Experiments were performed to see if any change takes place in the anthocyanin pigment, cyanin-3-monoside (Laurence, Price, Robinson & Robinson, 1938), during autolysis. Samples were exhaustively extracted with 90% (v/v) ethanol after various periods of autolysis, and measured portions boiled down *in vacuo* to remove ethanol, and treated with basic lead acetate. The lead precipitate was hydrolyzed for 1 hr. with N-HCl, and the amount of reducing sugar estimated. There was no significant change with time in the amount of reducing sugar (presumably mainly from the anthocyanin), which was taken as an indication that little change had taken place in the anthocyanin during autolysis. It is likely that, during the more drastic commercial fermentation, considerable changes in the anthocyanin do occur, which may lead to the development of tannin-like substances. Knapp & Hearne (1939) have reported the presence of leuco-anthocyanins in all parts of the cacao fruit, and these may be concerned in the residual tannin fraction.

The presence of *l*-epicatechin, closely related to cyanidin, in the cacao bean has been reported (Freudenberg, Cox & Braun, 1932). This may be the tannin-precursor found in the present investigation but so far direct evidence is lacking. The fact that a tannin-precursor is present is important, however, as the foregoing results indicate that unless the conditions during a commercial fermentation are mainly aerobic an increase in tannin, instead of the desirable decrease, may actually occur.

SUMMARY

1. When fresh cacao beans are minced and allowed to autolyze, there is a rapid decrease in the amount of tannin as measured by (*a*) precipitation with cinchonine sulphate, (*b*) total Stiasny precipitate, (*c*) residual Stiasny precipitate, (*d*) oxidation by permanganate (Lowenthal method).
2. A constant value was attained after about 54 min. by all these methods and it is suggested that autolysis divides the tannin compounds into two fractions—oxidizable and resistant.
3. The oxidizable tannin appears to be split into two approximately equal portions by cinchonine sulphate.
4. Evidence for the existence of a tannin-precursor was obtained by allowing minced beans to autolyze in water, when an increase in tannin occurred. The tannin-precursor is not soluble in 40% (v/v) acetone.
5. The resistant tannin fraction appears to consist of at least two components.

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Biochemical Characterization of the Actions of Chemotherapeutic Agents

2. A REACTION OF HAEMOLYTIC STREPTOCOCCI, INVOLVING PANTOTHENATE-USAGE, INHIBITED BY PANTOYLTAURINE, AND ASSOCIATED WITH CARBOHYDRATE METABOLISM

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(*Received* 13 *March* 1944)

The basis upon which pantoyltaurine was designed as an antibiotic agent (Snell, 1941; Kuhn, Wieland & Möller, 1941; McIlwain, 1942*a*; cf. Fildes, 1940), and subsequent investigations (McIlwain, 1944), suggested that the compound acted upon bacteria by affecting metabolic processes involving pantothenate. It is also suspected that other chemotherapeutic agents have their primary action upon

the metabolism of parasites, but in no case have the processes concerned been specified in detail. Such characterization could be expected to be of value in understanding the peculiarities and limitations of existing chemotherapeuticals and in suggesting new ones. This characterization has now been attempted in the case of pantoyltaurine.

During exploratory experiments the observation was made that the pantothenate concentration of streptococcal cultures normally diminished during their growth, but that this decrease was smaller in the absence of excess glucose or in the presence of pantoyltaurine. The disappearance of pantothenate was therefore followed in streptococcal suspensions containing various constituents of the growth media. Glucose catabolism was found to be associated with the process, and the acid formed during this breakdown was measured. The reaction in most experiments occupied a few hours at 37°; the streptococci used were grown with defined quantities of pantothenate, and the pantothenate of their cells was also taken into account. The concentrations of pantothenate and pantoyltaurine used in metabolic and growth experiments included those found *in vivo* during successful chemotherapy with the compound (McIlwain & Hawking, 1943).

EXPERIMENTAL

The streptococcal cultures, the conditions of their maintenance, and the method of enumerating viable organisms, were those described in Part 1 (McIlwain, 1944). A Group D strain was also used; all were freshly passaged β-haemolytic streptococci. The reagents were those of Part 1, except in the cases specified below.

Casein hydrolysate prepared by acid in the presence of titanous salts. The casein hydrolysate previously described contributed extraneous material to the harvested organisms and the following method of hydrolysis was chosen to avoid the 'humin' formation associated with the former preparation. The method was based on the findings of Sullivan & Hess (1937) (cf. also Lieben, 1943); the product is preferable for most bacteriological purposes in yielding a light-coloured medium which gives no precipitate on keeping or on making acid.

A mixture of concentrated hydrochloric acid (170 ml.) and technical 15% (w/v) titanous chloride (80 ml.) was added to casein (200 g.) in a conical beaker, mixed quickly before the liquid could be unevenly absorbed by the solid, and autoclaved for 45 min. at 120°. The solution was cooled in running water and taken to pH 6–6·5 with 40% NaOH (*c.* 240 ml.). A bulky, dark-coloured precipitate, mainly of titanium salts, separated. A small sample of the whole was boiled, filtered and tested by the addition of further small quantities of NaOH and HCl, and boiling; if a further precipitate formed, the bulk was adjusted in the sense suggested and a further specimen tested. The bulk, under conditions optimal for precipitation, was then boiled (frothing occurred initially and the precipitate lightened in colour) for 10 min., kept cool for 3 hr., and filtered through paper pulp on a Buchner funnel. Much of the phosphate of the casein was removed during precipitation.

Basal medium. A stock solution was prepared from the above filtrate by adding KH_2PO_4 (45 g.), oxalic acid (1 g.), NaOH to pH 7·6, water to 1000 ml., boiling gently for 5 min., keeping cold for at least 2 hr., and filtering through fluted papers. The reagents appeared to remove material contributed by the titanium solution as well as the calcium of the casein; the solution was kept with chloroform in the dark. The basal medium was prepared from it as required, by diluting 100 ml. to 700 ml., distributing in 70 ml. portions in plugged 250 ml. conical flasks, and autoclaving to sterilize and remove chloroform.

Growth medium C. The following solutions were mixed and made aseptically to a final volume of 100 ml. with M/18 phosphate buffer: basal medium (70 ml.), M-glucose (5 ml.), M/50 arginine and M/50 glutamine (2 ml.), Group *A* of McIlwain (1944) (5 ml.), M/2 $NaHCO_3$ (7 ml.), pantothenate and inoculum.

Growth. Freshly prepared medium C (100 ml.) was inoculated with approximately 10^6 organisms from approx. 24 hr. old serum-broth-agar cultures of the streptococci, and incubated at 37° in 5% CO_2-air. Unless otherwise described (cf. Table 2), incubation was continued for 45–48 hr. and the culture neutralized at 18, 24 and (if necessary) at 42 hr. For this purpose, 1 ml. was taken aseptically and titrated with N/20 NaOH to pH 7·6; the necessary volume of N-NaOH was then added to the bulk. The additions necessary were usually of 4–5 ml. and the total alkali added per flask about 10 ml.

After growth, the dry weight of organisms in 10 ml. of the culture (or pooled cultures) was determined after washing three times with water and drying at 100°. The bulk of the organisms was collected by centrifuging and washed three times with M/18 phosphate buffer of pH 7·6 (10 ml./100 ml. culture) or, when noted, with saline.

Reaction with pantothenate. The majority of the experiments were carried out in centrifuge tubes which contained in a volume of 5 ml.: (1) a suspension of streptococci, usually of 10–50 mg. dry weight, (2) phosphate buffer, commonly M/15 of pH 7·6, (3) the casein hydrolysate of McIlwain (1939) at a final concentration of 2%, (4) M/20 glucose, (5) 2×10^{-6}M-pantothenate, (6) pantoyltaurine or other inhibitors. The tubes, loosely stoppered, were incubated in air at 37°, usually for 4 hr. with occasional shaking. The bacteria were separated by centrifuging and the solutions titrated to their original pH with N/5 NaOH, made to a standard volume, and sterilized for assay by autoclaving at pH 6·5–7. The organisms remaining in the centrifuge tube were washed twice with buffer, pH 7·6, and kept for pantothenate assay.

In the majority of the present experiments no attempt was made to control the change in pH brought about by the organisms, as it was used for estimating their acid formation and the conditions were chosen to simulate those of the cultures in which disappearance of pantothenate was first observed. The final pH approached 5·3; pantothenate disappearance continued at pH 5 in the presence of the organisms, but not in their absence nor in the presence of certain inhibitors. It also occurred when the solution was maintained at pH 7·6 throughout the reaction.

Pantothenate assay. Microbiological methods, using the growth of *Proteus morganii* (N.C.T.C. no. 2818) and an occasional comparison using the Group G streptococcus, were employed (McIlwain & Hawking, 1943). The reference tubes contained 10^{-8} to 10^{-9}M-pantothenate. The quan-

tities of pantoyltaurine used in the present experiments did not interfere with the assay of the coincident pantothenate by *Pr. morganii*; when relatively high concentrations of streptococcal extracts were assayed, control experiments were carried out with addition of pantothenate to detect any inhibitory effect upon the growth of the test organism. Some values were confirmed by a photometric method to be described later.

Pantothenate liberation from organisms (Table 1)

Various methods of releasing pantothenate were carried out as follows. *Autoclaving* was at 120° for 20 min. *Autolyses* were carried out by suspending the washed organisms at different concentrations in buffers of varying pH, with a drop of benzene, and incubating at 37° for 1–3 days. *Digestion*: this was the procedure normally carried out and the conditions were based upon those of Cheldelin, Eppright, Snell & Guirard (1942) for the liberation of pantothenate from various materials. The washed bacteria (of about 30 mg. dry weight) in centrifuge tubes were suspended in M/10 acetate buffer, pH 4·7 (1 ml.); 2 % takadiastase (0·3 ml.) and 0·7 % purified papain (0·3 ml.), also in acetate buffer, were added and the whole incubated with a drop of benzene, at 37°, for 2 days, with occasional shaking. The solutions were then diluted, taken to pH 6·5–7 with NaOH, made to 5 ml., the tubes plugged and autoclaved, centrifuged, and the supernatant decanted sterilely for assay.

Two commercial specimens of takadiastase were found to contain satisfactorily small quantities of pantothenate (<2 mμ mol./g.) but it was found necessary to purify the papain. This was done by ethanol precipitation according to Hellerman & Perkins's (1934) method, which reduced the pantothenate content of the enzyme from 50 to <2 mμ mol./g. and doubled its gelatinase activity. The pantothenate of reagent controls in the normal digestion procedure described above was then less than could be assayed.

Table 1 shows that several procedures liberated approximately equal quantities of pantothenate from streptococci, those procedures using takadiastase tending to yield the highest quantities. Though the contribution of the enzymes to pantothenate liberation in the present experiments is doubtful, the enzymes have been included as a precaution (they increased the pantothenate obtained from certain tissues by Cheldelin *et al.* (1942)) and to render the results comparable with those of Thompson (1942). The present pantothenate-yields from streptococci are in fact notably lower than those recorded by Thompson for other bacterial species, but that this was not due to the technique employed was shown by examining certain of the organisms assayed by Thompson, when results comparable to his were obtained.

Benzene was added primarily to minimize contamination; in an experiment in which it was omitted, but which was not contaminated, the pantothenate liberation was no greater than in the presence of benzene. Benzene is known also to prevent the destruction of pantothenate by streptococci (see later); though there are further reasons (absence of glucose and casein) for the absence of this process during digestion, it was important to confirm that it did not occur, and this was done as follows. A batch of organisms (184 mg. dry weight) was divided into three portions; to one was added 0·5 ml. water, to another 0·5 ml. of 10^{-6}M-pantothenate and, to the last, 0·5 ml. of 10^{-5}M-pantothenate. All were digested and assayed by the standard procedures and the latter two found to contain both the native and added pantothenate.

Table 1. *Extraction of pantothenate from streptococcal cells*

Process	Batch of organisms*; dry weight (mg.)	Pantothenate extracted	
		mμ mol.	mμ mol./g. dry weight
Standing 24 hr. at 20° in 2 ml. phosphate buffer, pH 4·7 or 7·6	I; 25	0·10	4
Autoclaving at pH 4·7 or 7·0	I; 25	0·35	14
Autolysis 2 days at 37° in:			
(*a*) 5 ml. phosphate buffer, pH 7·6	II; 24	0·30	12·5
(*b*) 5 ml. phosphate, pH 4·7	II; 24	0·40	17
(*c*) 2 ml. acetate buffer, pH 4·7	III; 17	0·43	25
(*d*) 0·8 ml. acetate buffer, pH 4·7	IV; 42	1·25	30
Digestion 1 or 2 days at 37° in 2 ml. acetate buffer, pH 4·7:			
(*a*) alone or with papain	V; 50	1·4	28
(*b*) with takadiastase	V; 50	1·75	35
(*c*) with papain and takadiastase	V; 50	1·75	35
	VI; 24	0·65	27
	VII; 11	0·35	32

* Organisms: the Group G strain grown in medium C with pantothenate, 10^{-5}M. Details of processes are given in the text. The numbers used in designating batches of organisms in the different tables are independent and do not permit cross-reference.

Attempted liberation of pantothenate from culture fluid after growth. Portions of a culture fluid (100 ml.), in which 82 mg. dry weight of streptococci had been grown with 50 mμ mol. of pantothenate, were assayed after the following treatments: (i) filtration to sterilize; (ii) and (iii) autoclaving at pH 7·6 and 4·7 respectively; (iv) digestion at pH 4·7 with papain and takadiastase. In all cases, assay of the specimens gave values corresponding to < 1 mμ mol. of pantothenate in the whole of the culture fluid. The assays of culture fluids reported in the tables are after autoclaving at pH 7, without enzyme treatment.

RESULTS

A. *Disappearance of pantothenate from streptococcal cultures*

The disappearance of pantothenate was shown with the four strains of β-haemolytic streptococci (of Groups A, D and G) which were examined (Table 2). Disappearance of 50 mμ mol. of pantothenate from normal cultures of 100 ml. was almost complete. With increasing amounts of pantothenate, up to 1000 mμ mol./100 ml., the absolute amount disappearing increased; but so also did the proportion remaining. The pantothenate recovered from the organisms was in all cases small. Table 2 contains many results obtained during the growth of organisms for later experiments and illustrates the reasons for choosing the following conditions of growth in the majority of cases. (*a*) The cultures were neutralized during growth, since the yield of organisms was then nearly doubled. (*b*) Growth with the smaller quantity of pantothenate (50, not 1000 mμ mol./100 ml.) was preferred as the yield of organisms was the same as with the larger quantity, but less washing was required to separate them from pantothenate of their media. (*c*) Growth was continued until the second day as the yield of organisms increased, the pantothenate content of the media decreased and (see later) the majority of the organisms died.

In considering possible fates of the pantothenate, other than its metabolism by the organisms, the following points were examined: (*a*) *Stability of pantothenate during growth.* Previous observations by many authors suggested pantothenate to be stable under the temperature and pH conditions of the present experiments, and this was confirmed by incubating batches of media at pH 7·6 and at pH 5, which contained defined quantities of pantothenate. These were not found to be lost. (*b*) *Stability of pantothenate during sterilization of solutions.* Several solutions were assayed after autoclaving, or sterilizing-filtration, but no differences in their pantothenate contents were found. (*c*) *Stability of pantothenate of the organisms during digestion.* (*d*) *Absence of 'bound' pantothenate in the mother-liquors of growth.* Experiments, indicating that loss or apparent loss of the compound does not occur through these factors (*c*) and (*d*), have been described. (*e*) *Recovery of pantothenate from organisms.* The approximately equal results obtained under different conditions (the last five of Table 1) suggest the limit which is being approached to be a real one. Repetition of enzyme digestion yielded no further pantothenate. (*f*) *The buffer used in washing the organisms.* When tested separately this contained very little pantothenate.

Table 2. *Disappearance of pantothenate during growth of streptococci*

Organism and conditions of growth	Pantothenate/culture of 100 ml.: Added (mμ mol.)	After growth: In solution (mμ mol.)	After growth: In organism (mμ mol.)	After growth: Total; % of that added	Yield of organisms (dry wt.; mg./100 ml. of final culture)	Acid produced (m mol. NaOH needed/100 ml.)
Group A (Richards); culture neutralized and grown for 42 hr.	50	<1	0·5	**<3**	52	8·9
Group A (7188); neutralized; 67 hr.	50	<1	0·25	**<2·5**	16	4·1
Group D; neutralized; 67 hr.	50	1	0·25	**2·5**	55	9·8
Group G; neutralized; 18 hr.	50	2	0·5	**5**	53	5·7
Group G; neutralized; 42 hr.	50	<1	0·3	**<2·6**	80	9·7
Group G; neutralized; 45–48 hr.	50	<1	0·2–0·4	**<2·8**	Mean of 17 exps.: 77·2 (Standard deviation, 11·6)	Mean of 17 exps.: 10·72 (Standard deviation, 1·15)
Group G; not neutralized; 42 hr.	500	100	1·3	**20**	44	—
Group G; neutralized; 42 hr.	500	100	2·2	**20**	82	—
Group G; neutralized; 45–48 hr.	1000	300–500	1·3–2·4	**30–50**	Mean of 9 exps.: 78·4 (Standard deviation, 10·9)	Mean of 9 exps.: 10·18 (Standard deviation, 1·65)

The results of the remainder of this paper show that pantothenate disappearance is not a necessary concomitant of growth but can be influenced independently of many other processes then occurring.

B. *Dissociation of pantothenate metabolism from streptococcal growth and viability*

The results of Table 2 demonstrate that the disappearance of pantothenate from streptococcal cultures is not proportional to the amount of growth; 77–78 mg. of the Group G organism removed from 49 to 500 or 700 mμ mol. of pantothenate, according to the amount initially present. Also, results of growth with larger quantities of pantothenate (not quoted here) tended to show that the growth of a culture could precede much of the pantothenate-disappearance. The effect of streptococci, collected and washed, upon pantothenate in the presence of other constituents of the cultures was therefore examined. Table 3 gives the results of experiments in which conditions were varied so that the pantothenate remaining after reaction under different circumstances (which will be discussed later) varied over a range of 150-fold. Change in dry weight of the organisms was not, however, detectable. Their pantothenate content, also, did not increase. The usage of pantothenate in several experiments of Table 3 is 10 mμ mol., which during growth of the organisms themselves corresponds to an increase in mass of 16 mg. dry weight; any increase during the experiments was, however, of the order of 1 mg. Pantothenate disappearance in the presence of streptococci is thus not dependent upon growth of the organisms.

The organisms used during the experiments of Table 3 were, however, viable in the sense that they could be subcultured from the suspensions after exposure. As the suspensions contained many millions of organisms, a closer examination was necessary in order to find whether the reaction depended upon the presence of viable organisms.

Table 3. *The disappearance of pantothenate in washed streptococcal suspensions; its independence of growth*

(Each tube contained hydrolyzed casein, phosphate, streptococci and the additional substances listed below in a final volume of 5 ml. Exposure was at 37° and for 4 hr. except in the case indicated (*), which was for 1 hr. The first five tubes contained equal amounts of Suspension I, and the last of Suspension II, of different batches of the Group G streptococcus grown in pantothenate, 5 × 10⁻⁷ M.)

Additional substances			After exposure		
Pantothenate (mμ mol.)	Glucose (m mol.)	Inhibitors	Pantothenate in solution (mμ mol.)	Acid produced (m mol. NaOH to neutralize)	Dry weight of organisms (mg.)
		Suspension I			
0	0	0	<0·05	0	43·8
10	0	0	7·25	0	44·0
10	0·25	0	<0·10	0·106	44·8
*10	0·25	0	<0·15	0·070	45·1
10	0·25	Pantoyltaurine, 20 μ mol.	7·25	0·114	44·1
		Suspension II			
0	0·25	0	<0·07	0·118	35·2
10	0·25	0	1·25	0·116	35·2
10	0·25	Phenol, 20 μ mol.	6·5	0·064	36·9
10	0·25	Propamidine, 1 μ mol.	6·5	0·074	34·7
10	0·25	Pantoyltaurine, 20 mμ mol.	3·0	0·130	33·4

Table 4. *Glycolysis and the disappearance of pantothenate in streptococcal suspensions; their independence of viability*

(Each tube contained hydrolyzed casein, phosphate, glucose (0·25 m.mol.), pantothenate (10 mμ mol.) and preparations, in 5 ml., of the Group G organism (grown with pantothenate, 5 × 10⁻⁷ M) described below, and was incubated for 4 hr. at 37°.)

Organisms/tube			Pantothenate			Acid production		
			Remaining	Disappearance				
Preparation	Dry weight (mg.)	Number of viable organisms	in solution (mμ mol.)	mμ mol./g. dry weight	mμ mol./10⁸ viable organisms	M/5 NaOH used (ml.)	m mol./g. organisms	m mol./10⁸ viable organisms
Growth: 18 hr.; washed	14	8·3 × 10⁸	0·5	680	1·1	0·52	7·5	0·013
Growth: 18 hr.; washed	3·5	2·1 × 10⁸	9·0	290	0·5	0·15	8·6	0·014
Growth: 48 hr.; washed	25	2·3 × 10⁶	0·3	390	420	0·69	5·5	6·0
Growth: 48 hr.; washed	8·3	7·7 × 10⁵	4·0	720	780	0·33	8·0	8·6
Growth: 42 hr.; unwashed	20	5·7 × 10⁷	1·3	440	15	0·49	4·9	0·17
Growth: 42 hr.; washed	20	6·3 × 10⁷	1·5	430	13·5	0·41	4·1	0·13

This was carried out by determining, in cultures grown and collected differently, both the reaction with pantothenate and the numbers of viable organisms. Table 4 compares the pantothenate disappearance of different suspensions with two properties. It shows that while the disappearance per viable organism varied over a range of more than 1000-fold, that per unit of dry weight varied only $2\frac{1}{2}$-fold. The latter variation, moreover, was not entirely a property of the suspension but also depended upon the mass of organisms used (i.e. upon the present assessing not using methods necessary to kinetic investigations).

It was estimated that the suspension collected 18 hr. after inoculation contained at least 70 % of viable organisms, while that at 48 hr. contained about 0·1 %. Growth for the longer period was adopted as the standard procedure in the following experiments.

C. *Components necessary for metabolism of pantothenate by streptococcal suspensions*

The experiments of Table 5 showed that, of the constituents of the growth media in which disappearance of pantothenate was first observed, both glucose and the casein hydrolysate were necessary to pantothenate metabolism, though both could to some extent be replaced by related materials. Succinate and glycerol did not replace glucose, but disappearance of some pantothenate occurred when pyruvate was substituted. A mixture of amino-acids replaced the casein hydrolysate, though it was less effective. With organisms which had been washed with saline instead of the phosphate usually employed, replacement of the phosphate in the normal reaction-mixture by bicarbonate did not decrease pantothenate metabolism. The casein hydrolysate, however, contributed a little phosphate to the medium. With the saline-washed organisms, pantothenate disappearance was slightly increased by addition of a magnesium salt. The reaction proceeded anaerobically as well as aerobically (Table 6). Although it is dissociated from growth, pantothenate disappearance increases with time (Table 6).

D. *Effect of pantoyltaurine and other inhibitors upon pantothenate metabolism*

The present interest in the reaction of streptococci with pantothenate lies in its prevention by small quantities of pantoyltaurine. This has already been indicated in Table 3 and the effects with several different batches of organisms are given in Table 6. The phenomenon has been shown with 16 different batches of organisms, grown in the present media for periods varying from 1 to 3 days and with initial pantothenate concentrations of 5×10^{-7} to 10^{-5} M. No batch has failed to show the phenomenon, and the majority, grown with 5×10^{-7} M-pantothenate, showed the optimal effect recorded in Table 6 for suspension II. It therefore appears to be a charac-

Table 5. *Components necessary for pantothenate metabolism*

Components (other than pantothenate) in 5 ml.						Pantothenate (mμ mol.)			Acid production (m mol. NaOH required to neutralize)
Glucose or surrogate (μ mol.)		Casein hydrolysate or mixed amino-acids* (mg.)		Phosphate (275μ mol.) or bicarbonate (250μ mol.); $MgSO_4$ (4μ mol.)	Dry weight of organisms (mg.)	Added	After growth	Disappearance/g. dry weight	
Suspension I, washed with saline									
0		Hydrolysate,	50	Phosphate + $MgSO_4$	51	10	5–7	60–100	0
Glucose,	250	"	"	" "	51	10	0·25	190	0·202
"	"	"	5	" "	51	10	2·5	145	0·130
"	"	Amino-acids,	65	" "	51	10	2·0	155	0·120
"	"	Hydrolysate,	50	Bicarbonate "	51	10	0·15	195	0·20
"	"	"	"	Phosphate only	51	10	1·0	175	0·162
Suspension II, washed with phosphate buffer									
Glucose,	250	Hydrolysate,	50	Phosphate only	44	10	<0·05	>230	0·118
"	"	Amino-acids,	65	"	44	10	1·0	200	0·070
"	"	"	"	"	44	1	<0·05	> 20	0·074
Pyruvate		"	"	"	44	10	6·5	80	0
"		"	"	"	44	1	0·15	19	0
Suspension III, washed with phosphate buffer									
Glucose,	250	Hydrolysate,	50	Phosphate only	0	10	10	0	0
"	"	"	"	"	25	10	0·3	390	0·138
Succinate,	500	"	"	"	25	10	10	0	0
Glycerol,	500	"	"	"	25	10	10	0	0
0		"	"	"	25	10	8·5	60	0

Time of exposure, 4 hr.

* The group of 14 amino-acids of Gladstone (1939), in the proportions there given.

teristic of the organisms, though the degree of their reaction with pantothenate is variable, as is shown in Table 6. The amount of pantothenate in the organisms was always small. The maximum effect—the disappearance of little of the 10 mμ mol. of pantothenate in the presence of pantoyltaurine, while controls showed the disappearance of 99% of the substrate—was usually given by 50 to 100 mμ mol. of pantoyltaurine (see Table 6). Lesser effects are given by smaller quantities, and these are such that the quantities of pantothenate and pantoyltaurine which interact in producing the metabolic effect are of the same order of magnitude. The magnitudes are especially close if, as in the growth of certain bacteria (Snell, 1941), *l*-pantoyltaurine only is effective; the pantoyltaurine used in the present experiments was racemic.

Tables 3 and 6 show, however, that inhibition of reaction with pantothenate is not in itself a characteristic property of pantoyltaurine. It is the major inhibitory effect, which has yet been recorded, of the compound upon a metabolic process (cf. McIlwain, 1944) and is not given by its products of hydrolysis (Table 6), but is shown by benzene, by high concentrations of phenol and malonate, and by relatively low concentrations of propamidine and

Table 6. *Effects of inhibitors, etc. upon pantothenate metabolism*

Standard conditions of exposure, except when stated in the fourth column. All reaction-mixtures except the first initially contained pantothenate, 10 mμ mol.

Streptococci			Addenda/5 ml.		Pantothenate (mμ mol.) remaining		
Batch no.	Initial pantothenate content of their growth media (M)	Dry weight (mg.)	Inhibitors (μ mol.); or other special feature		Solution	Organisms	Acid produced (μ mol. NaOH to neutralize)
I	5×10^{-7}	54	None; no pantothenate		<0·05	0·05	62
			None		2·0	0·10	62
			Pantoyltaurine,	5	8–10	0·18	58
			”	0·5	8–10	0·25	66
			”	0·005	8–10	0·20	66
II	5×10^{-7}	28	None		<0·05	0·20	150
			Pantoyltaurine,	0·5	10	—	148
			”	0·15	10	—	136
			”	0·05	10	—	160
			”	0·015	1·0	0·25	148
III	10^{-5}	26	None		2·0	—	104
			Pantoyltaurine,	2	10	—	70
			”	0·4	10	—	78
			”	0·08	5	—	90
			”	0·02	4	—	94
			”	0·004	2	—	102
IV	5×10^{-7}	60	None		0·3	0·15	66
			Pantoyltaurine,	20	8·0	0·15	70
			Hydrolyzed pantoyltaurine,	20	0·5	0·15	72
			Phenol,	100	6·0	0·05	0
			Salicylate (see text),	100	0·2	—	82
			Propamidine,	4	2·0	0·20	50
V	5×10^{-7}	22	None		1·2	—	122
			Malonate,	100	5·0	—	0
			”	10	1·1	—	120
			Iodoacetate,	20	9·0	—	0
			”	2	9·0	—	0
			”	0·5	2·0	—	100
VI	5×10^{-7}	54	None		<0·15	0·15	150
			Sulphanilamide,	10	<0·15	0·20	142
			Benzene (saturated)		6·0	0·15	52
VII	10^{-5}	14	None		0·25	—	58
			Organisms heated 30 min. at 55°		8–10	—	0
			Organisms heated 30 min. at 65°		10	—	0
VIII	5×10^{-7}	68	Exposure: 4 hr. anaerobically		<0·10	—	62
			Exposure: 4 hr. aerobically		<0·10	0·10	56
			Exposure: 1 hr. aerobically		0·15	0·15	—
			Exposure: ½ hr. aerobically		3·8	0·10	—

iodoacetate. Salicylate and sulphanilamide are notable in having little effect at relatively high concentrations.

E. *Relation of pantothenate metabolism to glycolysis*

During the course of these investigations it was found that the effect of pantoyltaurine upon pantothenate metabolism could be more closely defined by taking into account also the acid formation from glucose, which is associated with the disappearance of pantothenate from streptococcal cultures and suspensions. Such glycolysis is a notable property of the present organisms; Table 2 shows that it is a major reaction during growth, and Tables 3 and 5, that the omission of glucose from the reaction mixture from which streptococci normally caused pantothenate disappearance, prevented or greatly reduced its disappearance. Acid production during metabolic experiments was therefore determined and is recorded in Tables 2–6.

The following points show a certain, but significantly incomplete, relationship between the degree of glucose metabolism and of pantothenate disappearance under these conditions:

(1) Acid formation from glucose, like pantothenate-metabolism, is independent of growth of the organisms (Table 3) and of their viability (Table 4) but varies with the dry weight of organisms in roughly the same manner as their pantothenate-metabolism (Table 4).

(2) The casein hydrolysate gave maximum effects both in acid formation and pantothenate metabolism; aerobic conditions are not necessary to either process; magnesium salts slightly increase both; added phosphate is necessary to neither. Amino-acids in place of the casein hydrolysate allowed both processes to proceed at a slower rate; pyruvate metabolism is related to that of glucose and the compound permitted a slow reaction with pantothenate (Table 5).

(3) Inhibition of both processes under the conditions of Tables 3 and 6 is complete or nearly so with iodoacetate, 2–20 μ mol., but not with 0·5 μ mol. It is considerable with malonate, 100 μ mol.; phenol, 100 μ mol.; with benzene, and after heating the streptococci to 55 or 65°. Sulphanilamide, 10 μ mol., and the lower quantity of malonate, affected neither process. In the instance recorded, salicylate affected neither process but on other occasions it has been found to affect both.

The correlations which have been described are only semi-quantitatively expressed, as the present experiments do not measure the course of pantothenate and glucose metabolism. The findings, however, show a major difference between the behaviour of pantoyltaurine and of other inhibitors. Results with the first three suspensions of Table 6 indicate that although the compound may depress glycolysis, this has not been found to occur to an extent of greater than 33 % and may not occur at all even with maximal inhibition of pantothenate metabolism. The relatively small effect of pantoyltaurine upon the more accurately measured *course* of glycolysis was previously reported (McIlwain, 1944). The peculiarities of the metabolic effects of pantoyltaurine are thus its inhibition of pantothenate-disappearance when present in extremely small concentrations, and the fact that inhibition of the process, even when brought about by relatively high concentrations of pantoyltaurine, occurs without a corresponding inhibition of glycolysis.

DISCUSSION

Previous findings have indicated that the need for pantothenate in streptococcal growth may be referred to its necessity to processes, probably enzymic, which are essential to growth of the organisms (McIlwain, 1942*b*). It is now suggested that the process by which pantothenate disappears from cultures, and by which it also disappears in the present metabolic experiments, may be one to which the actions of pantothenate and pantoyltaurine upon growth can be ascribed. This suggestion has been derived from the following considerations:

(1) The action of pantoyltaurine upon streptococcal growth is specifically connected with pantothenate (McIlwain, 1942*a*).

(2) Both growth and the metabolic process are very sensitive to pantoyltaurine, but of the two the metabolic process is the more sensitive. This order of sensitivity may not be entirely necessary to the causal relationship suggested above, but more satisfactory evidence for the relationship is afforded by it than if growth were to be the more sensitive. The order of sensitivity found is consistent with certain phenomena manifested in the course of growth (unpublished observations).

(3) Glycolysis, with which the metabolic process is associated, is a reaction especially important to the streptococci and pneumococci which are most sensitive to pantoyltaurine. Pantothenate has been found to be connected with carbohydrate metabolism in a variety of organisms: in *Pr. morganii* (Dorfman, Berkman & Koser, 1942; Hills, 1943), yeasts (Williams, Mosher & Rohrman, 1936; Teague & Williams, 1942) and animals (Wright, 1942). The latter investigation is of especial relevance to the present work, as it was found that administration of glucose to rabbits was followed by a lowering of their blood-pantothenate.

(4) Added pantothenate is, however, not necessary to glycolysis of the present organisms; Table 2 shows the reaction during growth to be unaffected by the presence of excess pantothenate in the culture

fluid, and Tables 3 and 6 that the glycolysis of suspensions is unaffected by the compound. A small stimulation only was found in previous experiments with the same organism (McIlwain, 1944) in which, in contrast with the present work, the course of glycolysis was measured. Pantothenate is always present in small quantities in the organisms; nevertheless, if free pantothenate is present, it is metabolized. As the association of the usage of added pantothenate with glycolysis is facultative, it is understandable that pantoyltaurine should be able, as is found, to depress pantothenate-usage without much effect upon glycolysis. The organism in presence of pantothenate and pantoyltaurine is then reverting to a condition similar to that obtaining when neither is present. This condition may be adequate for glycolysis but is not adequate for growth. The association of pantothenate-usage with growth is obligatory: growth is strictly limited by the quantity of pantothenate added when that is below a given concentration (McIlwain, 1944), and the limiting quantities are completely removed from the media. Pantoyltaurine in such cases prevents both the pantothenate metabolism and growth.

(5) To explain inhibition of growth by a process which preserves the growth-essential, pantothenate, it can be supposed that, in its functioning, pantothenate undergoes a cycle of changes, which is interrupted by pantoyltaurine, and of which at least one phase is relatively unstable in the presence of the reaction mixture.

SUMMARY

1. Pantothenate disappeared from streptococcal cultures and from certain reaction mixtures containing pantothenate through a process for which the organisms were necessary but which was independent of their growth and viability.

2. This metabolism of pantothenate was associated with the presence of glucose and a casein hydrolysate (or related materials) and perhaps of magnesium, but was independent of oxygen supply.

3. When glycolysis was prevented by omitting or adding various substances, or by treating the organisms, pantothenate metabolism usually also ceased.

4. The presence of pantoyltaurine is exceptional among the circumstances investigated, in that it causes inhibition of pantothenate metabolism without a corresponding inhibition of glycolysis.

5. Streptococci normally contain pantothenate and are capable of glycolysis in the absence of added pantothenate, but if it be assumed that both glycolysis and the presence of excess pantothenate are needed for the formation of a substance essential to streptococcal growth, and that such formation is inhibited by pantoyltaurine, then it is understandable that, as observed, (i) absence of added pantothenate inhibits growth, (ii) inhibition of glycolysis stops pantothenate metabolism, (iii) pantoyltaurine stops pantothenate metabolism and growth, but not glycolysis.

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A Quantitative Micro-method for the Separation of Inorganic Arsenite from Arsenate in Blood and Urine

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(*Received* 20 *March* 1944)

Studies on the metabolism of inorganic arsenic compounds in the animal body have been greatly hampered by the lack of a suitable quantitative method for the separation of trivalent from pentavalent inorganic arsenic in the body fluids. Precipitation methods for the separation of arsenite from arsenate in urine are unsatisfactory, as shown by Joachimoglu (1916), whose method was based on