cation of the enzyme with an over-all yield of 20% was effected.

3. A purified preparation of the enzyme attacked all three phosphoproteins studied, but had no appreciable action on glycerophosphate or casein phosphopeptone. The enzyme is hence a true phosphoprotein phosphatase, distinct from the acid phosphomonoesterase.

4. Optimum enzyme activity was found at pH 6·0 and at a substrate concentration corresponding to about 10 μmoles/ml. of casein phosphorus with 0·001 M thioglycollic acid as activator.

5. From the activation and inhibition studies it is deduced that sulphydryl and amino groups are essential for the activity of the enzyme. However the enzyme requires no dialysable coenzyme for its activity.

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The Synthesis of Coenzyme A by *Lactobacillus arabinosus* 17–5

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Washed, glycolysing suspensions of haemolytic streptococci cause a disappearance of pantothenate from the suspending medium (McIlwain & Hughes, 1944, 1945). As most of the pantothenate in animal and bacterial tissues exists in the form of coenzyme A (CoA) (Novelli, Kaplan & Lipmann, 1948) it seemed likely that the synthesis of CoA was the process responsible for the disappearance of pantothenate. Washed suspensions of *Lactobacillus arabinosus* 17–5 were found to take up pantothenate. The fate of pantothenate was studied in this organism because it requires pantothenate for growth and, unlike streptococci (McIlwain & Hughes, 1945) and *Escherichia coli* (Maas, 1952), it does not decompose pantothenate.

Pantothenate was added to washed suspensions and its disappearance was measured by microbiological assay. A major fraction of this pantothenate was converted into coenzyme A, which was determined by enzymic assay.

The part of this work in which cystine was shown to be essential for coenzyme A synthesis was communicated to the 2nd International Congress of Biochemistry (Pierpoint & Hughes, 1952).

EXPERIMENTAL

Materials. The acetylating enzymes required for the estimation of coenzyme A according to Handschumacher, Mueller & Strong (1950) and Lipmann, Kaplan, Novelli, Tuttle & Guirard (1947) were obtained from the livers of decapitated pigeons. The 4-aminoazobenzene was a chromatographically pure specimen prepared and given by Dr J. A. Miller (University of Wisconsin). Adenosine triphosphate (ATP) was prepared by the method of LePage (1949): the chromatographic procedure, described by Eggleston & Hems (1952), showed that 90% of the phosphate of the sample was present in the form of ATP.

Transacetylase was obtained from a strain of *Esch. coli* (National Collection of Type Cultures, strain 86) grown in 10 l. batches of medium containing 0·5% (w/v) Pronutrin (a commercial casein digest marketed by Herts Pharmaceuticals Ltd., Welwyn), 1% (w/v) glucose, 0·4% (v/v) boiled yeast extract, 0·4% (w/v) KH_2PO_4, 0·25% (v/v) salts mixture B of Barton-Wright (1946), 10% (v/v) tap water, pH 7·0. The cells were dried over P_2O_5 *in vacuo* and the enzyme extracted according to Novelli & Lipmann (1950). Acetyl phosphate was prepared by the method of Lipmann & Tuttle (1944), and found to be 87% pure by phosphate analysis (Berenblum & Chain, 1938) and by using succinic anhydride as a standard in the hydroxamic acid reaction (Lipmann & Tuttle, 1945).

The samples of CoA used as standards were various preparations from dog liver (Kaplan & Lipmann, 1948). Others were obtained commercially from Armour Laboratories, and had an activity of 10 Lipmann units/mg. Casein hydrolysate was prepared according to Barton-Wright (1946). Pantetheine was prepared and given by Drs Baddiley & Thain (1952).

Organisms. Lb. arabinosus 17–5 was grown in the semi-synthetic medium described by Barton-Wright (1946) for the assay of pantothenate. Satisfactory yields of CoA-deficient organisms (20 mg. dry wt./100 ml.) were obtained after 16–18 hr. growth at an initial pantothenate concentration of 1×10^{-7} M. Up to 5 times higher yields were obtained by increasing the pantothenate concentration to 1×10^{-2} M, but the cells contained more CoA and synthesized less on further incubation (Table 1). The cells were harvested by centrifugation, washed in 0·9% (w/v) NaCl and suspended in a solution containing 0·05 M glucose and 0·05 M phosphate buffer pH 6·5, in the saline of Krebs & Henseleit (1932).

Estimations of coenzyme A. To extract the intracellular CoA, the centrifuged cells were suspended in 0·5 ml. of water in stoppered tubes, and placed in a boiling-water bath for 10 min.

The assay routinely used was that of Handschumacher *et al.* (1950), which is based on the CoA-dependent acetylation of 4-aminoazobenzene. In addition the method of Kaplan & Lipmann (1948), in which sulphanilamide acts as acetyl acceptor, was used. This served to check the results obtained by the other method and also to calibrate freshly prepared samples of CoA in terms of the generally accepted Lipmann units.

Pantetheine has CoA activity when unfractionated pigeon-liver acetone powder is used as acetylating enzyme, owing to the presence of CoA-synthesizing enzymes (Govier & Gibbons, 1952). In order to check the results obtained when pantetheine was present in the incubation mixtures, estimations were made by the arsenolysis method of Stadtman, Novelli & Lipmann (1951) which is unaffected by pantetheine.

As shown in Table 2, known amounts of CoA added to cell suspensions were satisfactorily recovered by the method used. Addition of 0·1% (w/v) Na_2S, $9H_2O$ to the suspension during the extraction did not affect the recovery.

Estimations of pantothenate. Pantothenate was estimated essentially as described by Barton-Wright (1946), by measuring turbidimetrically the growth response of *Lb. arabinosus* 17–5. Combined pantothenate was estimated by assaying before and after treatment with a commercial sample of intestinal phosphatase and chicken-liver enzyme prepared and used in the manner described by Novelli & Schmetz (1951).

Conditions of incubation. Samples (1 ml.) of the cell suspensions were put into a series of 10 ml. centrifuge tubes, together with solutions of the substances to be tested, which had been adjusted to pH 6·5. The total volumes were made up with distilled water to convenient values, generally 2 ml., and the tubes incubated for periods up to 60 min. Unless otherwise stated all incubations were at 37°. Manometric experiments, in which glycolysis was followed by liberation of CO_2 from bicarbonate, showed that under these conditions lactic acid production continued linearly for approx. 90 min. There was no difference in the rate of glycolysis of pantothenate-deficient cells when incubated with and without pantothenate and cystine. Metabolic activities were stopped by cooling the tubes in freezing mixture. The cells were collected by centrifugation, the mother liquors decanted off and the tubes wiped dry with filter paper. When pantetheine was present in the incubated mixture, the cells were washed with fresh saline containing glucose and phosphate to remove surplus pantetheine. Glucose saline was used, as cells tend to lose coenzyme A when incubated in absence of glucose.

Table 1. *The effect of the concentration of pantothenate in the growth medium on the synthesis of coenzyme A by washed suspensions*

(Cells were collected after 16–17 hr. growth, and incubated under the standard conditions described in the text.

Concentration of pantothenate in the growth medium (M)	Yield of cells (mg. dry wt./100 ml.)	CoA content of cells (Lipmann units/g. dry wt.) Before incubation	After incubation
2×10^{-7}	29	30	750
10^{-4}	100	350	640
5×10^{-5}	105	700	800
10^{-2}	90	600	630

Table 2. *Recovery of coenzyme A*

(3·0 Lipmann units were added to suspensions of 4 mg. of cells of *Lactobacillus arabinosus*, heated and then assayed as described in the text.)

Heat treatment	% Recovery (Average of duplicate tubes) Expt. 1	Expt. 2
80° for 15 min.	100	107
100° for 5 min.	93	104
100° for 10 min.	106	105
100° for 15 min.	105	102

RESULTS

The effect of constituents of the growth medium upon coenzyme A synthesis by washed suspensions. Cells grown in the presence of 1×10^{-7} M pantothenate contained from 15 to 100 Lipmann units of CoA/g. dry weight compared with 600–900 units/g. dry weight in cells grown in an excess of pantothenate (10^{-3} M). Washed suspensions of the deficient cells synthesized an extra 50–100 units CoA/g. dry weight when incubated in the glucose-saline mixture with added pantothenate. This meant that the cells contained about 200 units CoA/g. dry weight, which is considerably less than the amount found in cells grown with adequate pantothenate, and it suggested that some other constituent of the growth medium was essential for maximum CoA synthesis. As casein hydrolysate increases the uptake of pantothenate by haemolytic streptococci (McIlwain & Hughes, 1945), its effect on CoA synthesis was tested. It increased both the rate of synthesis and the amount of CoA synthesized (Table 3). When synthetic mixtures of amino acids and single amino acids were tested for their ability to replace the casein hydrolysate, only cystine and cysteine were found to be

effective (Table 3). There was no additional increase in the amount of CoA synthesized when mixtures of amino acids or casein hydrolysate were incubated together with cystine. The effect of casein hydrolysate on the uptake of pantothenate found by McIlwain & Hughes was probably due to cystine.

The washed suspensions did not synthesize CoA when glucose was absent, indicating that CoA synthesis, like cozymase synthesis (Hughes & Williamson, 1952), depends on an energy supply. No other energy source was tested.

The omission of Mg^{2+} considerably decreased the amount of CoA synthesized (Table 4). This recalls a similar effect on the uptake of pantothenate (McIlwain & Hughes, 1945) and could be explained on the basis of the recent findings that at least one of the phosphorylating steps in CoA synthesis in liver depends on the presence of Mg^{2+} (Levintow & Novelli, 1952). Glutamic acid, which has been reported to stimulate the growth-promoting properties of pantothenate (King, Fels & Cheldelin, 1949) and to reverse the inhibitory effects of phenylpantothenone (Woolley, 1946) had no effect on CoA synthesis. Similarly, uracil, guanine, and adenine were without effect.

The effect of pantothenate and cystine concentration. The amount of CoA synthesized in glucose-saline containing 2×10^{-4}M cystine increased when the concentration of pantothenate was raised from 1×10^{-6} to 2×10^{-5}M (Fig. 1); there was no further increase in concentrations up to 1×10^{-3}M. The amount varied in a similar fashion when the panthothenate concentration was kept constant and the cystine concentration was varied from 1×10^{-6} to 5×10^{-5}M (Fig. 1). The amount formed was maximal when the concentrations of both cystine and pantothenate were greater than 2×10^{-5}M. For testing the effect of other conditions on CoA synthesis, the concentration of pantothenate and of cystine was generally 2×10^{-4}M. Under these conditions CoA synthesis continued linearly for 60 min. (Fig. 2), and ceased when the cells contained approximately 600–800 Lipmann units.

Table 3. *The effect of amino acids on coenzyme A synthesis*

(Each tube contained: 8·5 mg. dry wt. cells suspended in phosphate-saline containing glucose; $2{\cdot}5 \times 10^{-4}$M pantothenate; the amino acids indicated below in a final volume of 2·0 ml.)

Amino acid source added (final concentration)	CoA synthesized (Lipmann units/g. dry wt.)
None	109
5% (v/v) Casein hydrolysate	360
10^{-4}M Cystine	370
10^{-4}M Cystine; 5% (v/v) casein hydrolysate	350

Table 4. *The effect of* Mg *on synthesis*

(Each tube contained: 10 mg. cells suspended in the phosphate-saline containing glucose without Mg; $2{\cdot}5 \times 10^{-5}$M pantothenate; $1{\cdot}5 \times 10^{-4}$M cystine, and the additions listed below, in a final volume of 2·0 ml.)

Additions to mixture	CoA content of cells (Lipmann units/g. dry wt.) Before incubation	After incubation
None	50	265
$7{\cdot}7 \times 10^{-4}$M-$MgSO_4$	50	470

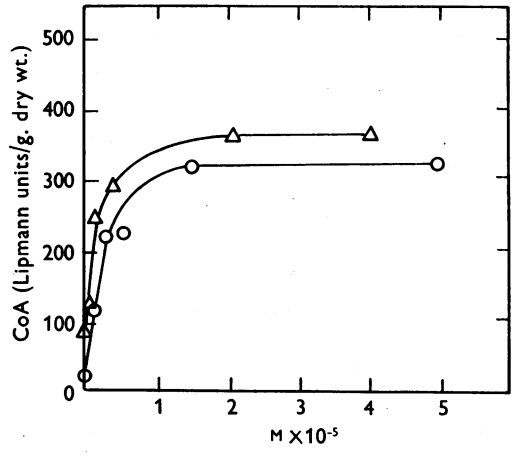

Fig. 1. Effect of pantothenate and cystine concentration on coenzyme A synthesis. Tubes contained 2 ml. of phosphate-saline containing glucose together with the following additions: O—O, 8·4 mg. of cells (batch A) in 2×10^{-4}M pantothenate and varying amounts of cystine; △—△, 13 mg. of cells (batch B) in 2×10^{-4}M cystine and varying amounts of pantothenate.

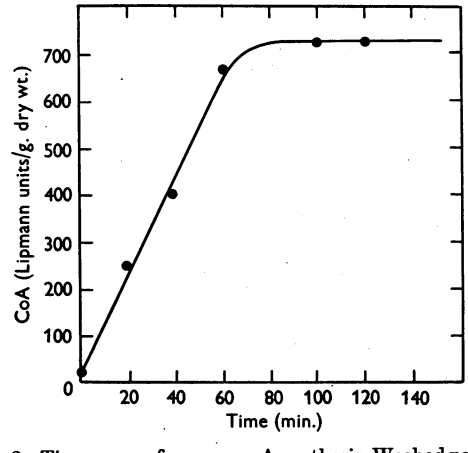

Fig. 2. Time curve of coenzyme A synthesis. Washed pantothenate-deficient cells (9·7 mg. dry wt.) were suspended in 2 ml. of the phosphate-saline containing glucose and 2×10^{-4}M cystine and 2×10^{-4}M pantothenate.

Table 5. *The effect of the age of cells on their ability to synthesize coenzyme A*

(For experimental details see text.)

Expt.	Age of cells (hr.)	Stage of growth	CoA content of cells (Lipmann units/g. dry wt.) Before incubation	After incubation	CoA synthesized
1	10	Beginning of log phase	190	300	110
	16	Log phase	240	440	200
	20	Stationary phase	260	530	270
	32	Stationary phase	0	300	300
2	9	Beginning of log phase	130	280	150
	12	Log phase	160	780	600
	18	End of log phase	135	685	550
3	17	End of log phase	230	480	250
	41	Stationary phase	42	272	230

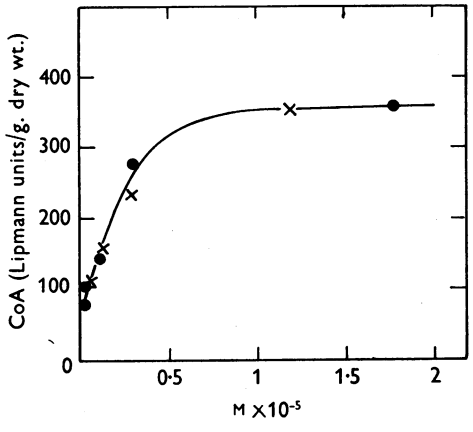

Fig. 3. Synthesis of coenzyme A from pantetheine. Deficient cells (8·0 mg. dry wt.) were suspended in the phosphate-saline containing glucose (final volume 1·8 ml.) with various amounts of cystine (●) or pantetheine (×). Those tubes containing cystine also contained 2×10^{-5} M pantothenate.

Table 6. *Comparison of coenzyme A estimations by arsenolysis and acetylation tests*

(Deficient cells (10 mg. dry wt.) were incubated in the phosphate-saline mixture with 10^{-4} M pantetheine, the cells collected, washed in fresh saline, and the CoA extracted and estimated.)

Method of estimation	CoA (Lipmann units/g. dry wt.) Before incubation	After incubation	Synthesized
Acetylation	100	600	500
Arsenolysis	400	950	550

The effect of age of cells upon coenzyme A synthesis. To test the effect of the age of cells on CoA synthesis, samples from large batches (1000 ml.) of the pantothenate-deficient culture were examined at intervals for their ability to synthesize CoA, as already described. The CoA content of the cells was approximately constant during the log phase of growth and decreased during the stationary phase, until almost all of the CoA had been lost (Table 5). The ability of the cells to synthesize was lowest at the beginning of the log phase, increased continuously during the log phase, and remained constant during the stationary phase (Table 5). This is in contrast to cozymase synthesis by this organism, which is greatest in the younger cells and decreases rapidly once the cells have ceased growing (Hughes & Williamson, 1952).

The effect of compounds related to cystine and pantothenate on coenzyme A synthesis. Cysteine and cystine were equally effective in increasing the rate and amount of coenzyme A synthesis. The amount of CoA synthesized by addition of an equivalent amount of SH-glutathione was only about 15% of that formed from cystine. It is uncertain whether this small effect of glutathione is due to contamination with cystine, or to slow hydrolysis. No effect was found when cystine was replaced by β-mercaptoethylamine, *N*-β-alanyl-β-mercaptoethylamine, taurine, cysteic acid, homocystine, methionine, *S*-methylcystine, thioglycollic acid, thioacetamide or ethanethiol.

Pantetheine completely replaced both pantothenate and cystine (Fig. 3), indicating that the role of cystine is to participate in the formation of the β-mercaptoethylamine residue of CoA. This result was confirmed by using the arsenolysis method of estimating CoA, which is not affected by pantetheine. A comparison of the two methods of estimation showed that although they gave similar values for the amount of CoA synthesized by a batch of organisms, the values obtained by the arsenolysis method for the total CoA content of the organisms were larger than those from the acetylation method (Table 6). This may be due to some material extracted from the cells which catalyses the non-enzymic decomposition of acetyl phosphate.

Effect of cystine on pantothenate uptake. When washed suspensions of deficient cells were incubated with amounts of pantothenate and cystine which did not limit synthesis, the ratios of pantothenate taken

Table 7. *The effect of cystine on the uptake of pantothenate*

(Washed cells were incubated in glucose-saline with pantothenate and cystine, as indicated below. After the incubation the cells were collected by centrifugation and washed in 0·9% saline. The CoA content of the cells, and the pantothenate content of the mother liquor were estimated.)

Expt.	Dry weight of cells/tube (mg.)	Cystine added (M)	Pantothenate Added (μg.)	Pantothenate Recovered (μg.)	Pantothenate Uptake (μg.)	CoA synthesized (Lipmann units/tube)	Pantothenate uptake (μg.) / CoA formed (Lipmann units)
1	13·8	$1·5 \times 10^{-3}$	2·85	1·40	1·45	2·0	0·73
		None	2·85	1·46	1·39	0·25	—
2	12	5×10^{-5}	2·93	1·22	1·7	3·3	0·55
		None	2·93	1·54	1·38	0·62	—
3	9·8	5×10^{-5}	6·8	3·5	3·5	4·3	0·82
		None	6·8	3·7	3·1	1·82	—

Table 8. *Pantothenate uptake in the absence of cystine*

(Deficient cells (15 mg. dry wt.) were incubated for 80 min. at 37° in 2 ml. of the phosphate-saline mixture, containing glucose and the additions described below. After incubation the cells were removed by centrifugation, washed twice in 5 ml. saline, and the pantothenate was estimated in the mother liquor and cell extracts.)

Additions	Pantothenate taken up by the cells (μm-moles)	CoA synthesized (μm-moles)	Pantothenate in extract (μm-moles): Before incubation with phosphatase	After incubation with phosphatase	After incubation with phosphatase + chicken liver
None	—	—	—	0·5	4·0
Pantothenate ($2·5 \times 10^{-5}$ M)	30	—	1·0	15	25
Pantothenate ($2·5 \times 10^{-5}$ M); cystine (5×10^{-5} M)	27	19·2	—	4	24

up/CoA formed (Table 7) agreed well with values of 0·65–0·71 μg. pantothenate/Lipmann unit reported for the pantothenate content of CoA (Lipmann *et al.* 1947; Beinert *et al.* 1953). Hence most, if not all, of the pantothenate taken up under these conditions is converted into CoA.

The amount of pantothenate which was taken up from the incubation medium was independent of the presence of cystine (Table 7). Thus, although cystine is essential for the conversion of pantothenate into pantetheine, and therefore to the formation of CoA, pantothenate may be bound in its absence. In an attempt to study the properties of the bound form, cells were grown in limiting amounts of cystine (10^{-5} M) and an excess of pantothenate (1×10^{-2}–1×10^{-3} M). However, the cells contained large amounts of free pantothenate, which were slowly released on washing with saline. Even after seven washings with 0·9% saline, pantothenate was still present in the supernatant, in quantities which were large compared to those of the bound form. This made a study of the bound form impossible.

More suitable material was obtained when growing cells were replaced by washed suspensions of pantothenate-deficient cells. These were incubated with glucose and pantothenate, in the absence of cystine, until 60–80% of the added pantothenate (50 μm-moles) had been taken up (60–80 min.). The cells were then separated and washed three times with saline, no pantothenate being detectable in the final washing. The cells were suspended in water and heated as described for the extraction of CoA. The extract contained no free pantothenate. When incubated with alkaline phosphatase (Novelli & Schmetz, 1951), 60–75% of the pantothenate that had been taken up by the cells was released (Table 8). If the incubation was carried out with phosphatase and chicken-liver enzyme 90–100% could be recovered.

When this was repeated with cells that had been incubated with glucose, pantothenate and cystine, only 10–20% of the growth-supporting activity of the bound pantothenate could be detected after incubation with phosphatase. This is probably due to the formation of pantetheine, which has about 10–15% of the ability of pantothenate to support the growth of *Lb. arabinosus* under the conditions used (McRorie & Williams, 1951), and which is formed from CoA on incubation with phosphatase (Brown & Snell, 1952). Incubation with phosphatase plus chicken-liver enzyme gave complete recovery (90–100%).

DISCUSSION

The route of coenzyme A synthesis in animal tissues is taken to be (see Levintow & Novelli, 1952):

(1) Pantothenate → pantetheine.

(2) Pantetheine + ATP → pantetheine 4-phosphate + ADP.

(3) Pantetheine 4-phosphate + ATP → pantetheine-adenosine nucleotide + pyrophosphate.

(4) Pantetheine-adenosine nucleotide + ATP → CoA + ADP.

As for the synthesis of CoA by bacteria, Craig & Snell (1951) suggest that (1) occurs at least in those bacteria in which pantetheine has greater growth-promoting activities than pantothenate. Even in organisms like *Lb. arabinosus*, which grow better on pantothenate than on pantetheine, this reaction may be a step in the synthesis of CoA. This is suggested by the fact that washed suspensions of the organisms utilize pantetheine as effectively as pantothenate plus cystine.

That β-mercaptoethylamine does not replace cystine, either for the growth of *Lb. arabinosus* or for the synthesis of CoA by washed suspensions, argues against the view that β-mercaptoethylamine is an intermediate in CoA synthesis. Experiments to be published in collaboration with Drs Baddiley and Mathias (Lister Institute) show that *N*-pantothenyl cysteine does not support either growth or the synthesis of CoA in *Lb. arabinosus*, or the growth of *Lb. helveticus* and *Proteus morganii*. However, since these papers have been prepared Brown & Snell (1953) report similar findings, but have shown that *N*-pantothenylcysteine is active as a growth factor for *Acetobacter suboxydans* and that it is probably a precursor of pantetheine in this organism.

The synthesis of CoA may not be the only reaction of pantothenate under the test conditions, as pantothenate disappears when cells are incubated in absence of cystine. The evidence so far available (release of pantothenate on addition of intestinal phosphatase) suggests that pantothenate undergoes phosphorylation in the absence of cystine. Whether this reaction occurs in the presence of cystine is uncertain.

SUMMARY

1. Glycolysing, washed suspensions of *Lactobacillus arabinosus* 17–5 grown on a medium deficient in pantothenate can synthesize 50–100 Lipmann units of coenzyme A/g. dry wt. from pantothenate. The addition of cystine increases the amounts of coenzyme A which can be synthesized to 600–800 Lipmann units/g. dry wt. The amounts of coenzyme A synthesized are related to the initial coenzyme A content of the cells; the most deficient cells synthesize most coenzyme A. Glucose and magnesium ions are essential for the synthesis.

2. Panthetheine can replace pantothenate and cystine. Cystine is presumably required for the formation of the β-mercaptoethylamine residue of panthetheine. It can be replaced by cysteine and partially by glutathione, but not by β-mercaptoethylamine, taurine, cysteic acid, homocystine, methionine, *S*-methylcystine, thioglycollic acid, thioacetamide and ethanethiol.

3. The ability of the cells to synthesize coenzyme A is smallest in cells harvested at the beginning of the log phase of growth, increases continuously during the log phase and remains constant for at least 42 hr. after the cells have ceased to grow.

4. Pantothenate is removed by the cells in the absence of cystine, and reappears on treatment with alkaline phosphatase. This suggests that pantothenate is phosphorylated in the absence of cystine.

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