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Biochemical Characterization of the Actions of Chemotherapeutic Agents

1. MEASUREMENT OF GROWTH OF STREPTOCOCCAL CULTURES THROUGH THEIR GASEOUS METABOLISM, AND THE EFFECTS OF PANTOTHENATE AND PANTOYLTAURINE UPON THE METABOLISM AND GROWTH

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During chemotherapeutical experiments with pantoyltaurine, large doses of haemolytic streptococci disappeared from rats, while in control experiments the animals succumbed to the infection (McIlwain & Hawking, 1943). *In vitro* pantoyltaurine did not kill the bacteria but delayed their growth. It has been argued elsewhere (McIlwain, 1943) that such bacteriostasis may afford an adequate basis for many chemotherapeutic actions without the assumption of additional effects of drugs upon the parasites *in vivo*, or of drugs upon defence mechanisms of the host. The action of pantoyltaurine thus represents a general problem in chemotherapy, namely, the characterization of the *in vitro* bacteriostasis associated with *in vivo* disinfection, and the assessing of its contribution to that process. Pantoyltaurine was prepared with the intention of interfering with the parasite's use of pantothenate. The latter, is present in the host, is essential to growth of the bacterium, and is capable, in sufficient quantity, of preventing the action of pantoyltaurine both *in vivo* and *in vitro*. The effects of both pantothenate and pantoyltaurine upon streptococcal growth *in vitro*, in relation to the general problem, have now been investigated.

A manometric method of measuring streptococcal growth was preferred to direct counting, or to opacity measurement, as the organisms are individually small but grow in tangled chains which precipitate quickly. The method permitted continuous readings in standard apparatus, without periodic sampling. Very similar conditions could be used for examining the effects of the reagents upon streptococcal metabolism; such effects are described here only in so far as they bear upon the use of the method for measuring growth. Because difficulties were encountered in the use of streptococcal respiration for growth measurement in pantothenate-limited solutions, and because the anaerobic glycolysis of the organisms involves a gaseous exchange many times that of their oxygen uptake, anaerobic conditions were adopted for growth. These may be more removed from circumstances *in vivo* than are aerobic conditions, but the difference is unusually small in the case of haemolytic streptococci, especially with respect to their growth in media containing glucose (cf. Hewitt, 1932). No difference in the concentrations of pantothenate and pantoyltaurine necessary for growth and inhibition under the two conditions has been found. The manometric method necessarily measures increase of metabolizing units and not of cell numbers. The two factors are known to increase at rates which are not entirely parallel (Huntingdon & Winslow, 1937); the same is true of cell mass and cell numbers. Increase in cell numbers approximately equal to increase in glycolysis was found during normal growth of the present organisms, when increases of 50- to 100-fold, progressing through the usual phases of growth, were observed. Further correlation of the manometric measure of growth with the usual bacteriological measure by increase in cell numbers has not been attempted as there is no certainty, except when a few cells only are concerned, that the pathogenicity of a normal culture is related to cell numbers rather than to cell mass

or metabolic activities. The finding of logarithmic phases in the present experiments makes it probable that, at least during those phases, growth in the usual sense was being measured.

The present experiments are confined to the effects of adding streptococci to initially known quantities of pantothenate and pantoyltaurine, whose concentrations were chosen to cover the range of values found in animals during treatment.

EXPERIMENTAL

(+) Calcium pantothenate was supplied by Merck and Co., Inc., and *dl*-sodium pantoyltaurine (which was assayed by the method of Snell, 1941*a*) by Glaxo Laboratories, Ltd. The majority of the present experiments were carried out with a group G strain of *Streptococcus haemolyticus* which showed typical β-haemolysis and of which relatively small intraperitoneal inocula were fatal to mice. I am indebted to Mrs Wilson Smith for its passage during the present experiments. It was maintained in blood broth, from which sub-cultures were made on serum agar for inoculation of the experimental media. In certain experiments its behaviour was compared with the Richards strain of streptococcus and another group A strain, but no difference in their reactions to pantothenate and pantoyltaurine were found.

Pantothenate-deficient media

The experimental media were based on a casein-hydrolysate medium by use of which pantothenate was identified as a growth factor for the present organisms (McIlwain, 1939), and differ from it mainly in employing bicarbonate in place of phosphate as buffering agent, and in the further addition of a pantothenate-free yeast preparation, which improved growth under manometric conditions.

Acid-hydrolyzed casein (20%). The previous (McIlwain, 1939) solution was used without treatment with oxalate, and kept with chloroform.

Yeast preparation. Autolyzed yeast extract (2 g., Marmite Food Extract Co. Ltd.) was dissolved in water (10 ml.); 5 ml. of this solution were autoclaved in a plugged tube at 120° for 20 min. with 2N-HCl (5 ml.) and 5 ml. with 2N-NaOH (5 ml.). The solutions were mixed, neutralized, decanted from a precipitate and kept aseptically.

Basal medium. Acid-hydrolyzed casein (50 ml.) with the yeast preparation (6·6 ml.) and water (to 200 ml.) was autoclaved.

Group A addenda. Cystine (0·24 g.); uracil, cytosine, thymine, and guanine (0·01 g. each) in 10N-HCl (2 ml.) were diluted somewhat and added to aneurin (5 mg.), nicotinamide (5 mg.), β-alanine (5 mg.), pimelic acid (10 mg.), 10^{-3}M-riboflavin (1 ml.), methionine (50 mg.), tryptophan (50 mg.), $(NH_4)_2SO_4$ (1 g.), NH_4Cl (1 g.), anhyd. $MgSO_4$ (0·2 g.), $Fe(NH_4)_2(SO_4)_2.6H_2O$ (0·5 g.), and glucose (5 g.). The solution was adjusted to 100 ml. with water and to an acid content of 0·2N. and filtered to sterilize.

Growth medium A. The following solutions were mixed aseptically to give a batch of a final volume of 30 ml.: 0·02M-arginine and 0·02M-glutamine, 0·6 ml.; basal medium, 7·5 ml.; group A addenda, 1·5 ml.; 0·5M-$NaHCO_3$, 2·8 ml.; water, glucose (usually 1·5 ml., M.), pantothenate, and inoculum as required. Incubation was in air or N_2 containing 5% of CO_2; the bicarbonate added was such as to form with the gas phase a buffer of pH 7·6. Phosphate was not added as such, in order to reduce retention of CO_2 in glycolysis; that contributed by the basal medium was found to be in excess of the organism's requirements.

Glycolysis medium B. Basal medium, 7·5 ml.; 0·2M-HCl, 1·5 ml.; 0·5M-$NaHCO_3$, 2·8 ml.; M-glucose, 1·5 ml.; pantothenate, pantoyltaurine, and organisms as required; water to 20 ml. The medium was distributed in 2 ml. lots, which after other additions were made to 3 ml. with water.

Metabolic experiments

Organisms were grown aerobically for 17–18 hr. in medium A, from about 10^8 cells/30 ml. and with 5×10^{-8}M-pantothenate, collected by centrifuging, and suspended in their own volume of saline. Portions (usually 0·5 ml.) were pipetted to Warburg vessels (conical, of about 20 ml., and with centre pots) already containing medium B and the required pantothenate and pantoyltaurine; the thermobarometer contained only a measured sample of the medium. The vessels, with yellow phosphorus in their centre pots, were equilibrated with 5% CO_2-N_2 at 37°. The evolution of gas under these conditions is referred to as a measure of glycolysis, and the symbol $Q_{CO_2}^{N_2}$ used to express the rate of evolution, in μl. of gas/mg. dry weight of cells/hr., without correction for resting metabolism.

For respiratory studies the organisms were similarly grown and collected but were suspended in one-third or one-sixth of their original volume of 0·2M-phosphate buffer, pH 7·6. Vessels contained this suspension, 1 ml.; basal medium, 1 ml.; pantothenate, pantoyltaurine, substrate, and water to 3 ml.; NaOH and paper in their centre pots; and were equilibrated with air at 37°.

Growth experiments

The method first attempted was a direct application to the present problems of the measurement of growth of bacteria through their respiration, previously employed by Greig & Hoogerheide (1941), Hirsch (1942) and Sevag & Shelburne (1942). Though the streptococci at present studied grew well in glucose broth under the conditions of manometric measurement of their respiration, little or no growth was obtained in several media containing defined quantities of pantothenate. Such media were necessary to the present investigation and contained complex constituents (casein, marmite, broth, or mixtures of these) which had been treated with acid or alkali under conditions which destroyed pantothenate. Many such media supported growth under ordinary bacteriological conditions but were inadequate for manometry. This was found to be due to the removal of CO_2 in respiratory measurements. Pappenheimer & Hottle (1940) found CO_2 necessary for streptococcal growth, but replaceable by adenylic acid and related compounds. Addition of such substances did not permit growth under the desired conditions with the present organisms, but labile material is evidently present in broth and yeast, which can do so. Oxaloacetate, capable of

gradual decomposition under the present conditions to yield CO_2, did not permit growth.

In search for alternative conditions, measurement of gas production from media containing glucose and bicarbonate buffer was chosen. Good growth was obtained and with the present organisms the method gave greater accuracy than could be obtained in standard apparatus by respiratory measurements, on account of the large gas production by the streptococci, which could be 15 times the oxygen uptake of the same suspension. Organisms grown in Medium A with 5×10^{-8} M-pantothenate were used as the experimental inoculum at 17–18 hr. The pantothenate content of the medium was then $< 5 \times 10^{-9}$ M and the organisms were used without separation from their culture medium, of which 1–2 % was transferred with the inoculum.

The procedure was to add the reagents to Warburg vessels, prepare a batch of medium A (sufficient for 10 portions of final volume 3 ml.) in 18 ml., take from it a sample for the thermobarometer, and inoculate and distribute the remainder. Phosphorus was added and the vessels equilibrated, zero time being taken as that at which they were placed in the thermostat (37°). Manometers were read to the nearest 0·5 mm. (*c.* 1 μl.) at 15 min. intervals; evolution of 5 μl./30 min. could be measured approximately and was found dependable; as the final readings were of 100–150 μl./15 min., growth was measured over a range of 40-fold increase (the total increase during an experiment being 50–100-fold) but with relatively low initial accuracy. The inoculum was chosen of such a size that, with 3 ml. of medium, the logarithmic phase covered the range of most accurate measurement with the present experimental arrangement. The original rate of gas production by the inoculum was then too small to measure and was computed from the known size of the inoculum, and its rate of gas production.

The Warburg vessels were drained and dried, inverted, at 100°, and left so until use. The reagents were initially sterile and were transferred to the vessels aseptically, but no attempt was made to sterilize the remainder of the apparatus. Nevertheless, no instance of contamination during the experiments, lasting from 2 to 8 hr., was encountered and in about one-half of the instances tested the solution of the thermobarometer, transferred immediately after the experiment to a sterile tube and incubated, remained without visible growth for 24 hr.

Viable counts

The roll-tube count technique was employed. Broth agar was found to be a suitable medium for enumeration of the group G organism; with the same suspension no more colonies formed in media containing serum and blood. In determining the effect of the reagents upon viability, organisms as used for inoculation of manometric experiments were diluted in medium B, so that the suspension could yield about 10^7 colonies/ml. A portion of this was incubated together with others containing pantothenate and pantoyltaurine. After given periods, dilutions were prepared by calibrated dropping pipettes and added to broth agar. This contained sufficient pantothenate to prevent the continued action of pantoyltaurine, and the colonies resulting after incubation were counted.

RESULTS

Metabolic processes, and their suitability for measuring growth

Relation to quantity of organisms. The rate of gas evolution from medium B by streptococci was constant during the first 75 or 120 min., after which growth sometimes occurred (Fig. 1). The rate corresponded to a $Q^{N_2}_{CO_2}$ of about 500 (Table 1). A suspension of organisms was chosen which evolved in 1 hr. about 350 μl., which is the total volume of gas evolved during the logarithmic phase of a typical growth experiment. The volume of gas evolved/hour by this suspension and by accurately prepared dilutions of it were: suspension A, 331; A/2, 170; A/4, 86; A/8, 44 μl. The decrease in $Q^{N_2}_{CO_2}$ with increasing rate of evolution is significant, but small, and was ignored in interpreting the growth experiments. Retention of CO_2 was found to occur in the present medium; it necessarily contains phosphates and amino-acids. The evolution of 300 μl. of CO_2 corresponds to about 10 % of that present as HCO_3^- and 5 % of that which could be expected (cf. Hewitt, 1932) to be produced from the glucose added.

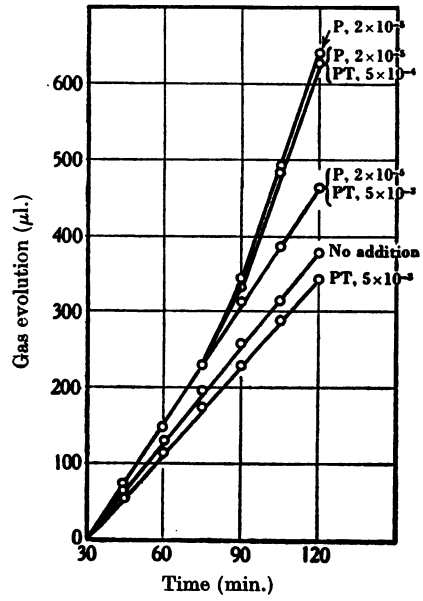

Fig. 1. Glycolysis and growth in the presence of pantothenate, pantoyltaurine, and mixtures of the reagents. Here and elsewhere: *P* = pantothenate; *PT* = pantoyltaurine; concentrations in molarities.

Relation to pantothenate and pantoyltaurine. Glycolysis was relatively unaffected by these compounds (Table 1). Any action of pantothenate was given by low concentrations, and was to accelerate the process, the maximum increase

Table 1. *Effects of pantothenate and pantoyltaurine upon anaerobic gas production by streptococci*

Exp.	Substances added: Pantothenate M × 10^7	Substances added: Pantoyltaurine M × 10^{-5}	$Q^{N_2}_{CO_2}$: Found	As percentage of effect with pantothenate: Without pantothenate	With mixtures	With pantoyltaurine
11	0	0	670	100	—	—
	3·3	0	673	—	—	—
	0	33	609	—	—	90
	3·3	33	677	—	100	—
	3·3	330	687	—	102	—
12	0	0	434	100	—	—
	330	0	432	—	—	—
	0	330	378	—	—	87
15	0	0	590	89	—	—
	33	0	662	—	—	—
	0	330	599	—	—	91
	0	3·3	602	—	—	91
	33	330	661	—	100	—
	33	3·3	670	—	101	—
19	0	0	590	84	—	—
	330	0	705	—	—	—
	3·3	0	701	—	—	—
	0	330	520	—	—	74
	3·3	75	682	—	97	—
	3·3	330	685	—	97·5	—
				Mean	99·5	86·5

Organisms were the group G strain, 17–19 hr. old and grown in medium A with pantothenate, 5×10^{-6}M; about 0·3 mg. (dry weight) was used per vessel, with medium B, 3 ml., containing glucose, 0·05M.

observed being one of 20%. Pantoyltaurine alone always caused a depression of 10–25% which was removed, completely or almost so, by low concentrations of pantothenate. In the presence of $3·3 \times 10^{-7}$M-pantothenate, no effects of pantoyltaurine in concentrations up to $3·3 \times 10^{-3}$M were found. As the major effect of pantoyltaurine upon growth is given by concentrations 200 times those of the coincident pantothenate, the effect upon glycolysis is readily dissociated from that upon growth. This permitted the present use of glycolysis for measuring growth. In the experiment of Fig. 1, growth occurred after 75 min. if pantothenate was present, but the concentration of pantoyltaurine which prevented the growth (PT, 5×10^{-3}M with P, 2×10^{-5}M) had no effect upon glycolysis. Comparably small effects of pantoyltaurine were found upon other processes: respiration with glucose (0·05M) as substrate was reduced by 20% by pantoyltaurine (0·02M); anaerobic gas production in the presence of pyruvate (0·02M) was inhibited by 18% by pantoyltaurine (0·02M) and restored by pantothenate (5×10^{-6}M).

Growth

The rate of gas production in growing cultures increased with time. Logarithms of the μl. of gas produced in successive periods of 15 min. (directly determined, or computed from longer periods while the evolution was small) were plotted against time. Experimental curves are shown in Figs. 2 and 4; they were characterized by the following values: (*a*) *Length of lag period.* This was formally considered to extend to times L at which extrapolations of the curves in their logarithmic phases reach values corresponding to that of the inoculum (cf. Lodge & Hinshelwood, 1943). The lag period was found to be small in normal growth, in spite of the aerobic growth of the inoculum; it was not reduced by its anaerobic growth. (*b*) *Acceleration of gas production* during the logarithmic phases, expressed as the time necessary for the culture to double its rate of gas production. The finding of a logarithmic phase enables this to be regarded as a reproduction time, which is necessarily a mean value for the culture and will express a balance between growth and any contemporaneous death; in a consideration of growth under abnormal conditions it must be remembered that the reproduction time primarily represents rate of increase of glycolyzing units, and its interpretation in terms of organisms and cell division may not be straightforward. In different experiments, the reproduction time of the normal organisms was between 26·5 and 28 min.; with growth in rich media, values of 21 min. were obtained. This difference is undesirable, but is much smaller (i.e. the conditions of experimental growth are more satisfactory) than in many comparable studies. (*c*) *Rate of gas evolution when a steady rate of evolution has been reached.* This stationary period

was followed for a short time only and so is not comparable with the stationary phase observed by ordinary bacteriological methods after many hours.

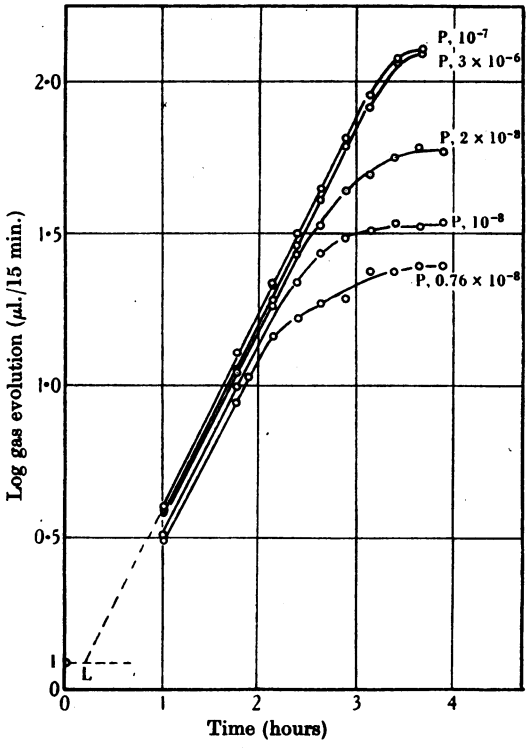

Fig. 2. The course of normal and pantothenate-limited growth. The extrapolation of the curve, *P*, 10^{-7} to *L* indicates the method of computing lag; *I* is the rate of gas evolution calculated for the organisms used as inoculum.

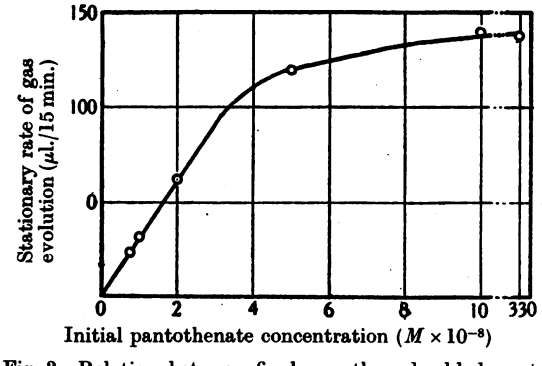

Fig. 3. Relation between final growth and added pantothenate, in absence of pantoyltaurine.

Effect of pantothenate. Growth did not occur in the absence of pantothenate; Fig. 2 shows the effects of suboptimal amounts upon growth. The lag and reproduction times are practically independent of pantothenate concentration though they tend to increase with decreasing pantothenate. The major effect is upon the value of the stationary population. This has reached its maximum value with 10^{-7}M-pantothenate; the relation of the value of the final rate of gas evolution to concentration of pantothenate is given in Fig. 3, which shows the two to be directly proportional for small values, and with higher pantothenate to reach a maximum at or below 10^{-7}M.

Effect of pantoyltaurine in the presence of pantothenate. Pantoyltaurine concentrations 100 times those of the coincident pantothenate prevented growth, in amounts measured in the present experiments, for 8 hr. or more. Growth could be started

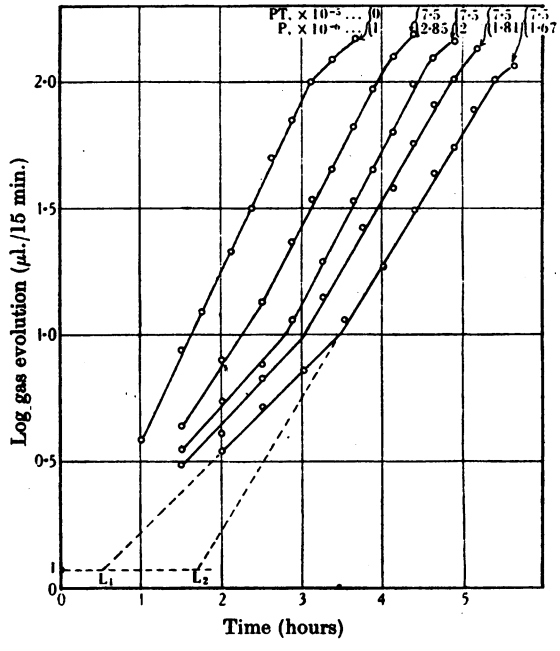

Fig. 4. The course of normal and pantoyltaurine-limited growth. Extrapolations (see Fig. 2): IL_1, lag of the initial, slow, phase and IL_2, of the second, fast, phase of growth in the presence of *P*, $1{\cdot}67 \times 10^{-6}$M and *PT*, $7{\cdot}5 \times 10^{-5}$M.

in an inhibited culture by adding further pantothenate. The specificity of the antagonism has been studied in detail in test-tube experiments, and the effects of only the following closely related compounds were examined here. Hydrolyzed pantoyltaurine (3×10^{-3}M; by 0·5N-HCl, 20 min. at 120°), taurine (3×10^{-3}M) and β-alanine (2×10^{-3}M or 2×10^{-4}M in addition to that present in the medium) were without effect upon growth in the presence of pantothenate (10^{-6}M). β-Alanine (2×10^{-3}M or 2×10^{-4}M, in addition to that already present) did not affect the inhibition caused by pantoyltaurine (10^{-4}M) in the presence of pantothenate (2×10^{-6}M).

The most interesting results were obtained with mixtures of pantoyltaurine and pantothenate, which only partly depressed growth. Fig. 4 illustrates this and shows that the presence of pantoyltaurine limits growth in a manner distinct from that associated with suboptimal amounts of pantothenate

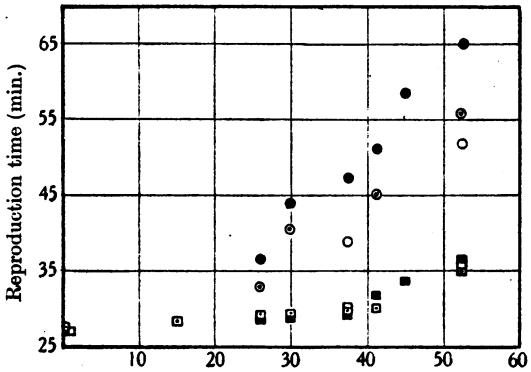

Fig. 5. Relation between reproduction time and quantities of pantothenate and pantoyltaurine. Circles: the initial, slow, process; rectangles: the second, fast, process. Black: *PT*, $7{\cdot}5 \times 10^{-5}$; spotted: *PT*, 3×10^{-4}; white: *PT*, $1{\cdot}2 \times 10^{-3}$ M.

alone. Growth may be delayed; its initial rate is lowered, though a more normal rate supervenes later, and the stationary rate of gas evolution reaches its normal value. The same general picture was given by other concentrations of pantothenate and pantoyltaurine; Fig. 5 summarizes their effect upon the reproduction time of the two phases and Fig. 6 their effect upon the lag of the second, fast, process.

Influence of pantothenate and pantoyltaurine on viability

The effects of pantothenate and pantoyltaurine upon viability were examined to assist in the interpretation of the slow phase of growth. The results

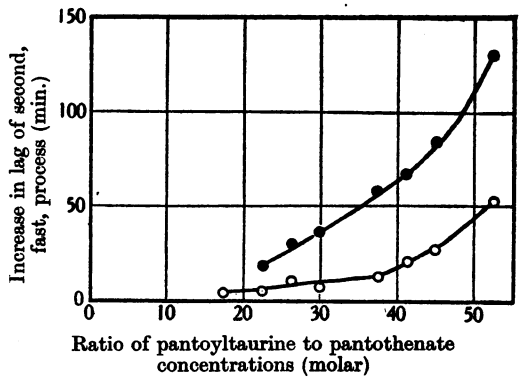

Fig. 6. Increase in lag of second, fast, process of pantoyltaurine-inhibited growth, with preponderance of pantoyltaurine. Black circles: *PT*, $7{\cdot}5 \times 10^{-5}$; white: *PT*, $1{\cdot}2 \times 10^{-3}$.

(Table 2) show that during periods comparable with those occupied by the present experiments, the conditions examined did not affect growth by killing organisms, which retained their usual ability to grow to visible colonies in the usual time. Growth has occurred in the uninhibited tubes of Exp. 2, Table 2.

DISCUSSION

Metabolic effects. An approach to understanding the actions of pantothenate and pantoyltaurine upon growth of streptococci may be made by attempting to find component systems of the or-

Table 2. *Viability of streptococci after incubation in the presence and absence of pantothenate and pantoyltaurine*

Exp.	Substances added	Conc. (M)	No. of colonies, at 48 hr., from 0·086 ml. of a 1/46,300 dilution of the suspension exposed; and from multiples (given in the denominator) of this volume: Initially	After 4 hr.
1	None	—	20, 53/2, 118/5, 249/10	24, 48/2, 119/5, 236/10
	Pantothenate	10^{-6}	—	21, 39/2, 85/5, 185/10
	Pantoyltaurine	10^{-3}	—	11, 48/2, 98/5, 215/10
			Initially	After 6 hr.
2	None	—	145/5, 158/5	131/5, 148/5
	Pantothenate	5×10^{-8}	—	162/5, 188/5
	Pantoyltaurine	10^{-5}	—	136/5, 124/5
	,,	10^{-6}	—	159/5, 168/5

Organisms were grown as described in Table 1 and exposed at 37° in medium B. No differences were noticed in the rates of growth of the colonies from previously exposed organisms, and the colonies did not increase in numbers after 48 hr.

ganism, which have equal or greater sensitivity to the reagents than has the overall process of growth. Metabolic reactions of the requisite sensitivity to pantothenate and pantoyltaurine have not been satisfactorily characterized in the present experiments, but evidence suggesting their existence has been obtained.

Such metabolic deficiency as was observed in the absence of pantothenate was made good by quantities of pantothenate small compared with those affecting growth. The deficiencies were not large; greater effects of pantothenate have been observed with *Proteus morganii* (Dorfman, Berkman & Koser, 1942; Hills, 1943). The inhibitory effect of pantoyltaurine upon the processes studied was also not large, but again was annulled by small quantities of pantothenate. This is the first finding of inhibition and antagonism by these compounds in a metabolic process, but the process concerned has not the characters of the hypothetical reactions through which pantoyltaurine may limit growth: it is a relatively minor effect and its inhibition by pantoyltaurine is antagonized by a much smaller proportion of pantothenate than will antagonize the effect of pantoyltaurine upon growth. The effects of pantoyltaurine and pantothenate upon metabolic processes in yeast, comparable with those studied above, were investigated by Teague & Williams (1942). The reactions in yeast were accelerated by pantothenate but no effect of pantoyltaurine was found. This difference from the present findings is understandable, as the growth of yeast, though it may be improved by pantothenate, does not in general require the compound to the same extent as do β-haemolytic streptococci, and the ratio of pantoyltaurine to pantothenate concentration needed for inhibition of the yeast studied by Snell (1941*b*) was much greater than that necessary for the present streptococci.

Growth experiments. The effects reported raise the following problems. Pantoyltaurine appeared to act by limiting the organism's use of pantothenate; this was the intention in preparing a metabolite analogue, and was confirmed by the inhibition of pantothenate-requiring organisms only, and the antagonism of such inhibition by pantothenate. (It was previously reported (McIlwain, 1942*b*) that *Proteus morganii* (requiring pantothenate) was not inhibited by ratios of pantoyltaurine to pantothenate of 10^5. Visible growth of two strains has now been found to be delayed by ratios of 10^5, and prevented by ratios of 2×10^5, under the conditions there described.) The two compounds have not been found to react with each other during growth, but their structural similarity makes competitive interaction possible. What, then, is the basis for the differing effects following decrease in pantothenate concentration and increase in pantoyltaurine? What, also is the reason for the more rapid phase of growth succeeding the initial, pantoyltaurine-inhibited one?

Directly comparable studies have not been encountered; those of Wyss (1941), Strauss, Lowell & Finland (1941) and Davies & Hinshelwood (1943) on the interaction of *p*-aminobenzoic acid and sulphanilamide afford only partial examples, as the organisms there examined did not require preformed *p*-aminobenzoate for growth. Suggestive evidence is, however, available from studies of the actions of unrelated substances which are required for, or which inhibit, growth. Of the growth-promoting substances whose effects upon *Bacterium lactis aerogenes* were investigated by Lodge & Hinshelwood (1939, 1943), some (like pantothenate in the present experiments) had their main effect upon the size of a stationary population; but magnesium salts and amino-acids mainly affected lag. Thus, although the characters of inhibition by pantoyltaurine are not such as to suggest that its effect upon growth is immediately through limitation of the organism's supply of pantothenate, they are characters which can be associated with reduced supply of other growth essentials. The pantothenate antagonism of pantoyltaurine inhibition shows such essential substances to be connected with pantothenate; a simple hypothesis suggested by the finding that pantothenate and pantoyltaurine affect metabolic processes is that the growth-limiting substances are ones for whose formation pantothenate is necessary, and whose formation pantoyltaurine inhibits.

The following points are related to this hypothesis:

(1) The concentrations of reagents which have been investigated are necessarily those which only partly inhibit growth. If this involves a partial inhibition of the hypothetical reactions the concentrations of whose products condition growth, these concentrations can still reach the value necessary for rapid growth after an interval, which increases with preponderance of pantoyltaurine. The duration of lag of the fast process in the presence of pantoyltaurine is so conditioned (Fig. 6). The possible contribution of bactericidal effects by the reagents during this phase has been discounted.

(2) If growth rates in the presence of inhibitory concentrations of pantoyltaurine are conditioned by the rate of formation of some intermediate, the equations of Wilson (1939), representing competitive enzyme inhibition, and of Gaddum (1937, 1943), representing competitive occupation of receptors, may be applicable to the present results. It is found, in agreement with those expressions, that the inhibitory effect of a given ratio of pantoyltaurine and pantothenate is not constant, but decreases with increasing absolute concentrations of

the reagents. The effect is shown both upon reproduction time of the initial, slow process (but not upon that of the fast process: Fig. 5) and upon the lag of the subsequent fast process (Fig. 6). It was not detected in previous qualitative experiments (McIlwain, 1942*a*).

(3) A further deduction from enzyme kinetics is that low pantothenate concentrations could themselves lower the rate of formation of the hypothetical intermediate, and hence the rate of growth. This is not definitely shown in the logarithmic phases of Fig. 2 (and clear logarithmic phases were not found with lower pantothenate concentrations), but that it occurs is made probable by the gradual approach to the stationary population in the lower three curves. In these cases pantothenate is limiting the stationary population and under such conditions it has been found to disappear from the culture medium. The rate of growth is lowered, finally to zero, during the disappearance; the two processes are connected, although the present experiments do not measure the connexion, since the pantothenate concentration is changing during growth and the lower concentrations necessarily correspond to very little growth.

(4) The expressions for the quantitative effects of competition, if applicable, would further suggest that the values of Fig. 5 connecting reproduction time with the reciprocal of pantothenate concentration in the presence of fixed amounts of pantoyltaurine would lie on straight lines of constant intercept on the ordinate. The values are not inconsistent with such a relation, but are derived from the least accurate portion of the growth curves. Wyss (1941) found conditions under which growth of *Esch. coli*, limited by sulphanilamide and *p*-aminobenzoate, consistently followed such expressions. The rate of uninhibited growth in his experiments was unusually slow and the entire course measured would seem to correspond to the initial phases of the present experiments. Davies & Hinshelwood (1943) found a later, more rapid phase in sulphanilamide-inhibited growth of *Bact. lactis aerogenes* and obtained growth curves similar to those of Fig. 3, though employing a technique entirely different from that of the present experiments. It is of additional interest that such effects are found with the present pathogenic organism, growing under almost optimal conditions, and that the quantitative effects of chemotherapeutic agents may be interpreted in ways similar both to those of general pharmacology and of biochemistry.

SUMMARY

1. The anaerobic evolution of gas by haemolytic streptococci from media containing glucose and bicarbonate was sufficiently regular and proportional to the quantity of organisms to be used as a measure of their growth. In some cases addition of pantothenate accelerated the evolution of gas and other metabolic processes; but adequate pantothenate was usually present during the growth experiments, which simulated *in vivo* conditions in their pantothenate and pantoyltaurine concentrations. Pantoyltaurine inhibited, by 10–15%, gas production from resting streptococci, and this inhibition was annulled by 1/10,000 of its concentration of pantothenate. This inhibitory action of pantoyltaurine did not interfere with the growth experiments, which concerned mixtures of one part of pantoyltaurine with 1/20–1/60 part of pantothenate.

2. Conditions were chosen for manometric measurement of streptococcal growth in media whose pantothenate content could be controlled by direct addition of the compound. Uninhibited growth then occurred with short lag, a well-marked logarithmic phase of almost optimal speed of growth, and reached a stationary value in 3–4 hr.

3. Reduction in the pantothenate content to levels suboptimal for growth had little effect on the duration of the lag period or on rate of growth, but reduced the stationary population; growth at concentrations less than 4×10^{-8} M was roughly proportional to the pantothenate added.

4. Pantoyltaurine depressed growth in the presence of excess pantothenate, not by an effect upon the stationary population (when growth occurred at all) but by slowing growth and increasing the lag period. The logarithmic period involved at least two phases: an initial one during which the rate of growth was much decreased, and a later, more rapid, one whose speed was less affected by the inhibitor.

5. It is concluded that pantothenate is of importance to the organism because of the products to which it normally leads, whose presence in adequate concentrations is necessary for normal growth. When pantoyltaurine affects growth, it is believed to lower the rate of formation of these products to such an extent that they limit the rate of growth of the organism.

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Synthesis of Vitamin C in Stored Apples

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Previously (Zilva, Kidd & West, 1938) we demonstrated that there was a change in the relative proportions of *l*-ascorbic and dehydroascorbic acids in Bramley's Seedling apples as the fruit developed, the equilibrium shifting in favour of *l*-ascorbic acid as the apple approached maturity. The general evidence made it reasonably probable that this change took place actually in the living tissue of the apple and not during analytical manipulation. In continuing this inquiry we discovered further that vitamin C was synthesized when apples were stored at 3° and that the capacity for synthesis was, as in the case of dehydroascorbic acid, high in the young but almost non-existent in the mature fruit. This was first observed in 1938 and the observation was confirmed every year subsequently, leaving little doubt that it is reproducible under our experimental conditions. In this communication an account of a characteristic experiment performed in 1942–3 is given.

METHODS

As already mentioned, in each of the four years preceding 1942 it was definitely established that there was a formation of vitamin C in young apples during storage. This was the case whether the comparison was made between the vitamin C concentration in the tissues of the fruits or between the contents of the entire apples before and after storage. In planning the final experiment not only were these two points borne in mind but an attempt was also made to ascertain whether the weight of the fruit at the time of picking, and the tree of origin, influenced this formation of the vitamin. In devising the experiment we were fortunate in having, on the statistical aspect of the problem, the valuable advice of Mr T. N. Hoblyn of East Malling Research Station, and the following scheme was adopted.

Sampling. The apples came off two 25-year-old three-quarter standard Bramley's Seedling trees on No. VI rootstock, grown at East Malling. The fruits were picked on 2 July, 20 July, 10 August and 12 October 1942, and were stored until 11 February, 25 February, 10 March and 6 April 1943, respectively.

The fruits were on each occasion picked from the two trees between 8 and 10 a.m. and were immediately sorted out into the following approximate weight groups: first picking—tree A, 18–19, 21–22, 24–25 g.; tree B, 18–19, 21–22, 24–25 g.; second picking—tree A, 39–41, 42–42, 44–45 g.; tree B, 43–45, 45–47, 48–49 g.; third picking—tree A, 65–68, 68–69, 70–71 g.; tree B, 62–62, 68–69, 70–71 g.; fourth picking—tree A, 119–122, 122–128, 128–132 g.; tree B, 122–124, 124–133, 134–138 g. Three apples from each weight group and each tree, i.e. 18 fruits of each picking, were analyzed immediately.

Storage. Similar samples, the individual weights being marked on each fruit, were simultaneously placed in store at a constant temperature of 3° and a humidity of 83% saturation. Three fruits from each weight group and each tree were removed from store at the times mentioned above and were analyzed immediately.

Analysis. The dry weight was determined as follows. The apples were cut into small pieces which were then dried in an oven at 50° in a vacuum. The drying was continued