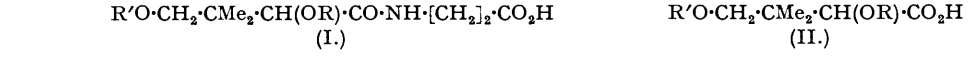
498. *Coenzyme A. Part II.* Evidence for its Formulation as a Derivative of Pantothenic Acid-4′ Phosphate.*

By J. Baddiley and E. M. Thain.

The behaviour of pantothenic acid-2′ and -4′ phosphates and the 2′ : 4′-diphosphate towards acid and alkali has been examined. This has facilitated the determination of hydrolysis conditions under which coenzyme A might be expected to give rise to such phosphates. Methods for the separation and detection on paper of these phosphates and related substances have been developed.

Mild alkaline or acid hydrolysis of coenzyme A gave a product indistinguishable on paper chromatography from pantothenic acid-4′ phosphate. The presence of both 2-mercaptoethylamine and adenosine-5′ phosphate residues is confirmed. Possible formulæ for the coenzyme are advanced.

The factor which participates in a number of biological acetylation processes, coenzyme A, has been shown from analyses of its highly active concentrates to be a derivative of pantothenic acid (Lipmann, Kaplan, Novelli, Tuttle, and Guirard, *J. Biol. Chem.*, 1947, **167**, 869). Recent investigations (De Vries, Govier, Evans, Gregory, Novelli, Soodak, and Lipmann, *J. Amer. Chem. Soc.*, 1950, **72**, 4838) have shown that besides pantothenic acid the molecule contains adenosine, a sulphur-containing substance probably related to cysteine, and two or three phosphate groups. A degradation product of coenzyme A, produced by the action of pigeon-liver extracts, was thought to be a phosphoric ester of pantothenic acid since it liberated pantothenic acid and phosphate on further enzymic breakdown (Novelli, Kaplan, and Lipmann, *J. Biol. Chem.*, 1949, **177**, 97). Although this degradation product has not yet been isolated and characterised it was thought that the correctness of these views could be checked by synthesis of the several possible pantothenic acid phosphates. As the liver degradation product was active in stimulating the growth of *Acetobacter suboxydans* (Novelli, Flynn, and Lipmann, *ibid.*, p. 493) a ready check of identity was available. In Part I of this series * we described the synthesis of pantothenic acid-2′ (I; R = PO_3H_2, R′ = H) and -4′ (I; R = H, R′ = PO_3H_2) phosphates, neither of which showed activity towards *A. suboxydans*. The two monophosphates,



(I.) (II.)

and the 2′ : 4′-diphosphate (I; R = R′ = PO_3H_2) were synthesised independently by King and Strong (*Science*, 1950, **112**, 562) using slightly different methods. These results, although in no way suggesting the absence of a pantothenic acid phosphate structure in coenzyme A, do indicate that the liver degradation product cannot be regarded as a simple monophosphate of pantothenic acid. Experiments described later in this paper eliminate the possibility of a pantothenic acid-2′ : 4′ diphosphate structure in coenzyme A itself and therefore also in the liver degradation product.

Observations made during the preparation of concentrates of " bound pantothenic acid " (*i.e.*, coenzyme A or related substances) from natural sources suggested that when in this bound form pantothenic acid is distinctly more stable to hydrolysis than is the free acid itself (Jukes, *Biol. Symposia*, 1947, **12**, 261; King, Fels, and Cheldelin, *J. Amer. Chem. Soc.*, 1949, **71**, 131; Neal and Strong, *ibid.*, 1943, **65**, 1659). A study of the stability of the amide bonds in our synthetic phosphates was undertaken in this connection. Alkaline hydrolysis was investigated, the extent of hydrolysis being measured by the amount of free amino-nitrogen, determined by Van Slyke's method (Peters and Van Slyke, " Quantitative Clinical Chemistry," 1932, Vol. II, p. 385), present in solution at given times. The results summarised in the Experimental section show that the amide bond in both phosphates is very much more stable to alkali than is that in pantothenic acid. In 0·5N-sodium hydroxide at 25° pantothenic acid hydrolysis obeyed first-order kinetics ($k = 1{\cdot}54 \times 10^{-4}$ sec.$^{-1}$), being 50% hydrolysed in 70 minutes. Under similar conditions the 2′-phosphate was 50% hydrolysed in 50 hours, but only 5% hydrolysis of the 4′-phosphate occurred in 24 hours. At 100°, however, appreciable

* The paper entitled " The Synthesis of Pantothenic Acid-2′ and -4′ Phosphates as Possible Degradation Products of Coenzyme A " by J. Baddiley and E. M. Thain (*J.*, 1951, 246) is regarded as Part I.

hydrolysis of the amide bonds in both phosphates occurred. First-order kinetics were not observed in either case. The rate for the 2′-phosphate was rapid at first (50% hydrolysed in about 10 minutes), but fell subsequently so that the time taken between 70% and 85% hydrolysis was 200 minutes. The 4′-phosphate was attacked smoothly but slowly, being only 20% hydrolysed in 330 minutes, a rate similar to the later stages of hydrolysis of the 2′-phosphate. The pantothenic acid amide bond in coenzyme A is about 30% hydrolysed in N-alkali at 100° in 180 minutes (King and Strong, *J. Biol. Chem.*, 1951, **189**, 325). This is in good agreement with our figures for pantothenic acid-4′ phosphate and differs quite markedly from those for the 2′-phosphate. The conclusion that a pantothenic acid-4′ phosphate structure may be present in the coenzyme is confirmed by observations described later in this paper.

As was shown in Part I (*loc. cit.*) the phosphate bonds in the synthetic phosphates are very stable to alkali and rather stable to acid. It seemed probable, then, that alkaline hydrolysis or even mild acid hydrolysis of coenzyme A should give rise to one of these phosphates if such a grouping were present in its molecule. According to the conditions necessary to hydrolyse other bonds in the coenzyme one may expect to obtain mixtures containing, not only a phosphate of pantothenic acid, but also a phosphate of pantoic acid (II) or its lactone both of which have lost the β-alanine residue. However, in view of the very small amounts of coenzyme A available to us for chemical study at this stage (a total of about 5 mg. was used in the work described in this paper), and also in view of the difficulties so far experienced in obtaining crystalline or otherwise characterisable derivatives of the phosphates of pantothenic and pantoic acid, the isolation of such degradation products and their chemical identification was not possible. Consequently, an investigation was made of the behaviour of these substances in paper chromatography, *n*-propyl alcohol–ammonia (Hanes and Isherwood, *Nature*, 1949, **164**, 1107) and *iso*butyric acid–ammonium *iso*butyrate–water (Magasanik, Vischer, Doniger, Elson, and Chargaff, *J. Biol. Chem.*, 1950, **186**, 37) proving satisfactory solvents. The phosphoric esters were detected as blue spots by spraying with perchloric acid and ammonium molybdate, followed by development with hydrogen sulphide (Hanes and Isherwood, *loc. cit.*). Adenine derivatives were also detected by inspection in ultra-violet light. The following were examined in both solvent systems : pantothenic acid-2′ and -4′ phosphate and -2′ : 4′ diphosphate, alkali hydrolysates of these, pantolactone-2 phosphate, adenylic acids a and b, adenosine-5′ phosphate and diphosphate, and inorganic phosphate.

Of the phosphates of pantothenic and pantoic acids the most rapidly moving in both solvent systems was pantolactone-2 phosphate. In *n*-propyl alcohol–ammonia this substance gave two clear spots, a fast-moving one which was shown to be the amide arising from the action of ammonia on the lactone and a slower one of the ammonium salt of pantoic acid-2 phosphate (II; R = PO_3H_2; R′ = H). After treatment of the lactone phosphate with ammonia, followed by paper chromatography, only one spot was observed, again corresponding with the amide, whereas pretreatment with barium hydroxide and then ammonium sulphate resulted in a spot of the ammonium salt of the acid. In the *iso*butyric acid system pantolactone-2 phosphate gave only one spot, the acidic conditions preventing opening of the lactone ring. Good separations of the pantothenic acid phosphates, both from each other and from adenosine-5′ phosphate, adenylic acids a and b, and adenosine diphosphate, were observed in both solvent systems. The relative positions of the pantothenic acid phosphates with respect to adenosine phosphates were reversed on changing from *n*-propyl alcohol–ammonia to *iso*butyric acid–ammonium *iso*butyrate. In neither system was it possible to effect more than partial separation of pantothenic acid-4′ phosphate from its product of alkaline hydrolysis [pantoic acid-4 phosphate (II; R = H; R′ = PO_3H_2)], although the very characteristic appearance of the double spot from pantolactone-2 phosphate was observed quite easily in hydrolysates of pantothenic acid-2′ phosphate.

The isomeric adenylic acids were separated readily on paper from the pantothenic acid phosphates. In *n*-propyl alcohol–ammonia all three were slower-moving than pantothenic acid-4′ phosphate. Isomers a and b (Carter and Cohn, *Fed. Proc.*, 1949, **8**, 190) were not separated in this system but moved slightly faster than adenosine-5′ phosphate. In *iso*butyric acid–ammonium *iso*butyrate the adenylic acid isomers moved considerably faster than pantothenic acid-4′ phosphate and, although isomers a and b were not separated, they moved noticeably faster than adenosine-5′ phosphate.

A mild alkali hydrolysate of coenzyme A from which inorganic phosphate had been removed by precipitation as its barium salt gave two phosphorus-containing spots from each of the two solvent systems. One corresponded in position with pantothenic acid-4′ phosphate or its hydrolysis product, pantoic acid-4 phosphate, and the other spot with adenosine-5′ phosphate.

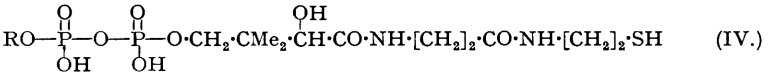
Shorter periods of hydrolysis sometimes resulted in the occurrence of an additional, slow-moving spot; this has not been identified yet but may represent a degradation product. The absence of purine derivatives at the positions occupied in both chromatograms by the pantothenic acid-4′ phosphate spots was ascertained by examination in ultra-violet light. The marked absence of spots corresponding to pantothenic acid-2′ phosphate, the 2′ : 4′-diphosphate, or their hydrolysis products eliminates the existence of such groupings in the coenzyme. In acid hydrolysates pantothenic acid-4′ phosphate was readily observed but little, if any, adenosine-5′ phosphate was present.

While these experiments should not be regarded as providing conclusive proof of the presence of a pantothenic acid-4′ phosphate residue in coenzyme A, at least they strongly suggest it. Confirmation should be forthcoming when more material is available and isolation of degradation products can be attempted. The presence of adenosine-5′ phosphate in coenzyme A hydrolysates confirms the findings of Lipmann (private communication). It was shown that no free adenosine-5′ phosphate was present in the sample of coenzyme before hydrolysis.

A phosphorus-free derivative of pantothenic acid has been isolated recently from natural sources (McRorie, Masley, and Williams, *Arch. Biochem.*, 1950, **27**, 471; Brown, Craig, and Snell, *ibid.*, p. 473) and shown to stimulate the growth of *Lactobacillus bulgaricus* (*Lactobacillus bulgaricus* factor, LBF). It has been proved by synthesis to be (III) (Snell, Brown, Peters,

$$HO{\cdot}CH_2{\cdot}CMe_2{\cdot}CH(OH){\cdot}CO{\cdot}NH{\cdot}[CH_2]_2{\cdot}CO{\cdot}NH{\cdot}[CH_2]_2{\cdot}SH \quad \text{(III.)}$$

Craig, Wittle, Moore, McGlohon, and Bird, *J. Amer. Chem. Soc.*, 1950, **72**, 5349). An enzymic degradation product of coenzyme A was indistinguishable from (III) in its ability to stimulate the growth of this organism, hence a close chemical relationship between coenzyme A and (III) can be assumed. If this degradation product of the coenzyme is the 2-mercaptoethylamide (III) of pantothenic acid, then the " cystein-like " substance described by De Vries *et al.* (*loc. cit.*) as a component of the coenzyme should be 2-mercaptoethylamine. An acid hydrolysate of coenzyme A was neutralised and subjected to steam-distillation on a micro-scale. The distillate contained a substance which gave both a ninhydrin and a nitroprusside reaction. Paper chromatography in butyl alcohol–acetic acid–water showed that these two reactions were given by a single substance corresponding in position on the paper with synthetic 2-mercaptoethylamine. This confirms the close relationship between (III) and coenzyme A. Synthetic 2-mercaptoethylamine was prepared for these studies by a convenient modification of Gabriel's method (*Ber.*, 1889, **22**, 1137; 1891, **24**, 1110) involving the hydrolysis of *N*-2-phthalimidoethylthiuronium bromide.



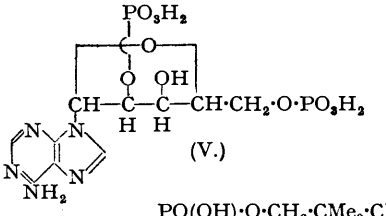
(IV.)

Novelli, Kaplan, and Lipmann (*Fed. Proc.*, 1950, **9**, 209) have shown that the coenzyme is probably a dinucleotide since it is split by a dinucleotidase. Its marked alkali lability is also consistent with this view. On the basis of this and the foregoing evidence we have proposed formula (IV) for coenzyme A (Baddiley and Thain, *Chem. and Ind.*, 1951, 337). The group R is adenosine, possibly bearing an additional phosphate group. If coenzyme A contains only two phosphate groups per molecule, then the position of attachment of the pyrophosphate group to the adenosine residue would be 5′, as indicated by the presence of adenosine-5′ phosphate in hydrolysates of the coenzyme. If three phosphate groups are present, however, the situation is less clear. It is very unlikely that an additional phosphate group could be attached at position 2′ in the pantothenic acid residue since we would expect then to detect pantothenic acid-2′ : 4′ diphosphate in coenzyme hydrolysates, both phosphate groups in this diphosphate being stable under the hydrolytic conditions we employed. There remain positions 2′ and 3′ in the adenosine residue as the most probable points of attachment of an additional phosphate group. Although it is not possible to differentiate at this stage between these, we favour 2′ by analogy with the formula recently proposed for coenzyme II (triphosphopyridine nucleotide) (Kornberg and Pricer, *J. Biol. Chem.*, 1950, **186**, 557). In this connection it would be of interest to study the nature of the products of dinucleotidase action on coenzyme A. If one product were adenosine-2′ : 5′ diphosphate (V), then the above formal relation between the two coenzymes would be established. The failure to detect formation of adenosine-5′ phosphate from coenzyme A by the action of a dinucleotidase (Lipmann, Kaplan, Novelli, Tuttle, and Guirard, *ibid.*, p. 235) would be explained in this way. King and Strong (*ibid.*, 1951, **189**, 325), contrary to the findings of Novelli, Kaplan, and Lipmann (*loc. cit.*),

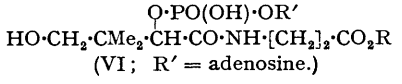
observed only a small destruction of activity when coenzyme A was treated with a dinucleotidase. They concluded that a pyrophosphate linkage was not present and suggested a partial formula (VI) containing a disubstituted monophosphate grouping. Now the phosphate bond in the adenosine phosphates is considerably less stable to alkali than that in pantothenic acid-4′ phosphate. Consequently, if in the structure postulated by King and Strong the phosphate linkage involved only the 4′-position in pantothenic acid and the 5′-position in adenosine, then hydrolysis should give rise to pantothenic acid-4′ phosphate and adenosine, not as we have observed in coenzyme A hydrolysates adenosine-5′ phosphate. (Their assignation of the phosphate to the 2′-position in the pantothenic acid moiety is presumably arbitrary and, as we have shown, incorrect.) However, a structure (VII) would be expected to give on hydrolysis both pantothenic acid-4′ phosphate and adenosine-5′ phosphate and would be unaffected by dinucleotidase action. Final decision between formulæ (IV) and (VII) for coenzyme A must await further investigation. The position of attachment of the pantothenic acid phosphate part to the adenosine-5′ phosphate in (VII) is still undecided.

The evidence presented so far does not exclude the possibility of cyclic phosphate structures for coenzyme A. Such cyclic phosphates of pantothenic acid are not known, consequently it is not possible to predict their behaviour under the hydrolytic conditions of our experiments or their behaviour on paper chromatography.

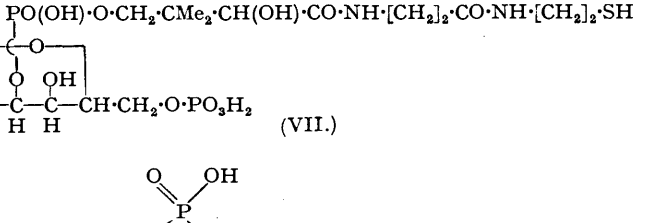
The structure of the liver degradation product is still uncertain. The inability of pantothenic acid-4′ phosphate to stimulate the growth of *A. suboxydans* might be interpreted in one of two ways. First, this degradation product may contain a cyclic phosphate [*e.g.*, (VIII)], which would suggest the presence of such a group in coenzyme A, or, secondly, it may be a derivative of (III) bearing a phosphate group on the primary hydroxyl group. The synthesis of these phosphates is under investigation.



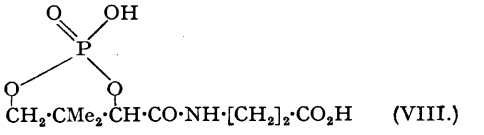
(V.)



(VI; R′ = adenosine.)



(VII.)



(VIII.)

Lynen and Reichert (*Angew. Chem.*, 1951, **63**, 47) have suggested that the acetylating activity of coenzyme A involves the terminal thiol group. They suggest that the coenzyme accepts an acetyl group at this position and transfers it to the substrate. In its mercaptoacetyl form coenzyme A presumably contains its acetyl in an " energy-rich " state. Lynen and Reichert provide evidence that such an acetyl group is present in their coenzyme A preparations obtained from yeast. Insufficient material has been available to us for acetyl determinations on coenzyme A prepared from *Streptomyces fradiae* as described by De Vries *et al.* (*loc. cit.*). It would seem reasonable, however, to expect a variable acetyl content depending to some extent on the metabolic state of the tissues or organism at the time of isolation. The expected lability of such an acetyl group might result in the isolation of a coenzyme A which, although highly active in acetylation systems, does not itself contain any acetyl group.

The experiments described in this paper were performed on a sample of coenzyme A with an acetylating activity of 180 units/mg., as measured by its capacity to acetylate sulphanilamide (Kaplan and Lipmann, *J. Biol. Chem.*, 1948, **174**, 37). Analytically pure samples of the coenzyme have not yet been described and until such are available it is not possible to rule

out the occurrence of hitherto unidentified groups in the molecule. The possible occurrence of more than one coenzyme A must also be considered. In this connection we have shown that paper chromatography of our sample of the coenzyme showed the presence of two rather slow-moving phosphate fractions, both of which were active in the acetylation of sulphanilamide. The total recovery of activity from the paper was low, however, and the method is as yet insufficiently refined for the isolation and chemical investigation of the different fractions.

Experimental.

Hydrolysis of the Amide Bond in Pantothenic Acid and Pantothenic Acid Phosphates.—Calcium pantothenate (13 mg.) and the barium salts of pantothenic acid-2′ and -4′ phosphates (25 mg.) were severally dissolved in 0·5N-sodium hydroxide (10 ml.), and the solution kept at 25° (thermostat). Aliquots (1 ml.) were analysed for free β-alanine by Van Slyke's method (*loc. cit.*).

Pantothenic acid.

Time (min.)	15	47	75	106	152	232	307
Hydrolysis, %	12·5	37·8	51·8	59·2	71·7	86·2	93·1

Pantothenic acid-2′ phosphate.

Time (hr.)	2	5	7	29·2	31·5	49·6
Hydrolysis, %	4·8	9·2	11·3	40·9	42	52·7

Under similar conditions pantothenic acid-4′ phosphate was only 5% hydrolysed after 24 hours.

A plot of log (decay) against time derived from these figures gave a straight line for pantothenic acid indicating first-order kinetics, $k = 1{\cdot}54 \times 10^{-4}$ sec.$^{-1}$ at 25°, but a curve was obtained for pantothenic acid-2′ phosphate probably owing to simultaneous hydrolysis of the phosphate complicating the rate of hydrolysis.

In view of the slow hydrolysis of the pantothenic acid phosphates the temperature was increased to 100°, conditions otherwise being the same, and smooth curves were obtained confirming the greater stability of pantothenic acid-4′ phosphate.

Pantothenic acid-2′ phosphate.

Time (min.)	5	10	15	38	70	80	140	270
Hydrolysis, %	28·7	51·0	55·0	67·1	69·9	75·2	80·1	89·0

Pantothenic acid-4′ phosphate.

Time (min.)	37	80	120	240	330
Hydrolysis, %	4	7·4	8·1	15·5	19·5

Hydrolysis of Coenzyme A and Pantothenic Acid Phosphates, and Examination of the Products by Paper Chromatography.—Coenzyme A, adenosine-5′ phosphate, pantolactone-2 phosphate, and the barium salts of pantothenic acid phosphates (*ca.* 0·5 mg.) were severally heated in sealed tubes with N-hydrochloric acid (0·1 ml.) or 0·3N-barium hydroxide (0·1 ml.) at 100° for 0·5—2 hours.

In acid hydrolyses the solutions were evaporated in a vacuum and the residues dissolved in 0·3N-barium hydroxide (0·1 ml.) to precipitate any free phosphate; thereafter acid and alkaline hydrolyses were treated similarly. Carbon dioxide was passed through the solutions until precipitation was complete and the solutions were boiled for 1—2 minutes to destroy any barium hydrogen carbonate. Barium carbonate was removed by centrifugation and sufficient ammonium sulphate added to precipitate barium from soluble barium salts. Barium sulphate was removed by centrifugation and the solutions evaporated in a vacuum.

The dried residues were dissolved in small volumes of water, usually about 0·05 ml.; then sufficient of these solutions to contain about 10 μg. of phosphorus was chromatographed on Whatman's No. 1 filter paper with (*a*) *n*-propyl alcohol–ammonia–water (6 : 3 : 1) (Hanes and Isherwood, *loc. cit.*) and (*b*) *iso*butyric acid–ammonium *iso*butyrate–water (*iso*butyric acid, 10 vols.; 0·5N-aqueous ammonia, 6 vols.) (Magasanik *et al.*, *loc. cit.*). Washing the paper with acids was unnecessary in the chromatography of phosphate esters of pantothenic acid since there was no serious tailing of the spots. The only advantage of washing was the increased speed of running, which had little effect on the observed R_F values. Since no special temperature control was employed the R_F values varied within small limits. Those given below are for room temperature (*ca.* 20°) and are averages of many runs.

Phosphates were detected by perchloric acid–molybdate spray (Hanes and Isherwood), and adenine derivatives by their fluorescence in ultra-violet light.

When the hydrolysis had been carried out in glass tubes a " ghost spot " appeared in the same place on the paper, probably due to silicates, irrespective of the nature of the substance hydrolysed or even whether any phosphate was being hydrolysed at all. These spots appeared a day or two after the papers had been developed and thus were readily distinguished.

The synthetic-2′ and -4′ phosphates of pantothenic acid were found to be homogeneous, but some samples of the -4′ phosphate gave in addition to the main spot a much weaker, faster-moving one (R_F 0·8 in *n*-propyl alcohol–ammonia), probably due to a small amount of material from which the benzyl

or phenyl groups had been only partly removed by hydrogenolysis. Pantothenic acid diphosphate prepared by King and Strong's method (*loc. cit.*) contained small amounts of pantolactone phosphate.

Substance.	PrOH–NH_3. R_F.	$Pr^i \cdot CO_2H$–NH_3. R_F.
Pantolactone-2 phosphate	0·55 (amide) 0·38 (NH_4 salt)	0·57
Pantothenic acid-2′ phosphate	0·45	0·40
,, -4′ ,,	0·33 0·36 hydrol.	0·37
,, diphosphate	0·13	0·18
Adenosine-5′ phosphate	0·26	0·44
Adenylic acid a	0·30	0·56
,, b	0·30	0·56
Coenzyme A	0·07, 0·13	—
Adenosine diphosphate	0·18	—
Adenosine	0·73	0·79
Adenine	0·65	0·90

Detection of 2-Mercaptoethylamine in Coenzyme A.—Coenzyme A (1·0 mg.) was hydrolysed with 5N-hydrochloric acid (0·2 ml.) at 100° for 3 hours (sealed tube). The hydrolysate was evaporated in a vacuum, the residue dissolved in water (0·5 ml.) containing sodium sulphite (*ca.* 1 mg.), the pH adjusted to 8 (Na_2CO_3), and the mercaptoethylamine steam-distilled in a gentle stream of nitrogen. The distillate was acidified with acetic acid and evaporated in a vacuum, and the dried residue divided into two parts and run on paper in butyl alcohol–acetic acid–water together with an authentic sample of 2-mercaptoethylamine. The dried paper was divided and one half sprayed with ninhydrin, and the other with sodium sulphite–cyanide–nitroprusside. Spots were recorded with both sprays corresponding to 2-mercaptoethylamine, $R_F = 0{\cdot}26$.

Biological Tests.—Two samples of coenzyme A, one of which weighed 0·225 mg. (38 units), were run down a paper chromatogram with *n*-propyl alcohol–ammonia for 40 hours. The paper was dried and divided down the centre, and the unweighed sample developed for phosphate with the perchloric acid–molybdate reagent. Portions of paper corresponding to the two main spots on the developed chromatogram, which were 7 cm. apart, were cut out of the paper containing the weighed sample of coenzyme A and eluted with water for 3 hours, together with a portion of paper taken from between the two spots, it having been observed that there was a very faint spot at this position. The eluates were evaporated in a vacuum and the residues analysed for coenzyme A activity by Kaplan and Lipmann's method (*loc. cit.*). The fastest-moving spot contained 7 units, the slowest 0·8 unit, while a slight activity of 0·6 unit was observed in the middle spot (this however may have arisen from " lagging " of the active fast spot).

N-2-*Phthalimidoethylthiuronium Bromide.*—A solution of *N*-2-bromoethylphthalimide (101 g.) and thiourea (35 g.) in absolute alcohol (250 c.c.) was refluxed for 4½ hours. The solid which separated was filtered off from the cooled mixture, washed with a little cold alcohol, and dried. The *thiuronium* salt (104 g.) was sufficiently pure for subsequent conversion into 2-mercaptoethylamine. Recrystallised from alcohol it formed prisms, m. p. 243° (Found : C, 39·9; H, 3·5; N, 12·5. $C_{11}H_{12}O_2N_3SBr$ requires C, 40·0; H, 3·6; N, 12·7%).

N-2-*Phthalimidoethanethiol.*—To the thiuronium salt (25 g.), dissolved in the minimum amount of boiling water, a solution of sodium hydroxide (3·1 g.) in water (50 c.c.) was added. Boiling was continued for 5 minutes. The oil which separated solidified on cooling. The solid was filtered off, washed with water, and dried in a desiccator (13 g.). Recrystallised from aqueous alcohol it had m. p. 76°. Gabriel (*loc. cit.*) records m. p. 79—80° for *N*-2-phthalimidoethanethiol prepared by the action of potassium hydrogen sulphide on *N*-2-bromoethylphthalimide. 2-Mercaptoethylamine was prepared by hydrolysis of the thiol in hydrochloric acid according to Gabriel's method.

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