media

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Most media suggested for the estimation of pteroyl glutamic acid (PGA), using *Lactobacillus casei* as a test organism, contain hydrolysed casein plus tryptophan and cystine as a nitrogen source. Snell (1944) found that the growth response of this organism to PGA was increased by supplementing such media with DL- α -alanine (hereafter referred to as alanine). Dolby & Waters (1944) reported that leucine, isoleucine and threonine behaved in a similar manner. Subsequently a number of workers have recommended the inclusion of alanine (1–2 mg./

10 ml.) in such media (Tepley & Elvehjem, 1945; Kreuger & Peterson, 1945, and Kodicek & Mistry, 1949).

During experiments on the formulation of a medium for the estimation of PGA with *Lb. casei*, it was found that addition of alanine to a medium containing a commercial brand of 'vitamin-free' casein hydrolysate, resulted in good growth in the absence of added PGA. When alanine was omitted no growth was obtained unless PGA was added. This unexpected behaviour on the part of the test

organism, was attributed to one or more of the following possible causes: (1) The ability of the organism to grow in a medium of a certain amino-acid content rich in alanine, in the absence of PGA. (2) The presence in the casein hydrolysate of a trace of PGA insufficient to produce growth without the addition of alanine, but sufficient to produce growth if alanine was present. (3) The presence of PGA in the inoculum, carried over from the inoculum medium. (4) The use of a culture of *Lb. casei* which had either developed atypical characteristics with regard to its PGA requirement, or which had become contaminated with another glucose fermenting organism with no PGA requirement.

Tests were made to investigate these possibilities and during the course of these L-glutamic acid was also found capable of stimulating the growth of *Lb. casei* in a PGA-free medium.

EXPERIMENTAL

The test organisms used were obtained from two cultures of *Lb. casei* A.T.C. 7469 maintained by different laboratories. One culture had been maintained in yeast-water-glucose-agar medium and the other in liver-tryptone-agar medium.

The inocula were prepared by transferring the organism from the stab culture to 10 ml. of PGA-free medium to which was added 2 μ mg. PGA (Stokstad & Hutchings, 1947). After incubating for 20–24 hr. at 37° the cells were centrifuged out and washed with 10 ml. of saline. This process was repeated until the cells had been washed (a) four times, (b) five times. The cells were finally suspended in saline and the suspension diluted to contain approximately 20 million organisms/ml. One drop of this suspension was added to each tube for the tests. The media used contained as a nitrogen source either (a) a commercial acid-hydrolysed casein, stated to be free from vitamins and peptides, which was used in a concentration of 50 mg./10 ml.

of medium together with 2 mg. DL-tryptophan and 0.8 mg. of L-cystine or (b) the following crystalline amino-acids: L-cystine, DL-tryptophan, DL-leucine, DL-isoleucine, DL-valine, DL-methionine, L-tyrosine, DL-phenylalanine, DL-threonine, L-lysine HCl, L-arginine, DL-serine, DL-norleucine and glycine, 2 mg. of each/10 ml. of medium plus 4 mg. of DL-aspartic acid and DL-histidine. The media differed from each other in some cases in their buffer and vitamin content as shown in Table 1. Growth was measured in terms of acid production after 96 hr. incubation at 37°. Four replicates were made of each test, the results shown in the graphs being the mean values. The linearity of graphical relationships was confirmed statistically.

The tubes of medium prior to inoculation were prepared aseptically and sterilized by heating in an autoclave at 5 lb. pressure for 5 min.

RESULTS

Preliminary tests showed that cells prepared by four or five washes from either culture, produced an identical amount of growth in media containing either of the two types of nitrogen source used without addition of PGA but with additional alanine. It was therefore concluded that the growth, obtained in the absence of added PGA, was due neither to the use of a culture which had acquired an atypical PGA requirement as a result of continued subculture in one particular type of medium, nor to the presence of PGA in the inoculum.

No organisms other than *Lb. casei* were found in those tubes which showed growth in the absence of added PGA. In the case of tests made in the amino-acid medium, the organisms growing in the tubes containing the lowest concentrations of alanine and L-glutamic acid used, were found, when stained, to differ in appearance from the normal organism. The cells had a clubbed appearance and showed no palisade arrangement. On subculture in glucose-

Table 1. *Composition of media used in tests*

(Nitrogen source as indicated in the text. pH 6.8.)

Component	Quantity in 10 ml. of medium				
	1	2	3	4	5
Sodium acetate (anhydrous) (mg.)	36	60	—	—	—
Potassium acetate (mg.)	—	—	36	300	300
Glucose monohydrate (mg.)	200	200	200	300	300
Uracil, guanine, adenine and xanthine—of each (mg.)	0.1	0.1	0.1	0.1	0.1
Aneurin, nicotinic acid, riboflavin and calcium D-pantothenate—of each (μ g.)	5	5	5	10	10
Pyridoxine hydrochloride (μ g.)	10	10	10	25	25
p-Aminobenzoic acid (μ g.)	1	1	1	2	2
Biotin (μ mg.)	4	4	4	4	4
Salt solution 'A' (ml.)*	0.05	0.05	0.05	0.05	0.05
Salt solution 'B' (ml.)*	0.05	0.05	0.05	0.05	0.05
Sodium chloride solution 0.2% (w/v) (ml.)	0.05	0.05	0.05	0.05	0.05
Water to	10 ml.	10 ml.	10 ml.	10 ml.	10 ml.

* Salt solution 'A' and 'B', Microbiological Panel of the Society of Public Analysts (1946).

yeast medium, however, they produced cells having the morphological and biochemical characteristics of *Lb. casei* A.T.C. 7469 (Kodicek & Pepper, 1948). Organisms in tubes containing a higher concentration of these amino-acids, showed the normal rod-shaped appearance but no palisade arrangement except when they were subcultured in glucose-yeast medium. Organisms in tubes containing the highest concentrations of these amino-acids used had the normal appearance of the organism and also showed palisade formation.

Fig. 1. The effect of PGA and alanine on the growth of *Lb. casei* in pteroyl glutamic acid (PGA)-free media containing a casein hydrolysate nitrogen source. Curve A, $\circ$, medium no. 1, responses to PGA additions; curve B, $\bullet$, medium no. 2, responses to PGA additions; curve C, $\triangle$, medium no. 3, responses to PGA additions; curve D, $\square$, medium no. 3, responses to alanine additions without PGA.

The liability of old cultures of *Lb. casei* to show pleomorphism and irregular beaded staining, as reported by Kodicek & Pepper (1948), is a characteristic of the *Lactobacillus* group in general.

Variations in morphology are also reported (Topley & Wilson, 1945) to be produced by growing the organisms in artificial media as distinct from media of natural origin. These observations suggest that the occurrence of changes of morphology in an organism when grown in a certain medium is an indication that that medium is deficient in substance(s) necessary for normal growth.

Tests made in media 1, 2 and 3 (Table 1) containing casein hydrolysate gave the responses, shown in Fig. 1 (curves A-C), to additions of PGA. These media gave low blank titres and good agree-

ment between replicates. The relation between log dose of PGA and response was found to be linear in the dose range 0.2–1.0 μ mg. PGA/10 ml. The addition of alanine to medium 3 in the absence of PGA resulted in growth, the amount of growth obtained by the addition of 1 mg. alanine/10 ml., exceeding that produced by 1 μ mg. of PGA/10 ml. (Fig. 1, curve D). A similar effect was obtained by adding alanine to medium 4 (Table 1), having the same amino-acid content as the three previous media, but differing in vitamin content (Fig. 2).

Fig. 2. The effect of alanine on the growth, response of *Lb. casei* to additions of pteroyl glutamic acid (PGA) in a PGA-free medium (no. 4) containing a casein hydrolysate nitrogen source. Curve A, $\circ$, 0.02 mg. alanine/10 ml. medium; curve B, $\bullet$, 0.04 mg. alanine/10 ml. medium; curve C, $\triangle$, 0.08 mg. alanine/10 ml. medium; curve D, $\blacktriangle$, 0.16 mg. alanine/10 ml. medium.

The relation between log concentration of alanine (log dose) and growth response was also found to be linear, over the range examined, namely 0.02–0.16 mg./10 ml. of medium. Addition of increasing amounts of PGA to medium 4 containing different amounts of alanine, produced equal increases in growth response irrespective of the amount of alanine present over the range 0.02–0.16 mg. alanine/10 ml., though the growth in the blank test (no PGA) increased with increasing alanine content, i.e. the curves relating PGA addition and growth, over the range 0.2–0.8 μ mg. PGA/10 ml., were parallel at all the concentrations of alanine examined (Fig. 2). This means that in the assay of PGA with these media the blank increases markedly with increasing alanine content of the medium, and that

the response produced by a mixture of PGA and alanine in this medium is the summation of the effects of each.

In the ranges investigated, the ratio of the doses of alanine and PGA producing equal growth is approximately 250,000:1. Samples containing alanine and PGA assayed with this medium for their PGA content would probably not contain enough alanine, relative to the amount of PGA present, to interfere seriously with the assay. It is shown later that tests made with the medium containing amino-acids as a source of nitrogen indicate that a change in the amino-acid content of the medium may specifically increase the response to alanine, so that the relative amounts of PGA and alanine producing the same response are not so widely separated. The apparent potencies of natural materials will, therefore, be liable to vary when assayed for their PGA content with *Lb. casei* using media of different amino-acid content as stated by Snell (1944).

Snell (1944) found that the addition of 2 mg. of alanine/10 ml. of medium increased the growth response to PGA by a maximum of 80%.

Kreuger & Peterson (1945) found that the addition of 1 mg. of alanine/10 ml. of medium increased the growth response to PGA by 10%.

The response obtained in the present experiments to the traces of PGA possibly present in the casein hydrolysate (represented by the blank titre in the tests) was 1.2 ml. 0.1N-sodium hydroxide; the response to this same amount of PGA obtained when 1 mg. of alanine was added to the medium was 14 ml. 0.1N-sodium hydroxide. This is approximately ten times greater than that obtained by Snell (1944), although the concentration of alanine was half that used in the tests he described.

The effect of addition of alanine in the present experiments is so much greater than the effect quoted by Snell that it is considered that the growth produced by alanine in these experiments cannot be due to traces of PGA in the casein hydrolysate. This conclusion was confirmed by the tests made with the amino-acid medium.

Further tests on the effect of alanine on the growth of *Lb. casei* in PGA-free media were made in medium 5 (Table 1) containing a mixture of amino-acids as the source of nitrogen. Tests were also made to determine the extent to which the growth-producing effect of alanine depended on the amount of L-glutamic acid present—this amino-acid being part of the PGA molecule, and therefore possibly connected with the role of PGA in the nutrition of *Lb. casei*.

The results showed that the amount of growth produced by a fixed amount of alanine depended on the amount of L-glutamic acid present in the medium up to a maximum L-glutamic acid content of

5 mg./10 ml. It was also found that in the presence of a fixed amount of L-glutamic acid growth was increased by increasing the quantity of alanine added. No growth was obtained in the absence of

Fig. 3. The effect of alanine and L-glutamic acid on the growth of *Lb. casei* in a pteroyl glutamic acid (PGA)-free medium (No. 5) containing a mixture of crystalline amino-acids as a nitrogen source.

Curve A, ○,	0 mg. L-glutamic acid/10 ml. medium	} Set 1
Curve B, ●,	1.0 " " " " "	
Curve C, ■,	2.5 " " " " "	
Curve D, +,	5.0 " " " " "	
Curve E, ×,	10.0 " " " " "	
Curve F, △,	1.5 " " " " "	} Set 2
Curve G, ▲,	2.0 " " " " "	
Curve H, □,	2.5 " " " " "	

Curves A-E and F-H were obtained in separate experiments at different times.

L-glutamic acid even when as much as 5 mg. of alanine was added to 10 ml. of medium, neither was any growth obtained when 5 mg. of PGA was added to 10 ml. of medium in the absence of L-glutamic acid and alanine. In the media containing 1.5, 2.5

and 5 mg./10 ml. of L-glutamic acid there was a statistically linear relationship between the responses and log. dose additions of alanine.

DISCUSSION

These results confirm the stimulatory effect of additions of alanine on the growth of *Lb. casei* in the presence of PGA in a medium containing hydrolysed casein, and show that in this medium growth occurs in the absence of PGA when alanine is added to the medium. In a medium consisting of a mixture of amino-acids the amount of growth produced by addition of alanine is influenced by the amount of L-glutamic acid in the medium.

It is possible that other amino-acids are involved either in stimulating growth in the presence of alanine, or in retarding growth in its absence. Hutchings & Peterson (1943) report that alanine is able to counteract the inhibitory effect on growth of histidine and isoleucine, producing a combination which has a marked stimulatory effect. Camien & Dunn (1950) have recently shown that glycine and serine also have an effect on the use of alanine by *Lactobacilli*. The effect of added alanine will, therefore, be also influenced by the quantity of these amino-acids in the medium.

From the tests made it appears that the amino-acid content of PGA-free media could be adjusted to give the optimum response to additions of alanine or L-glutamic acid and it might be possible to use *Lb. casei* in such media for the assay of these amino-acids.

Growth of *Lb. casei* in the absence of PGA has also been reported in the presence of other nitrogenous organic compounds. Stokstad (1941) observed that thymine would support half maximum growth of *Lb. casei* in PGA-free media containing adenine in concentrations of 3 µg./10 ml. medium. It was also found that guanine and other purines had the same effect. It is possible that in the cases where growth was restricted to half maximum, limitation of growth was caused by an insufficiency in the medium of the other components necessary for growth, possibly L-glutamic acid or alanine.

The reason for the failure of addition of alanine to stimulate the growth of *Lb. casei* in the absence of PGA in the media used by those workers recommending this addition, may have been that the casein hydrolysate used in their media had a different amino-acid content from the one used in the present tests. Alanine added to some PGA-free media may be used in neutralizing certain growth-restricting factors, for example histidine and isoleucine.

It follows, therefore, that it is not sufficient to add fixed quantities of amino-acids to media containing

protein hydrolysates; each medium has to be considered separately and the optimum amounts of supplementary amino-acids determined.

The amino-acid composition of a casein hydrolysate depends on the conditions of hydrolysis and the method used for removing the vitamins. Thus, Lugg (1938) and Boyd & Logan (1942) have reported that when casein is hydrolysed by acid the threonine, tyrosine, cystine and serine are destroyed to varying extents depending on conditions of hydrolysis. Bailey, Chibnall, Rees & Williams (1943) have also reported that the conditions of hydrolysis will also determine the degree to which the amino-acids have been racemized. This racemization will also affect the growth response since with some amino-acids only the L-forms are active in promoting growth (Kuiken, Norman, Lyman, Hale & Blotter, 1943). Treatment of the hydrolysate with adsorbing agents such as activated charcoal and fuller's earth to remove the vitamins will also cause a certain amount of selective adsorption of the amino-acids, with consequent alteration in their final proportions. Even with media prepared from the crystalline amino-acids slight variations in the final amino-acid content may result from differences in the method of autoclaving the final media and from differences in the purity of amino-acids obtained from different commercial sources.

SUMMARY

1. The titres of the blanks in media used for the microbiological estimation of pteroyl glutamic acid (PGA) with *Lactobacillus casei* depend on the amino-acid content of the media and in particular on the amount of L-glutamic acid and DL- α -alanine present.
2. Unrestricted growth of *Lb. casei* was obtained in PGA-free media containing certain combinations of L-glutamic acid and DL- α -alanine indicating that PGA could not be considered an essential growth factor for *Lb. casei* under those conditions.
3. The amount of DL- α -alanine needed in media used for the estimation of PGA must be determined by experiment for each medium with a different amino-acid content, such as may arise, for example, from the use of casein hydrolysates of different origins.
4. The possibility of using PGA-free media of a defined amino-acid content for the assay of DL- α -alanine and L-glutamic acid is suggested.

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The Action of Some Water-soluble Poly- α -amino-acids on Blood Clotting

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The action of naturally occurring acidic and basic substances of high molecular weight, such as heparin and protamine, on blood clotting is well known (for review see Chargaff, 1945) and the study of the anticoagulant effect of these substances has added much to the understanding of the blood-clotting mechanism. The recent synthesis of water-soluble poly- α -amino-acids (for review see Katchalski, 1950) makes possible the study of the action of polymers of well defined structure on blood coagulation. As the poly- α -amino-acids represent materials of relatively high molecular weight, composed of amino-acid residues and linked by peptide bonds, the study of their action on blood coagulation may serve to elucidate some aspects of the mechanism of action of simple proteins, such as protamine, on blood coagulation.

The water-soluble poly- α -amino-acids may be classified according to their electrochemical properties into neutral, basic and acidic poly-amides. In the present study poly-DL-alanine (Astbury, Dalglish, Darmon & Sutherland, 1948), the only known neutral water-soluble poly- α -amino-acid, was chosen as representative of the neutral type; the basic poly- α -amino-acids are represented by

synthetic poly-lysine (Katchalski, Grossfeld & Frankel, 1948) and poly-ornithine (Katchalski & Spitnik, 1949) and the acidic poly- α -amino-acids by the naturally occurring poly-D-glutamic acid (Hanby & Rydon, 1946) and the synthetic poly-aspartic acid (Frankel & Berger, 1949).

It has been found that neutral and acidic poly-amino-acids do not retard clotting of human blood at a concentration of about 100 $\mu\text{g./ml.}$ blood. Basic poly-amino-acids, on the other hand, cause a marked prolongation of clotting time at the same concentration. The clot-decelerating effect of the basic poly-amino-acids, poly-lysine and poly-ornithine, can be neutralized by the acidic poly-amino-acids as well as by heparin. The basic poly-amino acids act as antiheparins *in vitro*, similarly to protamine. Experiments with poly-L-lysine and poly-DL-lysine showed that variation of the steric configuration does not change significantly their effect on blood clotting. Lysine, ornithine and aspartic acid monomers were found to have no influence on blood clotting. The mode of action of the basic poly-amino-acids on blood clotting was investigated. The similarity of their action to that of protamine will be pointed out.