# The Phosphorylation of Pantothenic Acid by *Lactobacillus arabinosus* 17–5

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It was shown previously (Pierpoint & Hughes, 1952, 1954) that during glycolysis, washed suspensions of *Lactobacillus arabinosus* 17–5 synthesize coenzyme A (CoA) from pantothenate and cystine, and that in the absence of cystine no CoA is synthesized, although the pantothenate is taken up and retained by the cells. The bound form of pantothenate can be extracted from the cells but is available as a growth-factor only after digestion with intestinal phosphatase. It was suggested therefore that, in the absence of cystine, pantothenate is phosphorylated. The present paper is concerned with further studies on the phosphorylation reaction and identification of the product as pantothenic acid 4′-phosphate. The formation of the 4′-phosphate is of especial interest because, although it forms a part of the CoA molecule (Baddiley & Thain, 1951*a*), it cannot be used by bacteria (King & Strong, 1951) or by animal tissues (King & Strong, 1950) for the synthesis of CoA. The significance of the phosphorylation is discussed in the light of recently suggested routes of CoA synthesis (Hughes, 1953; Novelli, 1953; Brown & Snell, 1953).

## EXPERIMENTAL

*Sources of phosphorylating enzyme.* Dried preparations of *Lb. arabinosus* were prepared from cells that had been harvested after 16–18 hr. growth on the Nossal's (1951) medium and washed in 0·9% saline. They were thinly spread out on Petri dishes and dried over $P_2O_5$ in a desiccator continuously exhausted at 0·3 mm. Hg. When stored in the refrigerator, these preparations retained their phosphorylating activity for at least 3 months, though all activity was lost after 14 months at −14°.

Cell-free extracts were prepared by crushing washed cells, without abrasive, in the press described by Hughes (1951). If such extracts were not to be used immediately they were frozen and stored at −15°.

*Measurement of enzyme activity.* The ability of the various enzyme preparations to phosphorylate pantothenate was measured by following the disappearance of pantothenate from incubation mixtures as judged by microbiological assay with *Lb. arabinosus*. This was checked at frequent intervals by measuring the recovery of pantothenate after digestion with intestinal phosphatase. The method is based on the previous findings that phosphorylated pantothenates are inactive as growth factors for *Lb. arabinosus* but are hydrolysed to free pantothenate by the phosphatase (Pierpoint & Hughes, 1954). The standard conditions generally used were as follows. Samples of the material to be tested (0·1–0·5 ml.) were pipetted in 6 × ½ in. test tubes containing pantothenate ($4 \times 10^{-4}$ M), ATP (adenosine triphosphate) ($4 \times 10^{-4}$ M), 0·1 M-Na–K phosphate buffer (pH 7·0), 0·1 ml. salts *B* (Barton-Wright, 1952) and 0·2 ml. NaF (0·2 M) in a total vol. of 2·0 ml. After mixing, the solutions were incubated at 37° and samples (0·2 ml.) taken at intervals depending upon the activity expected. The samples, in 10 ml. corked centrifuge tubes, were placed in a boiling-water bath for 10 min., diluted with 4 ml. water and centrifuged to remove protein. The pantothenate was estimated in the supernatant by microbiological assay (Pierpoint & Hughes, 1954).

*Source and use of other enzymes.* The intestinal phosphatase was a sample from Armour and Co. Ltd.; 0·1 ml. of a solution (10 mg./ml.) was added to 0·2 ml. of 0·1 M aminotrishydroxymethylmethane (tris) buffer (pH 8·0) and the sample of pantothenic acid phosphates containing between 0·4 and 1·2 μg. of bound pantothenic acid in 0·2 ml. water. After incubating for 3 hr. at 37°, the solution was diluted with 4 ml. of water, centrifuged if necessary, and the free pantothenic acid estimated in appropriate samples. The presence of 0·5 M fluoride in some of the samples of enzymically prepared pantothenate phosphate did not affect the action of the phosphatase.

*Nucleotide phosphate estimation.* ATP and ADP (adenosine diphosphate) were estimated by the method described by Krebs & Hems (1953). The adenosine derivatives were first separated from each other and from the other phosphate-containing compounds by two-way chromatography in *iso*propyl ether–formic acid, and *iso*butyric acid–ammonia–ethylenediaminetetraacetic acid (EDTA). The nucleotide-containing spots were detected by u.v. light and the phosphate spray of Hanes & Isherwood (1949). They were cut out, wet-ashed and analysed for phosphorus. If the solutions for estimation contained protein they were first heated on a boiling-water bath for 30 sec. The precipitated protein was removed by centrifuging and the estimation performed on the supernatants. This procedure did not affect the recoveries of ATP.

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*Chromatography of pantothenic acid phosphates.* This was performed on Whatman no. 1 filter paper which had been washed in EDTA (Eggleston & Hems, 1952). A 25 $\mu$l. sample containing total P at a concentration of about $10^{-3}$ M was put on paper in 5 $\mu$l. spots from an Agla micrometer syringe (Burroughs Wellcome and Co.) and dried by a current of cold air from a hair drier. The chromatograms were irrigated in a descending stream of either the *iso*butyric acid–ammonia–EDTA solvent of Krebs & Hems (1953) or the *n*-propanol–ammonia–water solvent of Hanes & Isherwood (1949). After 16–18 hr. the papers were removed and dried in a hot oven. The position of the phosphates was located by the acid molybdate spray of Hanes & Isherwood (1949).

*Chemicals.* The pantothenic acid phosphates used in this work had been prepared as described by Baddiley & Thain (1951*a*, *c*). With the exception of pantothenic acid 4′-phosphate, they were chromatographically homogeneous in both the solvents used. The 4′-phosphate contained a small amount (approx. 5%) of a faster moving phosphate-containing spot which was also observed by Baddiley & Thain (1951*c*). The ATP used was prepared by the method of LePage (1949). All the other chemicals used were commercial samples. Solution *B* (Barton-Wright, 1952) was used as a convenient source of inorganic ions.

## RESULTS

### *Phosphorylation of pantothenic acid by cells of* Lb. arabinosus 17–5

Vacuum-dried preparations of cells which had been grown in a pantothenic acid-deficient medium caused a disappearance of pantothenic acid when incubated in the presence of ATP, phosphate buffer (pH 7·1) and inorganic ions; no disappearance occurred when ATP was omitted from the mixture (Fig. 3). The pantothenic acid could be completely recovered by incubating the reaction mixture with intestinal phosphatase (Fig. 1). The same effect occurred with vacuum-dried preparations of cells grown on pantothenic acid-rich media. In this case, however, incubation with phosphatase resulted in recoveries of pantothenic acid in excess of that added (Fig. 2), indicating the initial presence of pantothenic acid phosphate derivatives in such cells.

Cell-free extracts of *Lb. arabinosus* prepared in a bacterial press (Hughes, 1951) also catalyse the phosphorylation. The reaction was further studied in dried cells, which could be more conveniently prepared. The enzyme system could not be extracted from the dried cells by leaving them for periods up to 3 days with water at 5°.

*Effect of fluoride on the phosphorylation of pantothenic acid.* Sodium fluoride (0·02 M) caused a 40% increase in the amount of pantothenic acid phosphorylated (Fig. 2). This is probably due to the inhibitory effect of this ion on the ATPases present in dried cells. Evidence in support of this was obtained by incubating cells with ATP, inorganic

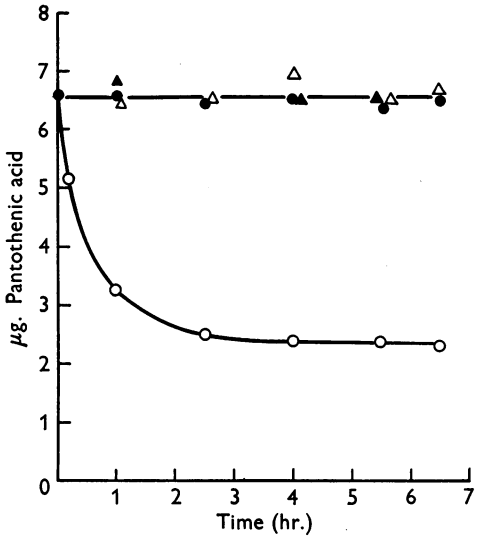



Fig. 1. The phosphorylation of pantothenic acid by dried pantothenate-deficient cells of *Lb. arabinosus*. Each tube contained 40 mg. dried cells, 0·02 ml. solution *B*, 0·5 ml. phosphate buffer (0·5 M, pH 7·1), pantothenic acid (7·3 $\mu$g.) and either water alone (●, ▲) or 1·0 ml. ATP (0·02 M), (○, △). Solutions were incubated at 37° and pantothenate was estimated before (●, ○) and after (▲, △) digestion with phosphatase.

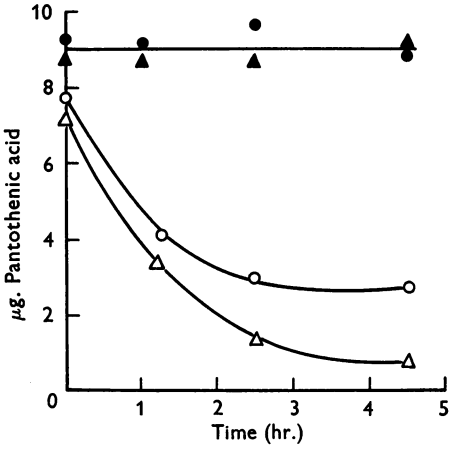



Fig. 2. Effect of fluoride on the phosphorylation of pantothenic acid by dried preparations of *Lb. arabinosus*. Cells were grown on excess of pantothenate. Dried cells (40 mg.) were incubated with 1·0 ml. ATP (0·05 M), pantothenic acid (7·3 $\mu$g.), 0·02 ml. solution *B*, 0·5 ml. of phosphate buffer (0·5 M, pH 7·0) and either water to 3·35 ml. (○, ●) or 0·35 ml. NaF (0·2 M) and water to 3·35 ml. (△, ▲). Pantothenate was estimated before (○, △) and after (●, ▲) digestion with phosphatase.

ions and phosphate buffer, in either the presence or absence of fluoride, and adding pantothenic acid 4·5 hr. later. Phosphorylation occurred only in those solutions that contained fluoride. The dried cells did not catalyse the phosphorylation when both ATP and pantothenic acid were added after the incubation, suggesting that ATP, but not the fluoride ion, has a 'protective' action on the enzyme involved.

Table 1. *Effect of pantothenic acid concentration on the amount of phosphorylation*

Tubes containing 20 mg. dried cells, 0·5 ml. ATP (0·02 M), 0·1 ml. solution *B*, 0·2 ml. NaF (0·2 M), 0·25 ml. phosphate buffer (0·5 M, pH 7·1), pantothenic acid and water to 1·7 ml. were incubated for 6 hr. and pantothenate was assayed as described.

| Concn. of pantothenic acid ($\mu$M) | Amount phosphorylated ($\mu$g.) | Amount phosphorylated (%) |
|---|---|---|
| 18 | 6·1 | 83 |
| 88 | 32·3 | 98 |
| 440 | 47·0 | 28 |
| 880 | 74·0 | 22 |

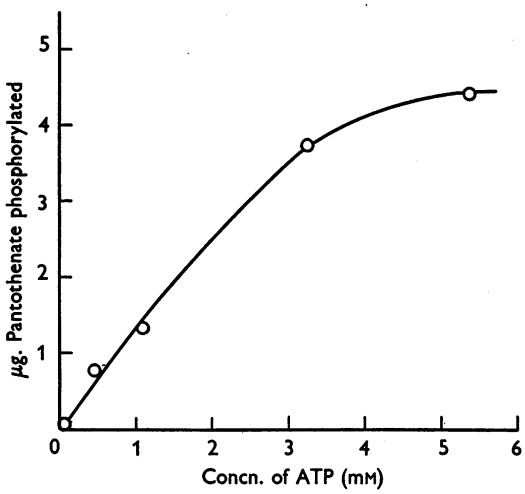



Fig. 3. Variation of phosphorylation of pantothenic acid with ATP concentration. Dried cells (40 mg.) were incubated for 6·5 hr. at 37° with pantothenate and various amounts of ATP and the remaining free pantothenate was assayed.

*Effect of concentrations of ATP and pantothenic acid on the phosphorylation.* Increasing the concentration of pantothenic acid from $1·8 \times 10^{-5}$ M to $8·8 \times 10^{-4}$ M increased the amount of phosphorylation that occurred in the presence of NaF (Table 1). The concentration of ATP used in early experiments was approximately optimal for the conditions under which it was used (Fig. 3). In most subsequent experiments on phosphorylation, the concentrations of pantothenic acid and ATP used were $4 \times 10^{-4}$ M. Under these conditions the ATP did not limit the reaction, and the large proportion of added pantothenic acid that was phosphorylated allowed its disappearance to be measured accurately.

Omitting inorganic Mg, Mn and Fe ions from the system reduced the amount of phosphorylation by 20%. Addition of cysteine or cystine in quantities up to 10 times the equivalent amount of pantothenic acid did not affect the amount of phosphorylation or the recovery of pantothenic acid after phosphatase digestion. This is in contrast to the reaction in intact deficient cells, where the addition of cysteine reduces the amount of pantothenate recovered by digestion with phosphatase because of the synthesis of CoA (Pierpoint & Hughes, 1954).

*Product of the phosphorylation reaction*

The enzymic phosphorylation of pantothenic acid may result in the formation of one of five possible compounds. These are pantothenic acid 2′-phosphate, pantothenic acid 4′-phosphate, pantothenic acid 2′:4′-cyclic phosphate, pantothenic acid 2′:4′-diphosphate and the acyl phosphate pantothenoyl phosphate (Fig. 4). The following comparison of the properties of the enzymically produced material with samples of the synthetically prepared monophosphates of pantothenic acid supports the conclusion that the 4′-phosphate is formed by the enzyme.

Pantothenic acid

$HO.CH_2.CMe_2.CH(OH).CO.NH.CH_2.CH_2.CO_2H$

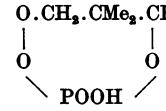
Pantothenic acid 2′-phosphate

$HO.CH_2.CMe_2.CH(O.PO_3H_2).CO.NH.CH_2.CH_2.CO_2H$

Pantothenic acid 4′-phosphate

$H_2O_3P.O.CH_2.CMe_2.CH(OH).CO.NH.CH_2.CH_2.CO_2H$

Pantothenic acid 2′:4-cyclic phosphate




Pantothenic acid 2′:4′-diphosphate

$H_2O_3P.O.CH_2.CMe_2.CH(O.PO_3H_2).CO.NH.CH_2.CH_2.CO_2H$

Pantothenoyl phosphate

$HO.CH_2.CMe_2.CH(OH).CO.NH.CH_2.CH_2.CO.OPO_3H_2$

Fig. 4. Phosphates of pantothenic acid.

(1) *Action of phosphatase on pantothenic acid phosphates.* King & Strong (1951) and Baddiley & Thain (1951*a*) report that the phosphates listed above, with the exception of the 2′:4′-cyclic compound, are hydrolysed by intestinal phosphatase. Table 2 shows the effect of intestinal phosphatase on synthetic phosphates under conditions in which 90–100 % of the enzymically produced phosphate is converted into pantothenic acid. Although none of the compounds was completely hydrolysed, D(+)-pantothenic acid 4′-phosphate was hydrolysed to a major extent (60–75 %). Increasing the time of incubation did not increase the degree of hydrolysis, but apparently decreased it (Table 3) owing to the instability of pantothenic acid at pH 8·3. The low results obtained with the racemic compounds are due to the fact that the pantothenic acid assay is specific for the D(+)-form.

(2) *Stability of pantothenic acid phosphates.* The phosphates of pantothenic acid are more stable than pantothenic acid in acid or alkaline solution at 100° (Baddiley & Thain, 1951*b*), and measurement of the rate of hydrolysis thus affords a method of distinguishing between 2′- and 4′-phosphates. In alkaline solution (0·5N-NaOH) the phosphate groups of both compounds are stable, while the amide linkage in the 2′-phosphate is more labile than that of the 4′-derivative. Fig. 5 shows the results of comparing the stability of the enzymically produced phosphate with a sample of synthetic pantothenic acid 4′-phosphate. The behaviour of the two compounds is very similar, and the stability towards alkali differentiates both from the 2′-phosphate.

Lack of any strictly comparable data on the stability of the cyclic 2′:4′-monophosphate and the 2′:4′-diphosphate permits no further conclusions to be drawn. In alkaline solutions, at least, the latter

Table 2. *Effect of intestinal phosphatase on pantothenic acid phosphates*

The various phosphates in 0·2 ml. tris buffer (pH 8·3) were incubated for 3 hr. at 37° with intestinal phosphatase solution, approx. 5 units (Schmidt & Thannhauser, 1943). Free pantothenate was assayed as described.

| Expt. | Substrate | Amount added (μg. bound pantothenic acid) | Pantothenic acid liberated (%) |
|---|---|---|---|
| 1 | D(+)-Pantothenic acid 4′-phosphate | 0·88 | 60 |
| | DL-Pantothenic acid 4′-phosphate | 0·88 | 30 |
| | Enzymically prepared phosphate | 0·39 | 92 |
| 2 | D(+)-Pantothenic acid 4′-phosphate | 0·44 | 60 |
| | DL-Pantothenic acid 4′-phosphate | 0·44 | 28 |
| | D(+)-Pantothenic acid 2′-phosphate | 0·44 | 11 |
| | DL-Pantothenic acid 2′-phosphate | 0·44 | 6·0 |
| | D(+)-Pantothenic acid 2′:4′-phosphate | 2·5 | 0·4 |
| 3 | D(+)-Pantothenic acid 4′-phosphate | 0·44 | 74 |
| | D(+)-Pantothenic acid 2′-phosphate | 0·44 | 18 |
| | D(+)-Pantothenic acid 2′:4′-phosphate | 1·25 | 0 |
| | D(+)-Pantothenic acid 2′:4′-phosphate | 0·62 | 0 |

Table 3. *Time course of phosphatase action on pantothenic acid 4′-phosphate*

Either D- or DL-pantothenic acid 4′-phosphate ($10^{-5}$M) was incubated with phosphatase and the free pantothenic acid liberated and assayed as described.

| Substrate | Pantothenic acid liberated (%) 1·5 hr. | 3 hr. | 6·5 hr. | 19·5 hr. |
|---|---|---|---|---|
| D-Pantothenic acid 4′-phosphate | 56 | 52 | 52 | 26 |
| DL-Pantothenic acid 4′-phosphate | 26 | 27 | 26 | 8·5 |

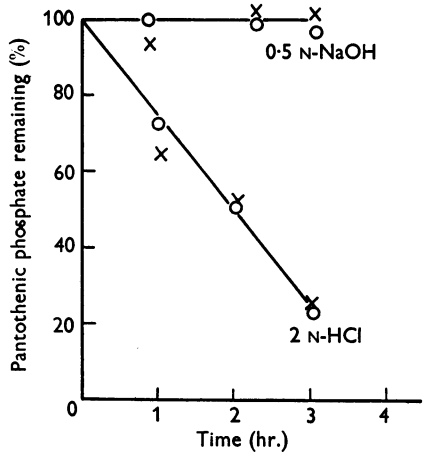



Fig. 5. Stability of pantothenic acid phosphates. 0·5 ml. 2·5N-NaOH or 0·5 ml. of 10N-HCl was added to 0·196 ml. of $10^{-5}$M synthetic DL-pantothenic acid 4′-phosphate (×), or 0·174 ml. enzymically produced (○) phosphate ($10^{-5}$M with respect to bound pantothenic acid). The volumes were adjusted to 0·25 ml. with water, and the tubes placed in a boiling-water bath for various times. The amounts remaining undecomposed were measured by assaying pantothenic acid released by phosphatase.

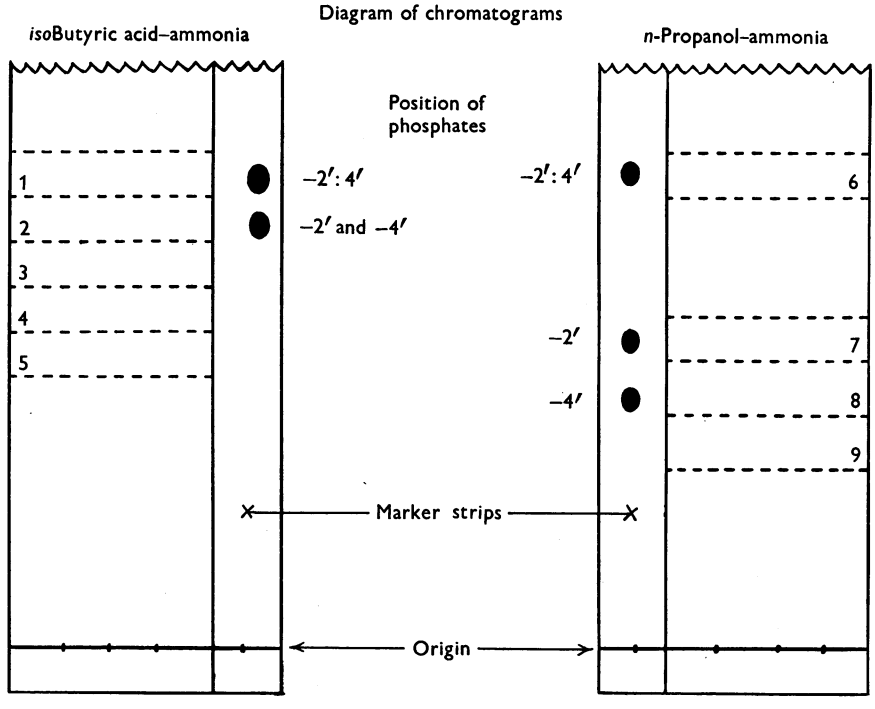
compound would be expected to behave like the 4′-phosphate, into which it is rapidly converted under these conditions (Baddiley & Thain, 1951*c*).

(3) *Chromatography of pantothenic acid phosphates.* The solution in which pantothenic acid was phosphorylated contained large amounts of other materials, which were removed as follows. The reaction mixture was heated on a boiling-water bath for 4 min. and the precipitated protein removed by centrifugation. Barium hydroxide (0·3N) was added and insoluble barium phosphates were removed by centrifuging. The solution was then heated at 100° for 5 min. to destroy the remaining pantothenic acid. The solution was adjusted to pH 6 with sulphuric acid; after barium sulphate had been removed by centrifuging, the

Table 4. *Chromatographic identification of the phosphorylated pantothenic acid*

Cells (100 mg. dry wt.) were incubated overnight with the following solutions: 4·5 ml. ATP (0·02M), 2·2 ml. phosphate buffer (0·5M), 1·2 ml. NaF (0·2M), 0·07 ml. solution *B*, 0·6 ml. pantothenic acid ($10^{-4}$M). The pantothenic acid phosphate was concentrated as described in the text and chromatographed for 16 hr. The positions of synthetic phosphates were detected on the chromatograms and the areas indicated in the diagram cut out and eluted with water. The eluates were assayed for pantothenic acid before and after digestion with alkaline phosphatase.

| Expt. | Final solvent | Area on chromatogram | Corresponding phosphate | Pantothenic acid liberated by phosphatase (μg.) | Proportion of total pantothenic acid recovered (%) |
|---|---|---|---|---|---|
| I | *iso*Butyric acid–ammonia | 1 | 2′:4′-cyclic | 0·03 | 12 |
| | | 2 | 2′- and 4′- | 0·26 | 88 |
| | | 3 | — | 0 | 0 |
| | | 4 | — | 0 | 0 |
| | | 5 | — | 0 | 0 |
| II | *n*-Propanol–ammonia | 6 | 2′:4′-cyclic | 0 | 0 |
| | | 7 | 2′- | 0 | 0 |
| | | 8 | 4′- | 0·12 | 97 |
| | | 9 | — | 0·004 | 3 |
| III | *n*-Propanol–ammonia | 6 | 2′:4′-cyclic | 0 | 0 |
| | | 7 | 2′- | 0·044 | 18 |
| | | 8 | 4′- | 0·196 | 80 |
| | | 9 | — | 0·004 | 2 |



solution was passed through a column of Amberlite IR-120 resin to remove cations, and was then freeze-dried. For chromatography, the solid was dissolved in a little water and neutralized with ammonia. Chromatography of the product showed that it contained a number of phosphates, some of which absorbed in the u.v. region (Baddiley & Mathias, 1954), and that the enzymically formed pantothenic acid phosphate was spread over a wide range (2–3 in.) of the chromatogram. Consequently, the material was further purified by chromatographic separation in *iso*butyric acid–ammonia–EDTA (Krebs & Hems, 1953), and elution of those areas of the chromatogram that contained the phosphate-bound pantothenic acid. When the eluted material was run again in the *iso*butyric acid–ammonia–EDTA solvent it behaved like either the 2′- or the 4′-phosphate. (These cannot be distinguished in this solvent.) In *n*-propanol–ammonia–water it behaved like the 4′-phosphate (Table 4). No synthetic 2′:4′-diphosphate was available to use as a marker spot in these studies, but it has been shown (Baddiley & Thain, 1951*b*) to move much more slowly than any of the other pantothenic acid phosphates in both the above solvents. Its $R_F$ value in *n*-propanol–ammonia–water was 0·13, compared with 0·45 for the 2′-phosphate and 0·33 for the 4′-phosphate. The corresponding values in *iso*butyric acid–ammonia–water were 0·18, 0·4 and 0·37.

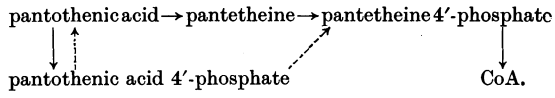
## DISCUSSION

The evidence presented makes it reasonably certain that the enzyme system which has been studied catalyses the formation of D(+)-pantothenic acid 4′-phosphate. Although this compound has not been isolated in quantities sufficient for the application of conventional chemical analysis, it has been shown that its stability in acid and alkali, and its chromatographic behaviour, correspond with those of a synthetic sample of this material. There is some discrepancy in the behaviour of the synthetic phosphate and the enzymically produced material, in that only 75% of the pantothenic acid content of the former is liberated by intestinal phosphatase under conditions where all (90–100%) of the pantothenic acid of enzymically formed material was released. This discrepancy may be due to partial racemization of the D(+)-pantothenic acid during the synthesis. King & Strong (1950) and Baddiley & Thain (1951*a*) report that 90–100% of the pantothenic acid of both 2′- and 4′-phosphates could be liberated by the action of intestinal phosphatase. The conditions used by the former workers were slightly different from those used in the present work.

Although ADP was formed during the phosphorylation of pantothenate by dried cell preparations, the amounts formed were very much larger than expected. This is due to the presence in the preparation of ATPases which are only partly inhibited by fluoride. It is not certain therefore whether ATP donates phosphate to pantothenate directly.

*Phosphorylation of pantothenic acid and CoA synthesis.* Hoagland & Novelli (1954) have shown that in liver CoA can be synthesized from pantetheine in three steps: (1) pantetheine + ATP → pantetheine 4′-phosphate + ADP; (2) pantetheine 4′-phosphate + ATP → dephospho-CoA + pyrophosphate; (3) dephospho-CoA + ATP → CoA + ADP.

These authors did not test the preparation which phosphorylates pantetheine for its ability to phosphorylate pantothenate. Since pantothenic acid 4′-phosphate is utilized neither by liver for CoA synthesis (King & Strong, 1951) nor as a growth factor for bacteria, it is now generally assumed that in the synthesis of CoA the phosphate is attached to 4′-carbon only after pantothenate has been converted into pantetheine. The present observations do not rule out the possibility that pantothenic acid 4′-phosphate is an intermediate in CoA synthesis in bacteria. Its inactivity with intact cells may be due to the inability of certain phosphoric esters to enter the cell. Since the formation of pantothenic acid 4′-phosphate has been detected only in the absence of cystine, it seems feasible that it might serve as a storage form of pantothenate. The two alternative roles are shown in the following scheme:




## SUMMARY

1. Dried cells of *Lb. arabinosus* catalyse the disappearance of pantothenic acid when incubated with ATP and phosphate buffer.

2. Phosphatase treatment results in a complete recovery of the pantothenic acid, indicating that the substance is being converted into a phosphoric ester. Sodium fluoride increases the extent of phosphorylation, probably owing to its inhibition of ATP-splitting enzymes present in dried cells.

3. The pantothenic acid derivative that is formed has been concentrated, and its properties have been compared with those of the synthetic pantothenic acid monophosphates. Its hydrolysis by intestinal phosphatase, stability to acid and alkali, and chromatographic properties suggest that it is pantothenic acid 4′-phosphate.

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# Studies on Sulphatase

## 11. THE PURIFICATION AND PROPERTIES OF THE ARYLSULPHATASE OF *ALCALIGENES METALCALIGENES**


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A strain of *Alcaligenes metalcaligenes*, which was isolated from an inter-tidal mud, possessed considerable arylsulphatase activity, and a preliminary purification of the enzyme has been made (Dodgson, Melville, Spencer & Williams, 1954). The present work describes the further purification of the arylsulphatase and, from consideration of its properties, gives some indication of its possible functional groups.

## MATERIALS AND METHODS

*Arylsulphatase substrates.* The potassium salts of *p*-nitrophenyl (NPS), *p*-acetylphenyl (APS) and nitrocatechol (NCS) sulphates were prepared by the method described previously (Dodgson *et al.* 1954).

*Other substrates.* Potassium chondroitin sulphate was prepared from tracheal cartilage by a modification of the method of Einbinder & Schubert (1950). The potassium salts of *p*-nitrophenyl phosphate and phenyl acetate were commercial samples.

*Determination of arylsulphatase activity.* Enzyme activity was determined by the method previously described (Dodgson *et al.* 1954) and was expressed in terms of nitrogen, as determined by the micro-Kjeldahl method of Markham (1942), or protein, as determined by the method of Lowry, Roseborough, Farr & Randall (1951).

*Determination of nucleic acid.* Ribonucleic and deoxyribonucleic acids were estimated by the method of Schmidt & Thannhauser (1945). The ratio of the spectrophotometric readings at 280 and 260 mμ. was used to locate nucleic acid in the electrophoresis experiments (Warburg & Christian, 1941).

* Part 10: Dodgson, Rose & Spencer (1955).

## EXPERIMENTAL AND RESULTS

### *Preparation of enzyme concentrate*

The enzyme was concentrated to stage *B* as previously described (Dodgson *et al.* 1954). Attempts to purify the enzyme further by ammonium sulphate fractionation and adsorption techniques with ion-exchange resins, alumina, calcium carbonate and benzoic acid were of little value since losses in total enzyme activity were not compensated by the degree of purification achieved. However, paper electrophoresis of the stage *B* enzyme suggested that considerable purification was possible by the use of this technique.

*Electrophoresis experiments.* The enzyme solution (0·1 ml. of a solution of a 0·1 g. stage *B* in 0·6 ml. of 0·1 M phosphate buffer, pH 8·0) was run for 18 hr. on vertically held Whatman no. 100 filter paper at 110 v in the presence of 0·1 M phosphate, pH 8·0. The paper was dried at room temperature and cut into 1 cm. strips. Protein was located by staining with bromophenol blue (see Flynn & de Mayo 1951) and arylsulphatase activity was located, according to the method described by Dodgson, Spencer & Thomas (1955), by incubating each strip with 0·5 ml. of 0·0015 M NPS in 0·1 M phosphate at pH 8·75.