# ADVANCES IN ENZYMOLOGY

*AND RELATED SUBJECTS OF BIOCHEMISTRY*

Edited by F. F. NORD

FORDHAM UNIVERSITY, NEW YORK, N. Y.

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**Volume XVI**

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# CONTENTS

# THE STRUCTURE OF COENZYME A

By J. BADDILEY, *London, England*

CONTENTS



## I. Introduction

The discovery by R. J. Williams and his collaborators in 1933 that a naturally occurring substance which they called "pantothenic acid" would stimulate the growth of yeast (93) marked the opening of one of the more significant chapters in the history of biochemistry. The recognition that this vitamin was essential not only for other microorganisms but also for the growth and health of many animals indicated its fundamental importance in living processes. However, a considerable time elapsed before the mechanism of pantothenic acid action became clear. The more recent advances in our knowledge of this subject have developed from the important observation by Lipmann and his collaborators (55) that enzymic acetylation of amines required a coenzyme (coenzyme A, CoA), which was a derivative of pantothenic acid. Although acetylation of amines is itself an important biological process, the almost universal occurrence of pantothenic acid in living cells suggested that it possessed other functions. The discovery that CoA was essential for the acetylation of choline (54) strengthened this view. The great importance of this coenzyme was established by the observation that it was an essential participant in the transfer of "active acetate" during the synthesis of citric acid (71,72). The elucidation of the function of CoA in the synthesis of

citric acid (74,82,83) and the identification of "active acetate" as *S*-acetyl-CoA (60) were significant developments in our knowledge of the function of the coenzyme. A detailed description of the numerous biological processes in which CoA derivatives are now known to participate is beyond the scope of this article. These processes include the metabolism of pyruvate, fatty acids, fats, succinate, and porphyrin synthesis among others.

The structure of CoA is now fully established. This has been achieved by a combination of chemical, enzymic, and microbiological methods which will be described in the following chapters.

## II. Isolation

CoA was first detected in liver preparations (52), where it was shown to be necessary for the acetylation of aromatic amines. Subsequently, it was established (54) that a factor present in brain extracts which was responsible for the acetylation of choline (25,57,62) was identical with the liver coenzyme. Impure preparations, now known to contain not more than about 30% of CoA, were obtained from fresh hog liver by fractionation through mercury and barium salts (56). The coenzyme contents of these preparations were determined by a colorimetric procedure (34) based on the capacity of the extract to acetylate sulfanilamide in the presence of acetate and ATP. An improved method (30) has been used in more recent studies. The inconvenience of fresh liver as a source of the coenzyme stimulated a search for more accessible and richer sources. Microorganisms contain relatively large amounts of CoA and concentrates of about 60% purity have been obtained from *Streptomyces fradiae* by the use of chromatography on charcoal columns (24). The most readily available source of CoA, however, is yeast. Highly pure coenzyme has been prepared from dried brewers' yeast by a method which involves adsorption on charcoal and subsequent elution with pyridine (14). Commercial preparations of CoA probably originate from yeast.

When the coenzyme is extracted by any of the above methods it is believed to exist in the form of mixed disulfides with some of its own products of partial decomposition and with other naturally occurring sulfhydryl compounds. It follows that final purification to a homogeneous substance would be extremely difficult and would be accompanied by a considerable loss of biologically useful material. This difficulty has been overcome in two independent methods for the isola-

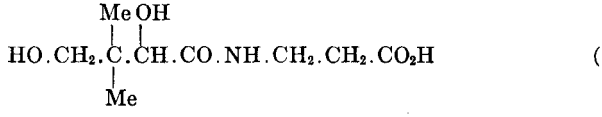
tion of pure, or nearly pure, CoA from yeast (14) and from *S. fradiae* (29). Both methods include a reduction step during which disulfides are reduced to sulfhydryl compounds, the coenzyme then being freed from its contaminants by precipitation. In the route from *S. fradiae* zinc and acid effect the reduction, whereas in the yeast route the reducing agent is the sulfhydryl form of glutathione.

## III. Structure

### A. RECOGNITION AS A DERIVATIVE OF PANTOTHENIC ACID

When CoA concentrates were subjected to a thorough vitamin analysis no vitamins of the B group were detected in more than trace amounts. However, when the clarase-papain treatment used in its purification was prolonged the pantothenic acid content increased. It was also found that β-alanine was liberated by acid hydrolysis. Eventually, pantothenic acid itself was liberated by the combined action of intestinal phosphatase and an enzyme present in liver, both of which were known to inactivate the coenzyme (55,69). The pantothenic acid content was determined by microbiological methods and later analyses of pure CoA indicated that one pantothenic acid residue was present in the coenzyme molecule (24). This and other quantitative data given below have been confirmed on samples of pure CoA from other sources (14).

The structure (I) was proposed for pantothenic acid by Williams and his collaborators (93). This has been fully established by several syntheses (48,78,84). The details of this work and the earlier history of the vitamin have been reviewed before in this series (92) and will not be discussed further here.



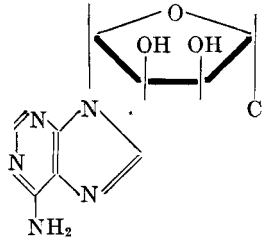
(I)

Although CoA is a derivative of pantothenic acid, and the intact coenzyme stimulates the growth of chicks in accordance with its pantothenic acid content, it is inactive as a substitute for the vitamin in microbiological tests (55). From a comparison of the CoA activities of various tissues with their pantothenic acid contents after double enzyme treatment (see above), it has been concluded that most, if not all, cellular pantothenic acid exists in the form of CoA (69). This

explains earlier observations that the vitamin frequently occurs in "bound forms" which are unavailable to bacteria (33,37,63,64,92). It would appear that these bound forms are in fact CoA or mixtures of the coenzyme and its products of partial decomposition. This has been established beyond doubt for the pantothenic acid derivative which stimulates the growth of *Acetobacter suboxydans* (see later).

### B. NATURE OF PURINE AND SUGAR COMPONENTS

Early impure samples of CoA absorbed ultraviolet light very strongly at 260 mμ. That the absorption arose from the presence of adenine derivatives was shown by the isolation of this purine as its picrate from acid hydrolyzates (55). The ratio of adenine—determined from its ultraviolet absorption—to pantothenic acid was about 2:1. However, as purification proceeded the adenine content decreased, and analysis of the highly purified coenzyme indicated that it contained only one mole of adenine. The adenine present in the pure coenzyme could be entirely accounted for as some bound form of adenosine, 9-β-D-ribofuranosyladenine (II). This was demonstrated by liberation of the nucleoside from the coenzyme through the action of alkaline phosphatase. Adenosine was then determined by a specific deaminase (24,29).




(II)

It appears then that CoA, like a number of other coenzymes, is a nucleoside derivative. Furthermore, it resembles flavin-adenine-dinucleotide (FAD) and the pyridine-nucleotide coenzymes in that it is an adenosine derivative of a vitamin of the B group. This resemblance will become even more apparent when the nature of the linkage between the nucleoside and vitamin is discussed.

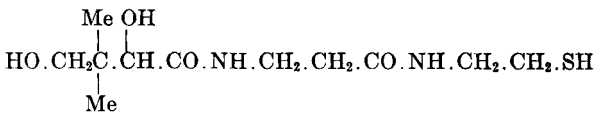
### C. RELATIONSHIP BETWEEN CoA AND PANTETHEINE

Besides pantothenic acid and adenosine, CoA contains phosphate groups and a sulfur-containing residue (24). Analyses of pure coen-

zyme show that only one of these sulfur-containing residues is present per mole of pantothenic acid (29). The sulfur is present in the thiol or disulfide state, since CoA gives a strong positive nitroprusside reaction. It was recognized that the thiol group was contained in a fragment which resembled cysteine in certain respects. However, differences were noted and its exact nature was not established until later.

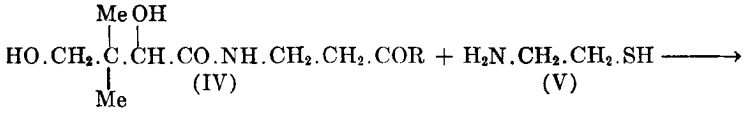
A considerable advance in our knowledge of the structure of CoA was forthcoming from a series of investigations on what seemed at first to be an unrelated topic. Snell and his collaborators found that *Lactobacillus bulgaricus* requires a growth factor which is normally present in yeast extracts (32,94). Subsequent assays showed that this substance (*Lactobacillus bulgaricus* factor or LBF) is widely distributed and is required for the growth of other microorganisms. Several chromatographically distinct forms of LBF are known to exist naturally (77). Details of the isolation and chemistry of the different forms of LBF have been discussed previously in this series (80). Briefly, it appears that the different forms of the factor are mixed disulfides of LBF with otherwise inert, naturally occurring sulfur compounds. The factor itself, LBF, was given the names "pantethine" and "pantetheine" which refer to the disulfide and sulfhydryl forms, respectively.

A possible connection between pantetheine and CoA was suspected when it was found (18,20,61) that the former was a derivative of pantothenic acid. It was also found that the sulfhydryl component of pantetheine is identical with 2-mercaptoethylamine. The structure of pantetheine (20) was represented as *N*-pantothenyl-2-mercaptoethylamine (III) and this was proved by synthesis.



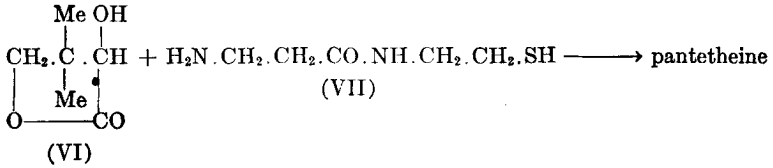
(III)

These syntheses, some of which have been discussed more fully before (80), fall into two general types. In the first type a derivative of pantothenic acid (IV) is allowed to react with 2-mercaptoethylamine (V) or its disulfide:




The first synthesis of pantetheine started from the methyl ester (IV: R = OMe) (81,95) but yields were low and purification was difficult. Modifications of this method utilize pantothenyl azide (95) (IV: R = $-N_3$) or ethyl pantothenyl carbonate (90) and 2-mercaptoethylamine or its disulfide. A further modification involves ethyl pantothenyl carbonate and ethyleneimine, and then reaction of the resulting acylated imine with a thio acid (79).

The second type of synthesis is more convenient for the preparation of reasonable amounts of pantetheine. In this route (8) pantolactone (VI) is condensed with *N*-β-alanyl-2-mercaptoethylamine (VII):




The peptide (VII) was first prepared from carbobenzyloxy-β-alanyl azide and 2-benzylthioethylamine. Benzyl groups were removed from the resulting product with sodium in liquid ammonia (8). Several alternative routes to the synthesis of the peptide (VII) or its disulfide have been introduced more recently. These involve protection of amino groups as carbobenzyloxy or phthalyl derivatives (16,41,85, 86).

The belief that pantetheine and CoA may be related biologically was strengthened by the observation that one of the products of the action of intestinal phosphatase on CoA, but not the intact coenzyme, stimulated the growth of microorganisms in a manner similar to that shown by pantetheine (18,19). Also, the activity of pantetheine was destroyed by the enzymes from chicken liver which liberate pantothenic acid from CoA. Furthermore, concentrates of LBF were partially converted into CoA by the action of tissue extracts (44), and synthetic pantetheine was also converted into CoA by incubation with adenosine triphosphate (ATP) and a pigeon liver extract (27).

Chemical evidence for the structural relationship between the growth factor and the coenzyme was obtained by acid hydrolysis of the latter. The acid hydrolyzate was subjected to steam distillation and it was shown that a steam-volatile substance containing both sulfhydryl and amino groups was produced. This was identified as 2-mercaptoethylamine by paper chromatography (4). These findings were confirmed in other laboratories (20,28). The whole of the sulfur present in highly purified CoA could be accounted for in the 2-mercaptoethylamine residue (28). From these and the biological considerations, then, it is clear that CoA contains within its molecule an intact pantetheine moiety.

## D. TYPE AND POSITION OF PHOSPHATE GROUPS

Three phosphate groups are present in CoA (24,29,68). Since the adenosine residue bears three hydroxyl groups and one amino group and the pantetheine residue bears two hydroxyls and one sulfhydryl group, all of which are capable of esterification with phosphate or pyrophosphate, the theoretically possible methods of combination are considerable. The successful determination of the type and position of these phosphate linkages has required the combination of enzymic and chemical methods.

Three problems arise in connection with the phosphate groups in CoA: the position of substitution in the pantetheine part, the position of substitution in the adenosine part, and the nature of the linkage between the two.

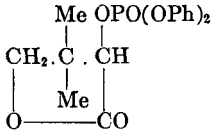
### *1. Position of Substitution in Pantetheine*

At an early stage in the history of the chemistry of CoA it became clear that pantothenic acid is bound in the molecule as a phosphoric ester. This followed from the observation that in order to liberate pantothenic acid from the coenzyme the combined action of an unidentified enzyme present in liver and an alkaline phosphatase was required (55,69). It is now known that the liver enzyme hydrolyzes the amide linkage between pantothenic acid and 2-mercaptoethylamine. The phosphatase used in these early experiments was a rather unspecific enzyme and gave little information about the nature of the phosphate group, other than that it must be attached to the pantothenyl residue.

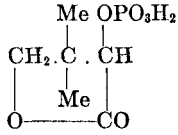
Unlike pantothenic acid, neither CoA nor one of the products ob-

tained from it by the action of a liver enzyme alone, will stimulate the growth of lactic acid bacteria. However, both the liver enzyme degradation product (67) and a pantothenic acid derivative from heart muscle (38) are more active than pantothenic acid itself in stimulating the growth of *Acetobacter suboxydans*. These two substances were believed to be identical, and it was thought at the time that they were simple phosphoric esters of pantothenic acid, particularly as they were both destroyed by the action of a phosphatase.

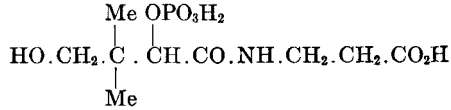
During attempts to elucidate the nature of this "*Acetobacter* stimulatory factor" (ASF) several phosphates of pantothenic acid were synthesized. In the synthesis of pantothenic acid 2′-phosphate (X) pantolactone was converted into its 2-diphenyl phosphate (VIII) by reaction with diphenyl phosphorochloridate.* Phenyl groups were removed by hydrogenolysis and the resulting pantolactone 2-phosphate (IX) was condensed with the sodium salt of β-alanine (3,39,42):



(VIII)



(IX)



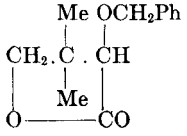
(X)

Better yields of pantothenic acid 2′-phosphate were obtained in the above synthesis by using β-alanine benzyl ester and hydrogenating the resulting product (3).

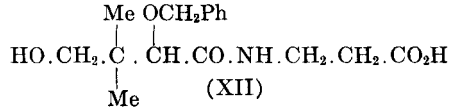
In an unambiguous synthesis of pantothenic acid 4′-phosphate (XIII) it was necessary to protect the hydroxyl group at position 2′. For this purpose pantolactone 2-benzyl ether (XI) was prepared. When this was heated with the sodium salt of β-alanine, pantothenic acid 2′-benzyl ether (XII) was obtained. Phosphorylation with diphenyl phosphorochloridate and removal of protecting groups by

* The nomenclature of phosphorus compounds employed in this article has been described in a report by the Chemical Society, London (*J. Chem. Soc., 1952,* 5122) and in *Chem. Eng. News, 30,* 4515 ff. (1952). This nomenclature has been agreed upon by the Chemical Society and the American Chemical Society.

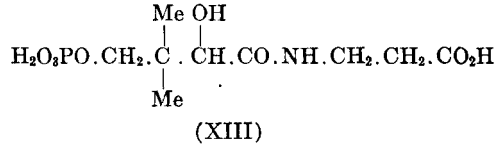
catalytic hydrogenolysis yielded pantothenic acid 4′-phosphate (XIII) (3):



(XI)

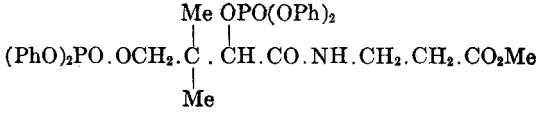


(XII)

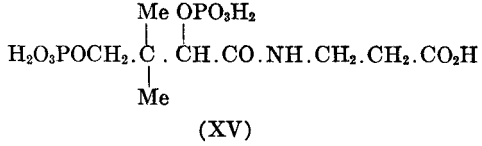


(XIII)

Pantothenic acid 2′,4′-diphosphate (XV) was synthesized from methyl pantothenate by phosphorylation of both hydroxyl groups with diphenyl phosphorochloridate to give the neutral ester (XIV). The four phenyl groups were removed by hydrogenolysis and the resulting methyl ester was hydrolyzed with alkali (43):

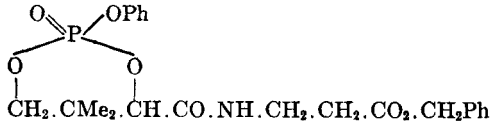


(XIV)

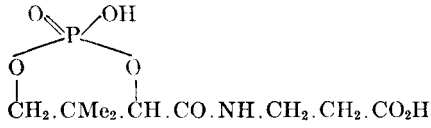


(XV)

The cyclic phosphate, pantothenic acid 2′,4′-hydrogen phosphate (XVII) was prepared by phosphorylation of benzyl pantothenate with phenyl phosphorodichloridate ($PhO.PO.Cl_2$) and removal of protecting groups from the resulting neutral ester (XVI) by hydrogenolysis (5):



(XVI)



(XVII)

None of these phosphates of pantothenic acid was able to stimulate the growth of either *A. suboxydans* or lactic acid bacteria. It follows then that ASF is not a simple mono- or diphosphate of pantothenic acid.

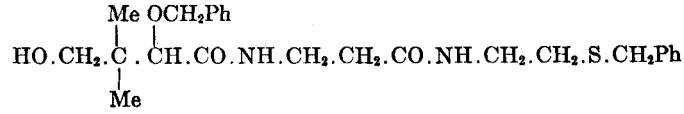
One of the marked characteristics of the pantothenic acid residue in CoA is its stability toward acids and alkali, when compared with the very labile free vitamin (33,37,44,63). The amide linkage in the synthetic phosphates of pantothenic acid is also very stable. However, the rate of alkaline hydrolysis of this linkage varies individually and it was found that the rate of hydrolysis of this linkage in CoA corresponded closely with that for pantothenic acid 4′-phosphate but differed from the rate for the 2′-phosphate (4).

The conclusion that a pantothenic acid 4′-phosphate structure is present in the coenzyme was substantiated in a study of the products of both acid and alkaline hydrolyses of CoA. It was shown by paper chromatography that the phosphate of pantothenic acid produced in this way was indistinguishable from synthetic pantothenic acid 4′-phosphate (4). This was confirmed later by the isolation and full chemical identification of this phosphate from an alkaline hydrolyzate of CoA (9). It is known that no phosphoryl migration occurs in pantothenic acid phosphates under these hydrolytic conditions.

Since pantothenic acid 4′-phosphate is inactive in stimulating the growth of *A. suboxydans* it follows that ASF is not simply a phosphate of pantothenic acid. It was suggested (4) that ASF is actually pantetheine 4′-phosphate (XX) and later developments have shown this to be correct. However, enzymic evidence on concentrates of ASF seemed to suggest that the 2-mercaptoethylamine residue is not present (65) and the possibility was considered that some small unidentified group is present in ASF and hence also in CoA.

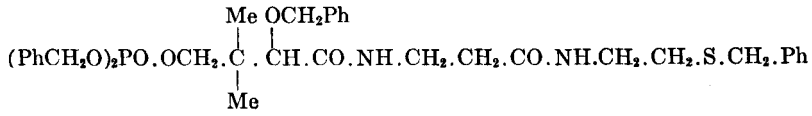
Proof for the nature of ASF and its identity with the degradation product from CoA was obtained through a chemical synthesis of pantetheine 4′-phosphate (11) and the isomeric 2′-phosphate (10). An unamibuous synthesis of the 4′-phosphate was effected in the fol-

lowing manner. Pantothenic acid 2′-benzyl ether (XII) was converted into pantetheine $O^{2'}$,*S*-dibenzyl ether (XVIII) by reaction with ethyl chloroformate followed by 2-benzylthioethylamine:

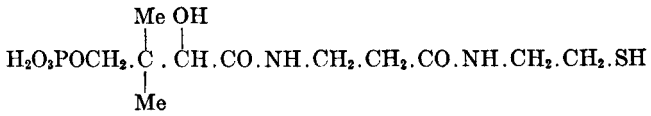


(XVIII)

The primary hydroxyl group in this compound was phosphorylated with dibenzyl phosphorochloridate [$(PhCH_2O)_2PO.Cl$], giving the neutral dibenzyl phosphoric ester (XIX). The four benzyl groups were removed with sodium in liquid ammonia, giving pantetheine 4′-phosphate (XX):



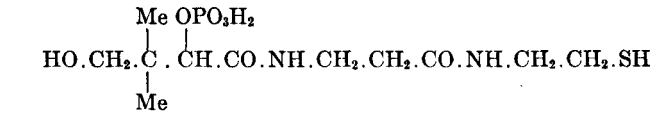
(XIX)



(XX)

A more convenient synthesis involves direct phosphorylation of pantetheine with dibenzyl phosphorochloridate, then removal of benzyl groups with sodium in liquid ammonia.

Pantetheine 2′-phosphate (XXI) was prepared by heating together pantolactone 2-phosphate (IX) and β-alanyl-2-mercaptoethylamine (VII):



(XXI)

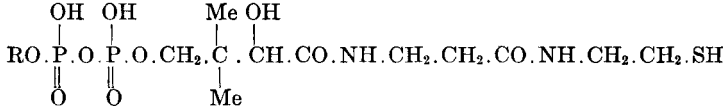
Pantetheine 4′-phosphate is readily converted into CoA by a mixture of enzymes from pigeon liver together with ATP. The 2′-phosphate and the cyclic phosphate of pantetheine (see p. 16) are completely inactive in this system. It was concluded that pantetheine

4′-phosphate is identical with the liver degradation product from CoA, and consequently also with ASF (13). Its identity with ASF was confirmed later by direct comparison with the natural material in growth studies on *A. suboxydans* (36).

### *2. The Pyrophosphate Linkage*

The presence of a pyrophosphate linkage in CoA was first demonstrated by the action of a pyrophosphatase from potato (70). Rapid inactivation of CoA was observed with this enzyme. These findings were not generally accepted at first (44), but it is now known that failure to repeat the enzyme experiments was due to incorrect control of pH during incubation (66). The action of a snake venom pyrophosphatase on CoA confirms the presence of a pyrophosphate linkage (88,89). Chemical support for the pyrophosphate structure has also been obtained (9) (see later).

The first correct partial formula incorporating the features described so far for CoA is shown below (6) (XXII):

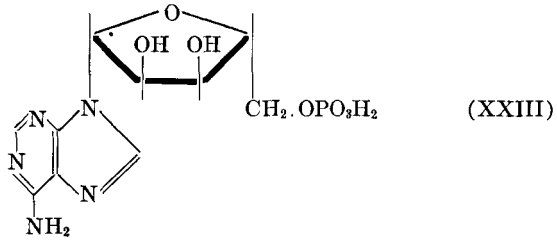


(XXII)

Whereas it was recognized that R is an adenosine residue the total number of phosphate groups in CoA was uncertain at that time and precise determination of the position of the pyrophosphate linkage on the nucleoside was not possible. A final solution of the problem was only possible after the nature of the nucleotide fragment had been clarified.

### *3. The Adenosine Phosphates from CoA*

Mild acid or alkaline hydrolysis of CoA yields a number of phos-



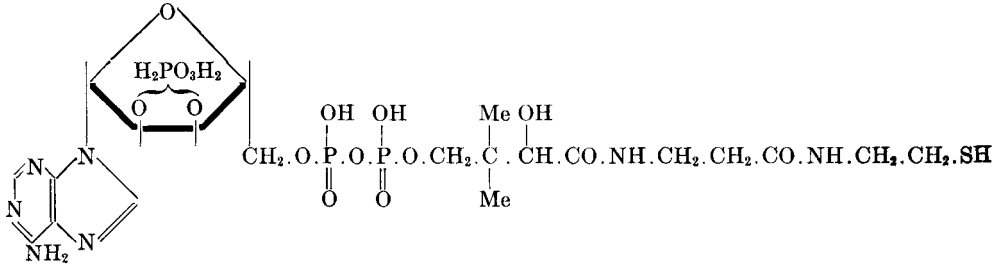
(XXIII)

phoric esters. One of these was identified as adenosine 5′-phosphate (XXIII) by comparison on paper with a synthetic sample (4).

The presence of a phosphate group at position 5′ in the adenosine moiety in the coenzyme has also been established by enzymic methods. When CoA is hydrolyzed by the pyrophosphatase from potato (45) one of the products is adenosine 5′-phosphate. This has been identified by the action of a specific "adenosine 5′-phosphate-deaminase" (68) and also by paper chromatography (73).

Nucleosides and nucleotides which are unsubstituted at positions 2′ and 3′ are oxidized by periodate to dialdehydes, which can be demonstrated on paper chromatograms by spraying them with Schiff's reagent (22). Nucleotides which bear phosphate groups at positions 2′ or 3′ are unaffected by this treatment. CoA does not give a color on paper in this test and consequently must be substituted at one or both of these positions. These findings, together with the evidence for the nature of the other linkages discussed below, led to the conclusion that a single phosphate group is present at either position 2′ or 3′ in the adenosine moiety in CoA (5). It was not possible on the above evidence to distinguish between these two possibilities.

It was found earlier (68) that of the three phosphate groups present in CoA one is a monoester and two are diesters (pyrophosphate). In the formula (XXIV) then advanced for CoA these features were incorporated, together with the foregoing evidence concerning the location of phosphate groups (5):

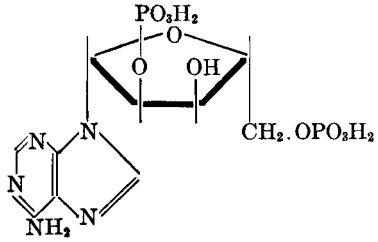


(XXIV)

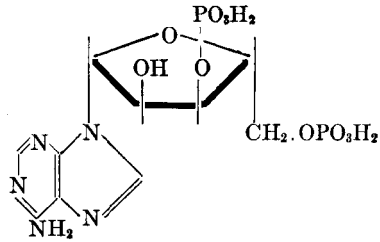
Chemical methods for locating the phosphate group in nucleoside 2′- and 3′-phosphates were not available at that time. However, the problem of the precise location of the primary phosphate in CoA has been resolved by enzymic methods which, considered together with recent evidence for the structure of the isomeric phosphates of adeno-

sine, have established that the monoester phosphate group in CoA occupies position 3′.

The two isomeric monophosphates of adenosine which are produced by alkaline hydrolysis of ribonucleic acid are known as adenylic acids *a* and *b* (23). It is now established that these are, respectively, the 2′- and 3′-phosphates of adenosine (17,35). A phosphatase has been isolated from barley which is specific for the hydrolysis of the *b* series of nucleotides and has no action upon either *a* or 5′-nucleotides. This enzyme readily hydrolyzes one phosphate group in CoA, and consequently this phosphate must be present on the *b* or 3′ position in the adenosine moiety (88,89). In this respect CoA differs from triphosphopyridine nucleotide (TPN). The latter contains an adenosine 2′,5′-diphosphate structure (XXV) (46) and is unaffected by the barley enzyme:




(XXV)

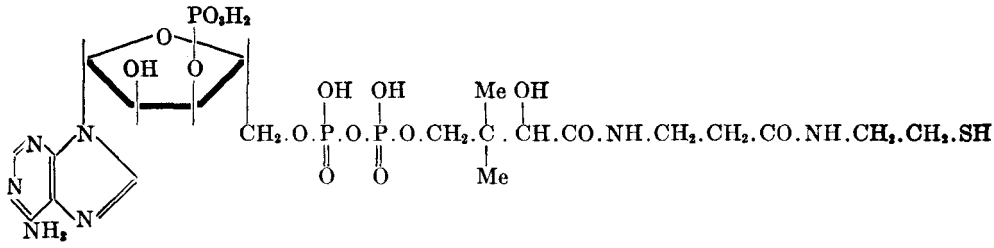



(XXVI)

Confirmation of the presence of an adenosine 3′,5′-diphosphate (XXVI) structure in the CoA molecule has been obtained by a study of the action of a snake venom pyrophosphatase (88,89). This pyrophosphatase hydrolyzes CoA giving adensoine 3′,5′-diphosphate (XXVI) itself. This differs in its behavior on paper from the 2′,5′-diphosphate obtained from TPN. Furthermore, the diphosphate from CoA gives adenosine 5′-phosphate (XXIII) after treatment with the barley enzyme and adenosine when treated with the barley enzyme and a 5′-nucleotidase. The diphosphate (XXVI) from CoA is deaminated by taka-diastase deaminase. This enzyme will attack adenosine 3′-phosphate but not the 2′-phosphate. The 2′,5′-diphosphate from TPN is unaffected by the deaminase.

It is apparent then, from the combined evidence of chemical hydrolysis, periodate oxidation, and enzymic hydrolysis, that CoA contains an adenosine 3′,5′-diphosphate structure.

The final structure for CoA which emerges from all these considerations is represented by (XXVII):



(XXVII)

Strong support for the pantetheine 4′-pyrophosphate structure shown in this formula was obtained during a study of the action of dilute alkali on the coenzyme (9). Besides pantothenic acid 4′-phosphate a cyclic phosphate was isolated which is identical with pantothenic acid 2′,4′-hydrogen phosphate (XVII). In this respect CoA shows some similarity to both flavin-adenine-dinucleotide and uridine-diphosphate-glucose. Both these coenzymes readily yield cyclic phosphates under alkaline conditions. The flavin coenzyme gives riboflavin 4′,5′-hydrogen phosphate (26) and the uridine coenzyme gives glucose 1,2-hydrogen phosphate (49,75). Cyclic phosphate formation occurs through intramolecular phosphorylation involving the pyrophosphate group and a suitably placed hydroxyl group. The similar behavior of CoA strongly supports the pyrophosphate structure. The cyclic phosphate grouping could not be present *per se* in the original coenzyme since no cyclic derivatives of pantothenic acid phosphates are formed under acidic conditions. These cyclic phosphates are fairly stable to acids.

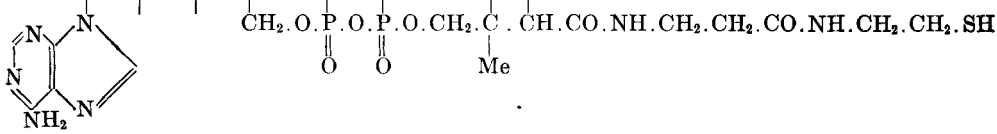
Another product of alkaline hydrolysis of CoA was shown to be the cyclic phosphate of pantetheine (or pantethine) (9). The structure of this degradation product was proved by synthesis. In the first route pantethine was phosphorylated directly with moist phosphoryl chloride whereupon the disulfide form of structure (XXVIII) was obtained. A second route depended upon a novel reaction. Pantolactone 2-diphenyl phosphate (VIII) was heated with an excess of β-alanyl-2-mercaptoethylamine (VII). Cyclization and loss of phenyl groups was accompanied by amide formation to give pantetheine

2′,4′-hydrogen phosphate (XXVIII) identical with the substance formed from CoA (10):

(XXVIII)

### *4. Enzymic Synthesis*

Good confirmation of formula (XXVII) for CoA has been obtained by enzymic resynthesis experiments. Enzymes have been separated from liver that convert pantetheine into CoA by the following route (31,50,51). First, pantetheine is converted into pantetheine 4′-phosphate by ATP. This then reacts with a second mole of ATP in the presence of a different enzyme to give inorganic pyrophosphate and 3′-dephospho-CoA (XXIX). Finally, a third enzyme together with ATP converts 3′-dephospho-CoA into CoA:



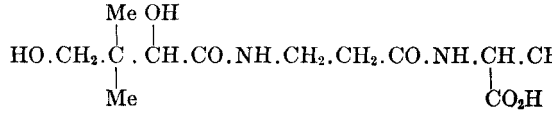
(XXIX)

It is clear from this enzymic synthesis, as also from the action of the *b*-nucleotidase and certain chemical properties, that the monoester phosphate is the one at position 3′ and that a 3′-pyrophosphate formulation for CoA is untenable. 3′-Dephospho-CoA was first formed by the action of a phosphomonoesterase on CoA (70) but only recently have its isolation been achieved and its properties studied (87,89). It is without activity in the routine phosphotransacetylase test for CoA but at higher concentrations it shows slight activity. It is, of course, active in the acetylation tests which incorporate a liver enzyme preparation since under these conditions it is converted into CoA.

Studies have been made on the earlier stages of CoA biosynthesis. These relate to the mechanism of pantetheine formation from panto-

thenic acid. *L. arabinosus* is known to produce CoA from pantothenic acid only if cystine is present in the medium. Cystine cannot be replaced by 2-mercaptoethylamine. Consequently, it appears that pantetheine is formed enzymically from pantothenic acid and cysteine, but not from pantothenic acid and 2-mercaptoethylamine (76).

Of the fairly large number of derivatives of pantothenic acid which have been examined as possible CoA precursors only pantothenyl cysteine (XXX) is more effective than pantothenic acid (21). This peptide has been synthesized by several different routes (2,15) and is now established as a pantetheine precursor in *Acetobacter suboxydans* (2,21,36). Furthermore, it is converted into pantetheine by an enzyme present in liver (31). Presumably the conversion involves a decarboxylase.



(XXX)

The course of the enzymic synthesis of CoA can be formulated in the following way:

pantothenic acid ⟶ pantothenyl cysteine ⟶ pantetheine ⟶ pantetheine 4′-phosphate ⟶ 3′-dephospho-CoA ⟶ CoA

There remain certain features of CoA biosynthesis which are not readily explained by the above scheme. These concern the synthesis of pantetheine from pantothenic acid. Pantetheine is not utilized by all microorganisms as a CoA precursor, when supplied in the medium. Furthermore, in the absence of cystine *L. arabinosus* will convert pantothenic acid into its 4′-phosphate (1). It would seem possible then that phosphorylation might, under certain circumstances, precede reaction with cystine. In this connection it has been shown that pantothenyl cysteine 4′-phosphate has about 60% of the activity of pantothenyl cysteine in stimulating the growth of *A. suboxydans* (2).

## IV. The Functional Group of CoA

No discussion on the structure of CoA would be complete without some consideration of the function of the coenzyme in natural processes and, in particular, the over-all mechanism of its action. However, the ramifications of the reaction processes now extend so widely

into different branches of biochemistry that it becomes impossible to consider CoA reactions here in any but the briefest general terms. The discovery by Lynen and his collaborators (60) of the nature of "active acetate" marked the beginning of our understanding of the mechanism of action of CoA. By the use of iodoacetate and nitroprusside they discovered that the sulfhydryl group in CoA becomes substituted during acetyl transfer. Furthermore, they were able to demonstrate the formation of an intermediate thiol acetate. This intermediate, *S*-acetyl-CoA, was identified as the so-called "active acetate." The reaction, in its broadest sense, is represented by the following scheme:

$$\mathrm{AcX} + \mathrm{CoA{-}SH} \longrightarrow \mathrm{HX} + \mathrm{CoA{-}SAc}$$

$$\mathrm{CoA{-}SAc} + \mathrm{HY} \longrightarrow \mathrm{CoA{-}SH} + \mathrm{AcY}$$

The source of acetyl groups (AcX) may be one of several types (*e. g.*, acetate in the presence of ATP, pyruvate, acetyl phosphate, etc.). HY is the substance which becomes acetylated in the scheme, and CoA-SH is the reduced form of CoA. It will be seen that this is regenerated continuously during the reactions.

It is now known that in addition to acetic acid a number of other carboxylic acids participate in enzymic transformations in the form of their thio esters with CoA. These include benzoic, succinic, and a number of hydroxy, keto, and unsaturated acids concerned in the synthesis and degradation of long-chain fatty acids and in glyceride synthesis. The biochemical significance of the enzymic reactions which these derivatives undergo has been discussed in recent reviews (53,58,59).

Finally, some consideration of the special chemical properties of thiol esters would seem to be appropriate. This class of compounds has been studied recently with the object of clarifying the nature of acyl transfers of the type shown by CoA derivatives. In particular it was intended to demonstrate chemical analogies for the enzymic processes. Thiol esters, especially those of 2-mercaptoethylamine (7,47,91) and pantetheine (12,40,79), are powerful acylating agents, being most reactive toward amines. In this respect they are considerably more reactive than their corresponding *O*-esters. Full details of the electronic transformations and nature of the transition states in acylation reactions of thiol esters are not known. Also, the exact nature of condensations involving the acetate methyl group of

acetyl-CoA is not fully understood. In this last type of reaction, however, it is probable that activation of the acetyl-CoA involves loss of a proton from the methyl group:

$$CoA-S.CO.CH_3 \rightleftharpoons CoA-S-CO.CH_2^{(-)} + H^{(+)}$$

It is interesting to note that the $\alpha$-hydrogen atoms of an acid become more acidic through binding of the carboxyl carbon to sulfur; consequently thiol esters of acids tend to release protons from the $\alpha$-position much more readily than do the corresponding *O*-esters (58).

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# COAGULATION OF THE BLOOD

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> *. . . an absolutely clear and exhaustive understanding of any single thing in the world would imply a perfect comprehension of everything else.* —SCHOPENHAUER

## CONTENTS

In the conclusion of the last review on this subject which appeared in *Advances in Enzymology* it was observed "that the field of blood clotting has developed to such an extent that in the future any thorough treatment would have to be restricted to certain pertinent parts of the whole complex in order to fulfill its purposes without becoming too voluminous." Finding myself in agreement with this, the material was selected on the basis of what seemed of the most interest to me at this time. The fibrinolysin mechanisms are not considered. In a previous review, reference was made to important books and monographs. It is now possible to add references to additional works which have appeared recently (4,18,22, 30,62,93,133,163,194,217,272,273,308,310,313).

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## I. Introduction

The basic chemical events which occur during the clotting of blood may be represented by a diagram such as Figure 1. The main ideas refer to the transformation of fibrinogen to fibrin and cofibrin on the basis of the enzyme activity of thrombin. This thrombin is derived from prothrombin. Many substances are involved in this transformation of prothrombin to thrombin, and they may be divided into two classes, namely, those acting in a positive way to bring about activation and those which function negatively to retard activation. The activators and inhibitors, like prothrombin itself, may have their origin in precursors. So in certain instances there is no interaction with another molecule until the first molecule itself has undergone a transformation and the second one may likewise have been altered. The details for such preliminary events have not as yet

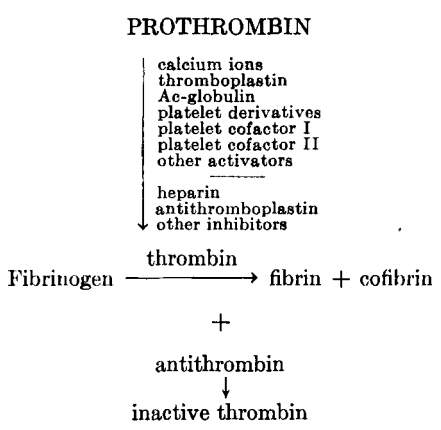


Fig. 1. A diagrammatic representation of the blood coagulation mechanisms.

been as well described as the conversion of fibrinogen to fibrin or the change of prothrombin to thrombin. In many instances the substances which play an active role in such chemical events are not found after the clotting mechanisms are spent. The best understood example is thrombin itself which is neutralized by antithrombin activity.

## II. Purification Work

### A. INTRODUCTORY REMARKS

Since there are so many substances that participate in the reactions that take place when blood clots, and since they all interact more or less simultaneously, it is impossible to deduce anything exact about the details of the chemical mechanisms that are involved. Nor is it possible to have much of an idea about the properties of these individual substances. For these reasons it is important to obtain these substrates in as purified a form as possible. Their properties can then be studied individually, and simple interactions may be described. As a first approximation it is even profitable to have concentrates of hypothetical substances in support of evidence for their existence. In most instances purification work is a major undertaking mainly because the clotting factors are represented in trace quantities together with mass quantities of albumen and globulin. In many instances their activity is easily destroyed by common

laboratory manipulations. Even when great progress has been made in obtaining a substance in essentially purified form continuous effort is required to keep supplies on hand, for these are complex molecules and they are not prepared by synthesis. The methods must, therefore, be practical and give good yields.

Despite these and other handicaps this approach to the problem of understanding the chemical mechanisms of blood coagulation is quite important.

## B. FIBRINOGEN

This is one of the first proteins ever separated from plasma and there are several procedures by which valuable preparations may be obtained. Only a few of these, however, yield a product which may be regarded as pure fibrinogen. Fibrinogen has been obtained in crystalline form (20,156).

The main principles employed for its preparation have been the following: (*a*) salting out with $(NH_4)_2SO_4$ and other salts (19,44,113,116,132,230,232,247), (*b*) precipitation by adding ether in the cold (148), (*c*) precipitation by fractionating with alcohol in the cold (221,222,300), and (*d*) the so-called freeze-thaw technique in which frozen plasma is thawed to 0°C., and the insoluble fibrinogen is collected by centrifugation (365).

There are certain advantages and disadvantages in the use of any one of these techniques. In general, the $(NH_4)_2SO_4$ fractionation procedures do not yield a product of high purity and it often contains other blood coagulation components associated with it. In the alcohol fractionation technique fibrinolysin as well as the antihemophilic factor are frequently found in variable quantities. With the freeze-thaw technique an unusually stable product is obtained and the product is easy to isolate. However, there is a great disadvantage in the need for rather large quantities of raw material. For example, it is quite convenient to work with 12 to 24 liters of plasma. Laki (162) has reworked a product obtained by alcohol fractionation techniques by again fractionating with $(NH_4)_2SO_4$. Likewise, fractionation with $(NH_4)_2SO_4$ of the fibrinogen obtained by freeze-thaw techniques can yield a very elegant fibrinogen product, which shows stability characteristics not observed with other products. Another useful technical manipulation was introduced by Lorand (187). Fibrinogen products are dialyzed against ammonium acetate or $(NH_4)_2CO_3$ or other suitable volatile salts. When these products are dried from the frozen state much of the salt sublimes and one obtains a dried fibrinogen preparation low in salt concentration. For certain studies in blood coagulation fibrinogen may be filtered through Seitz filters to remove certain undesirable components. For the same reasons adsorbing agents such as $BaSO_4$ may also be used.

### C. Ac-GLOBULIN

The first attempts to obtain this factor in purified form were made by Ware and Seegers (370). The activity was adsorbed on $Mg(OH)_2$, eluted by decomposing the $Mg(OH)_2$ with $CO_2$ under pressure, and then fractionated with $(NH_4)_2SO_4$ in the cold. The product was far from being a single substance and it was not possible to come to any exact conclusion about the physicochemical properties of Ac-globulin. The product was not free from prothrombin. The preparations were, however, very potent and it could be said that Ac-globulin represents less than 0.7% of the total plasma proteins. This established the fact that it is one of the plasma trace proteins. The chief difficulty with the method of purification was encountered with the $Mg(OH)_2$, which is quite alkaline and probably destroys much activity on the basis of pH changes. During the same year in which the work of Ware and Seegers appeared, Owren (250) fractionated plasma with the use of ether in the cold and was able to obtain a product largely free from prothrombin, the latter having been removed from the original plasma by Seitz filtration. The degree of purification was 100-150 times in terms of activity per milligram of nitrogen. On that basis it must be considered that a crude product was obtained.

Lanchantin and Ware (168) and Lewis and Ware (178,375) prepared human serum Ac-globulin. The plasma Ac-globulin was precipitated with acid after removal of prothrombin on $BaSO_4$. The plasma Ac-globulin was then converted to serum Ac-globulin by adding a small amount of thrombin. The accelerator was then adsorbed on Amberlite IRA-400, and eluted with 3% NaCl. They observe that, in contrast to plasma Ac-globulin, the active accelerator is adsorbed on $BaSO_4$ and on $Ca_3(PO_4)_2$.

Potent preparations of serum Ac-globulin were obtained from bovine serum by Ware and Seegers (371). They used approximately the same methods which they used for the preparation of plasma Ac-globulin. Although this preparation was also a crude product, it did serve to lend support to the view that serum Ac-globulin is something quite different from plasma Ac-globulin. Certainly further work on the purification of Ac-globulin is needed, not only to find more suitable and more convenient methods, but also to obtain the material essentially free from impurities for use in scientific studies and for characterizing its physicochemical properties.

## D. PROTHROMBIN

Descriptions of the preparation of prothrombin products by Fuchs (96) and by Mellanby (206) appeared in the same year. The preparations which they obtained were not very potent by present-day standards. Each description, however, contained most valuable information for future use.

Mellanby showed that prothrombin is precipitated from diluted plasma by acidification and later work (50) showed that the yield in this first precipitate could be raised to 100% by the simple expedient of keeping the salt concentration low. The work of Fuchs represented the important concept of purifying blood coagulation components by adsorbing them on inorganic salts from which they could subsequently be eluted. His work, however, involved direct adsorption from plasma which gives a rather low percentage of adsorption and many impurities accompanying the prothrombin. Another purification procedure involving direct adsorption on $Al(OH)_3$ has been described (224). Direct adsorption on barium salts has also been attempted (72,345). These direct adsorption and elution techniques, by and large, are limited in one respect or another for obtaining materials free from other clotting factors or for obtaining the highest activity that is possible. Fractionation of plasma with $(NH_4)_2SO_4$ (216) has also thus far not yielded a product of high specific activity.

An exhaustive study of the possibilities for the purification of prothrombin was made by the reviewer in an intensive study covering approximately 15 years of work (70,292,294,298,314,315). In the method devised the prothrombin is first precipitated quantitatively from diluted plasma, and then adsorbed on $Mg(OH)_2$, eluted by decomposing the $Mg(OH)_2$ with $CO_2$, fractionated with $(NH_4)_2SO_4$, precipitated isoelectrically, and finally additional impurities are removed by adsorption on $BaSO_4$. It was eventually possible to obtain a product suitable for scientific study. Confidence in the value of the product obtained by these methods was exhibited by establishing a routine for its production on a research basis and sending the purified prothrombin to many colleagues for their use.

Physicochemical characterization by Lamy and Waugh (166) gave the following data: $S = 4.84$, $D = 6.24 \times 10^{-7}$ sq. cm. per second, intrinsic viscosity = 0.041, and partial specific volume, $V = 0.070$. They point out that, with the exception of the value for the partial specific volume, this corresponds to the properties of plasma albumin. The electrophoretic mobility in the Tiselius apparatus corresponds approximately to $\alpha_2$-globulin (307). The electrophoretic pattern thus far has never corresponded exactly to that of a homogenous substance. Some material of slower mobility invariably remains. The material with slower mobility corresponds to that which first increases its quantitative representation when prothrombin changes to thrombin. In the ultracentrifuge the bovine materials

were remarkably uniform and the boundary spreading corresponded to that of homogeneous material. The method of purification was also applied to human materials and the activity obtained was even higher than that of bovine prothrombin (314). This human material, however, did not appear to be a single substance when examined in the ultracentrifuge. More work on purified human prothrombin is needed. The bovine preparation can at least be regarded as representing largely prothrombin itself. There are profound changes in the physicochemical properties of the material when it changes to thrombin. From the standpoint of considering the purity of the product the only remaining question seems to be whether many trace impurities of significance may remain. A careful study of the Ac-globulin content indicates that perhaps 0.2% of the total protein may be Ac-globulin (315). This is a significant amount of Ac-globulin but it can be removed by heating aqueous solutions of the product at 53°C. Prothrombin is apparently not altered by the heating if the salt content of the solution is very low (373). The factor VII content is evidently quite low inasmuch as the preparations do not correct the deficiency represented by factor VII deficient plasma (134), and Owen, Magath, and Bollman have concluded (243) from their critical studies that factor VII is not present in appreciable quantities.

Laki and associates have made a quantitative analysis of the amino acid composition of prothrombin (164). They find that 18 amino acids are represented. The sulfur is present as cystine (—S—S—) and methionine. This is of interest because Carter and Warner (43) have showed that agents commonly used to block the reactivity of —S—S— linkages inhibit the production of thrombin, whereas those that inhibit —SH groups do not. Glutamic acid, aspartic acid, and arginine are present in highest weight percentages. The amino acids are present in entirely different proportions than those found in plasma albumin. Thus, while the size and shape of the molecule may be like that of albumin, the fundamental composition is quite different. Prothrombin contains carbohydrate as an integral part of the molecule; at least part of this is glucosamine (298). Schultze and Schwick (291) believe that prothrombin contains heparin, but their supporting evidence for that view does not appear to be adequate. Recently (214) a method was devised for separating the carbohydrate from the remainder of the prothrombin and it appears to be a polysaccharide.

## E. THROMBOPLASTIN

First it seems appropriate to remark about nomenclature, as so nicely reviewed by Milstone (217). He states that Morawits introduced the word thrombokinase to designate the active substances of tissue juices and that Nolf used the term thromboplastin to refer to adjuvants. Even today we have no knowledge of the chemical transformation which these substances bring about. Consequently, Morawits and Nolf were clearly reserving the term thrombokinase for a substance involved in certain chemical reactions that were hypothetical and the term thromboplastic referred to other reactions that were equally hypothetical. The reviewer has been accustomed to use the word thromboplastin for substances from tissues like lung and brain, and to follow more nearly the usage of Howell (127). Apparently most investigators in this field are using the term in that sense. However, Biggs and Macfarlane (29) have regarded this as an incomplete thromboplastin and use the words blood thromboplastin for a combination of factor VII, antihemophilic factor, Christmas factor, Ac-globulin, and platelets.

Chargaff and associates (45,47) were able to isolate the thromboplastic protein from bovine lung extract by alternating high-speed centrifugation and redissolving of the precipitate. The molecular aggregate contains protein, lipide, nucleic acid, and carbohydrate. Their work has been repeatedly confirmed in other laboratories. The ease with which thromboplastin can be sedimented makes this a very convenient source of activator material in a variety of studies in blood coagulation. Contamination of the product with materials of the plasma can be greatly reduced if the lung is first perfused.

## F. FACTOR VII

### (SPCA, Cothromboplastin, Stable Conversion Factor, Convertin, etc.)

Concentrates have been prepared in three different laboratories by employing the technique of adsorption on barium salts and elution with sodium citrate or phosphate buffer. No exact information (9,63,155) is available on the comparative activities of products obtained in these laboratories.

In the work of Deutsch and Schaden (63) the electrophoretic pattern showed a major component plus two others. It could not be said which of these repre-

sented factor VII, although it was presumably the large peak. The electrophoretic mobility of this component was definitely less than that of prothrombin. It is important to note, in view of the uncertain role of factor VII in blood coagulation mechanisms, that these preparations were all obtained from serum and not from plasma, and that they undoubtedly contain several substances concerned with prothrombin activation. Recently McClaughry (198) used serum, free from prothrombin, and handled it exactly as is ordinarily done in the purification of prothrombin by the methods of Seegers and associates (292,298,314,315). He obtained a preparation with two major components in the electrophoretic apparatus which had a very powerful effect on the activation of purified prothrombin, the latter having been obtained from *plasma* by the same method of purification.

## G. PLATELET COFACTOR I

## (Antihemophilic Factor)

It is well known that fraction I obtained by cold ethanol fractionation of plasma (345) may contain activity effective in correcting the clotting defect of hemophilic plasma. The potency of the preparations is, however, variable and they have not been studied in terms of exact assay procedures.

Johnson, Smathers, and Schneider (136) have prepared concentrates from plasma by $(NH_4)_3SO_4$ fractionation techniques. Although they present a method for the quantitative assay of the material, again there are no exact data given concerning the degree of purification of their product. It is interesting, however, that they were able to obtain preparations from serum that were as potent as preparations obtained from plasma. Evidently, since the antihemophilic material is not found in serum, it could only be obtained in these preparations by dissociating it from an inhibitor. Laki and Lorand (160) found that cofactor I activity may be adsorbed on kaolin from acidified plasma. Later (191) they continued their work and were probably able to produce the most potent preparations thus far obtained. Even these, however, did not represent the pure material, and exact analytical data on potency are not given. The adsorption on kaolin is a most valuable technique and seems to be promising for large-scale production of this factor.

Brinkhous (39) and associates have also obtained fractions of plasma with antihemophilic activity and in the work of Tocantins (357) he separated the antihemophilic material from hemophilic plasma itself. He was primarily interested in demonstrating that it could be obtained from this source and not so much in presenting a method for preparing a high-quality product.

Spaet and Kinsell (337) have used ordinary $(NH_4)_2SO_4$ fractionation techniques to obtain cofactor I activity from bovine sources. It had approximately 70 times the cofactor I activity of fresh human plasma per milligram of protein. It is

difficult to state how this potency compares with other products. Paper electrophoresis indicated that the activity is associated with $\beta_2$-globulins. They point out that the antihemophilic factor of human plasma, as compared with bovine plasma, is unstable on storage and shows great loss when fractionation attempts are made. It seems likely that cofactor I can soon be available in sufficient quantity and purity for an exact description of its physicochemical properties.

### H. PLATELET COFACTOR II

(Plasma Thromboplastin Component or PTC, (3))

White, Aggeler, and Glendening (386) have prepared concentrates of this factor from plasma, but prefer to prepare it from serum. First the serum is acidified for the purpose of destroying the remaining prothrombin and factor VII. Then platelet cofactor II is adsorbed on $BaSO_4$ and is subsequently eluted with the use of sodium citrate solution. Data on purity are not available.

### I. INHIBITOR OF PLATELET COFACTOR I

Simple ether extraction of serum or plasma yields a material which acts as a powerful inhibitor of platelet cofactor I (143,357). When the material is transferred to aqueous solvents it is difficult to obtain adequate dispersion. Apparently the active principle is a lipide. In an ultrasonic apparatus the activity of suspensions can be greatly increased. When such suspensions are frozen they again lose much activity, presumably on the basis that large aggregates form. Thus far no one has described the chemical nature of the active material. An inhibitor can also be separated from brain and other tissues (357).

## III. Formation of Fibrin

### A. INTRODUCTORY REMARKS

The recent reviews by Laki (163) and Ferry (81) and the papers appearing in the recent symposium on the chemistry of prothrombin and fibrinogen (87,165,192,382) are valuable for consultation. The general perspective is perhaps well portrayed by the remarks of Lorand (189) who states:

"It is believed that the clotting of fibrinogen reveals a story that has a significance beyond the borders of blood coagulation itself, and it may be an indication of how certain principles are being utilized in building biological fibers. The mechanism represented by the fibrinogen-fibrin transformation may be a common pattern in the biogenesis of a number of proteins. The enzymically altered protein displays quite different properties from those of the primary one, although it differs very little from it in molecular and chemical constitution."

### B. PRELIMINARY OBSERVATIONS

The work on the interaction of thrombin and fibrinogen required, as a preliminary step, adequate amounts of purified fibrinogen and