Johnson, Mr. Charles Jordan, Jr., and Mr. W. F. Hain for skilled technical assistance. This work was supported in part by U. S. Public Health Service Research Grant GM-2907, from the Division of General Medicine, National Institutes of General Medical Sciences.

The Synthesis of Selenocoenzyme A[1,2]

Wolfgang H. H. Günther and Henry G. Mautner

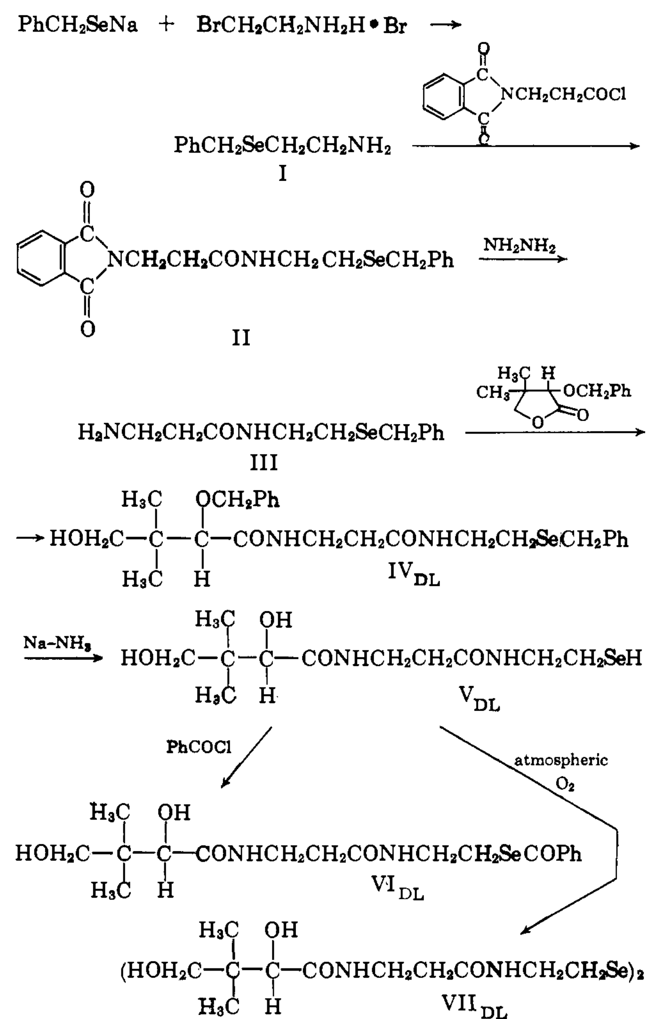
Contribution from the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut. *Received February 11, 1965*

The syntheses of 4'-phosphoselenopantethine, 3'-dephosphoselenocoenzyme A, isoselenocoenzyme A, selenocoenzyme A, and related compounds are described.

The numerous roles played by coenzyme A in metabolic pathways have been the subject of intensive research since the discovery of the requirement for a cofactor for the acetylation of choline in extracts of rabbit brain[3] and the acetylation of sulfanilamide in extracts of pigeon liver.[4] The structure of this cofactor, named coenzyme A by Lipmann, was established in the laboratories of Lipmann,[5-7] Baddiley,[8] and Snell.[9-12] Lynen and his co-workers[13,14] succeeded in characterizing "active acetate" as the acetylthiol ester of coenzyme A, thus demonstrating the vital nature of the single sulfur atom of this molecule to the functions of coenzyme A either in transferring acyl groups to suitable acceptors or in activating a methylene group adjacent to the thioacyl carbon to condensation reactions.[15]

Since the atomic radii of sulfur and selenium are very similar,[16] replacement of the sulfur of coenzyme A with selenium should not affect appreciably either the size of this complex molecule or its ability to fit enzymic receptor sites. However, while acyl derivatives of coenzyme A or of selenocoenzyme A may be considered as being isosteric, their reactivities and electron distributions might be expected to differ. Thus, it has been shown by the use of model compounds that selenol esters will undergo aminolysis much more readily than thiol esters[17,18] although hydrolysis proceeds at similar rates.[2,19] While selenocoenzyme A should have an ability to fit receptor sites similar to that of its sulfur analog, the possibility exists that the strength of binding to receptor sites, or the ability to induce conformational changes in enzymes subsequent to binding, might be different for isologs of this type. In the hope of gaining additional information about

Scheme I



(1) This work was supported, in part, by grants from the National Science Foundation (GB-1626) and the National Cancer Institute of the United States Public Health Service (CA-3937-07).
(2) Part of this work was presented before the Symposium on Coenzymes and Metabolic Pathways, Gordon Conference, Meriden, N. H., July 1964.
(3) D. Nachmansohn and A. L. Machado, *J. Neurophysiol.*, **6**, 397 (1943).
(4) F. Lipmann, *J. Biol. Chem.*, **160**, 173 (1945).
(5) F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle, and B. M. Guirard, *ibid.*, **167**, 869 (1947).
(6) G. D. Novelli and F. Lipmann, *ibid.*, **182**, 213 (1950).
(7) F. Lipmann, *Bacteriol. Rev.*, **17**, 1 (1953).
(8) J. Baddiley, *Advan. Enzymol.*, **16**, 1 (1955).
(9) G. M. Brown, J. A. Craig, and E. E. Snell, *Arch. Biochem.*, **27**, 473 (1950).
(10) E. E. Snell, *et al.*, *J. Am. Chem. Soc.*, **72**, 5349 (1950).
(11) E. E. Snell and G. M. Brown, *Advan. Enzymol.*, **14**, 49 (1953).
(12) W. L. Williams, E. Hoff-Jørgenson, and E. E. Snell, *J. Biol. Chem.*, **177**, 933 (1949).
(13) F. Lynen, E. Reichert, and L. Rueff, *Ann.*, **574**, 1 (1951).
(14) F. Lynen and E. Reichert, *Angew. Chem.*, **63**, 47 (1951).
(15) L. Jaenicke and F. Lynen, *Enzymes*, **3B**, 3 (1960).
(16) L. Pauling, "The Nature of the Chemical Bond," 3rd Ed., Cornell University Press, Ithaca, N. Y., 1960, pp. 246, 260.
(17) H. G. Mautner and W. H. H. Günther, *J. Am. Chem. Soc.*, **83**, 3342 (1961).
(18) H. G. Mautner, S. H. Chu, and W. H. H. Günther, *ibid.*, **85**, 3458 (1963).
(19) S. H. Chu, W. H. H. Günther, and H. G. Mautner, unpublished data.

Scheme II

$$\mathrm{I} + \mathrm{HOH_2C{-}C(CH_3)_2{-}C^*H(OH){-}CONHCH_2CH_2COCOC_2H_5}\ (\text{with } \overset{O}{\|}\ \overset{O}{\|})$$

↓

IV_{DL} $\quad \mathrm{HOH_2C{-}C(CH_3)_2{-}C^*H(OH){-}CONHCH_2CH_2CONHCH_2CH_2SeCH_2Ph}$ $\quad VIII_D$

(1) $(PhCH_2O)_2POCl$
(2) 50% CH_3COOH
(3) Na/NH_3
(4) O_2

$$\mathrm{(H_2O_3POCH_2{-}C(CH_3)_2{-}C^*H(OH){-}CONHCH_2CH_2CONHCH_2CH_2Se)_2}$$

IX_D and IX_{DL}

↓ X (adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate: NH_2, N, N, N, N, O, $CH_2OP(O)(O^-){-}N$ morpholine O, HO OH)

$$\mathrm{Adenosine{-}CH_2O\overset{O}{\overset{\|}{P}}(OH){-}O{-}\overset{O}{\overset{\|}{P}}(OH)OCH_2{-}C(CH_3)_2{-}C^*H(OH){-}CONHCH_2CH_2CONHCH_2CH_2Se)_2}$$

XI_{DL}, dephosphoselenocoenzyme A diselenide

the functions of coenzyme A on the molecular level, the preparation of its selenium isolog was undertaken.

It was reported recently[20] that injection of ^{75}Se-labeled selenite into rats resulted in the incorporation of some radioactivity into coenzyme A isolated from their livers. However, no proof was provided that replacement of sulfur by selenium, rather than addition of selenium to sulfur, had taken place, nor was the "seleno-coenzyme A" isolated. Since it is known that thiols can react with selenite to form S–Se addition products[21,22] which are often difficult to separate from the corresponding sulfur compounds, the preparation of selenocoenzyme A by biological means did not seem promising, and the chemical synthesis of this compound was undertaken.

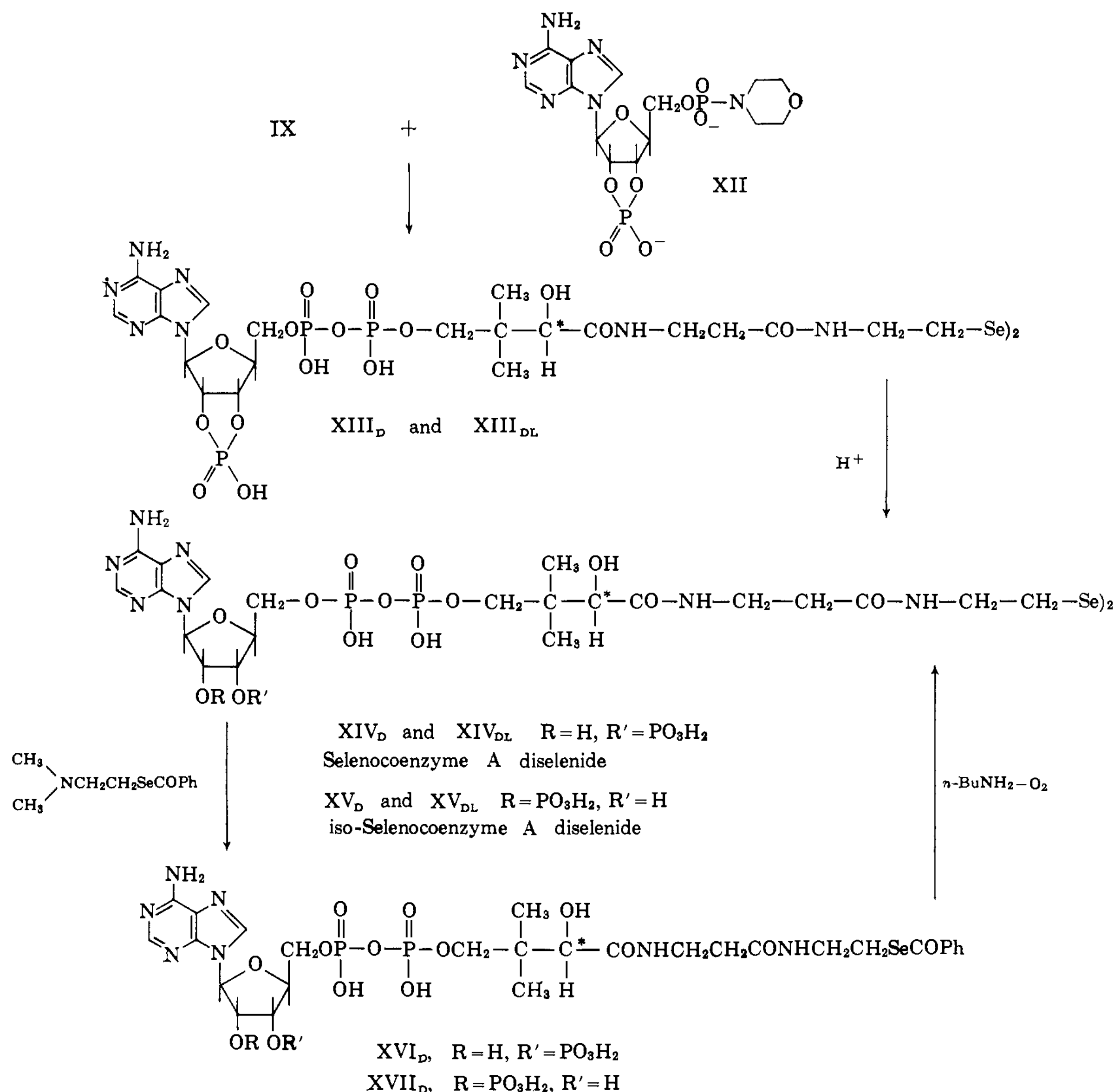
The synthetic scheme used was based on the synthesis of coenzyme A by Moffatt and Khorana.[23] Since the initiation of this work, further syntheses of coenzyme A have been reported by Michelson[24] and Gruber and Lynen.[25] The reaction sequence used for the synthesis of selenocoenzyme A is outlined in Schemes I–III.

The key compound for the synthesis of selenocoenzyme A was 4′-phosphoselenopantethine (IX_D), condensation of which with bis(4-morpholino-N,N′-dicyclohexylcarboxamidinium) adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (XII)[23] yielded the 2′,3′-cyclic phosphate $XIII_D$. Hydrolysis of the latter compound yielded a mixture of selenocoenzyme A (XIV_D) and isoselenocoenzyme A[26] (XV_D).

The synthesis of selenopantethine (VII_D) using the condensation of the mixed carbonic ester anhydride of D-pantothenic acid with selenocystamine was achieved previously in this laboratory.[27] Treatment of selenopantethine with dibenzyl phosphorochloridate resulted in the formation of ethyl acetate extractable material, presumably 4′-dibenzylphosphoselenopantethine. Partial hydrolysis of the phosphoric acid triester with 50% acetic acid and debenzylation with sodium in liquid ammonia yielded a product containing only traces of organic selenium as well as metallic selenium and hydrogen selenide. The difficulties encountered were unexpected in view of the effectiveness of sodium and liquid ammonia in debenzylating unsymmetrical benzyl

(20) K. W. Lam, M. Riegl, and R. E. Olson, *Federation Proc.*, 20, 229 (1961).
(21) J. A. Stekol, *J. Am. Chem. Soc.*, **64**, 1742 (1942).
(22) D. F. Peterson, *Proc. S. Dakota Acad. Sci.*, **30**, 53 (1951).
(23) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961).
(24) A. M. Michelson, *Biochim. Biophys. Acta*, **50**, 605 (1961).
(25) W. Gruber and F. Lynen, *Ann.*, **659**, 1 (1962).
(26) W. H. H. Günther, S. H. Chu, and H. G. Mautner, Abstracts, 143th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept. 1962, p. 10-O.
(27) W. H. H. Günther and H. G. Mautner, *J. Am. Chem. Soc.*, **82**, 2762 (1960).

Scheme III



monoselenides[28,29] and in view of the comparatively high yields obtained in applying the above reaction sequence to the synthesis of 4'-phosphopantethine.[23,30]

To circumvent the problems encountered in the debenzylation of the diselenide the synthesis of 2'-benzyloxybenzylselenopantetheine (IV$_{DL}$) was undertaken. Condensation of benzylselenol[31] with 2-bromoethylamine or ethylenimine yielded 2-benzylselenoethylamine (I), which in turn was treated with β-phthalimidopropionyl chloride to form benzylselenoethyl-β-phthalimidopropionamide (II). Removal of the phthaloyl group with hydrazine yielded benzylselenoalletheine (III), which could be condensed with DL-benzyloxypantolactone[30] to form the desired dibenzylselenopantetheine (IV$_{DL}$), a crystalline solid, in 82% yield.

Phosphorylation of this compound with dibenzyl phosphorochloridate, followed by partial hydrolysis and reduction with a stoichiometric amount of sodium in liquid ammonia, produced 4'-phosphoselenopantethine (IX$_{DL}$), which was isolated in 46% yield as the barium salt of the diselenide. Debenzylation of the crystalline 2'-benzyloxybenzylselenopantetheine was found to provide a more convenient method for preparing selenopantethine than the condensation method reported previously.[27]

The preparation of optically active D-selenopantethine 4'-phosphate proved to be more difficult since the direct phosphorylation of D-selenopanthethine was unsuccessful and since an attempt to prepare optically active 2-benzyloxypantolactone by the reaction of D-pantolactone with sodium and α-chlorotoluene yielded only racemic product.[23] Condensation of D-pantolactone with benzylselenoalletheine yielded a mixture of products, chromatographic separation of which proved to be very difficult. Condensation of 2-benzylselenoethylamine with the mixed carbonic ester anhydride of D-pantothenic acid, followed by phosphorylation with dibenzyl phosphorochloridate in the fashion described previously, yielded D-selenopantethine 4'-phosphate as the barium salt. This compound had $[\alpha]^{29}D$ $+8.4°$ as compared to literature values ranging from $+10.8$[30] to $+14.6°$[32] for the sulfur analog.

(28) E. Painter, *J. Am. Chem. Soc.*, **69**, 229 (1947).
(29) H. J. Klosterman and E. Painter, *ibid.*, **69**, 2009 (1947).
(30) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 1610 (1953).
(31) S. H. Chu, W. H. H. Günther, and H. G. Mautner, *Biochemical Prepn.*, 10, 154 (1963).

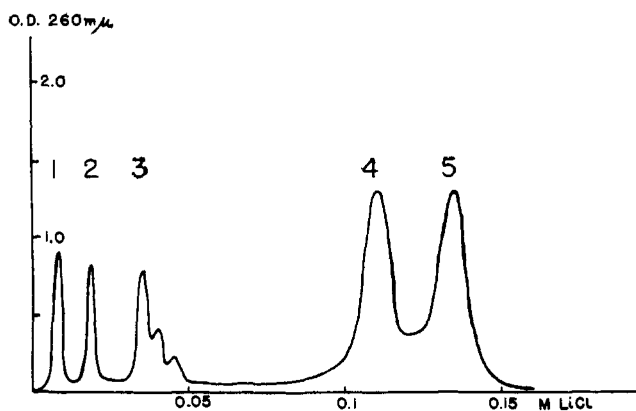


Figure 1. Chromatography of products in the synthesis of DL-dephosphoselenocoenzyme A. Column packed with DEAE Cl^- and eluted with a linear gradient of lithium chloride. Peak 4: the mixed diselenide of DL-dephosphoselenocoenzyme A and of DL-selenopantetheine 4′-phosphate; peak 5: the diselenide of DL-dephosphoselenocoenzyme A. For details see text.

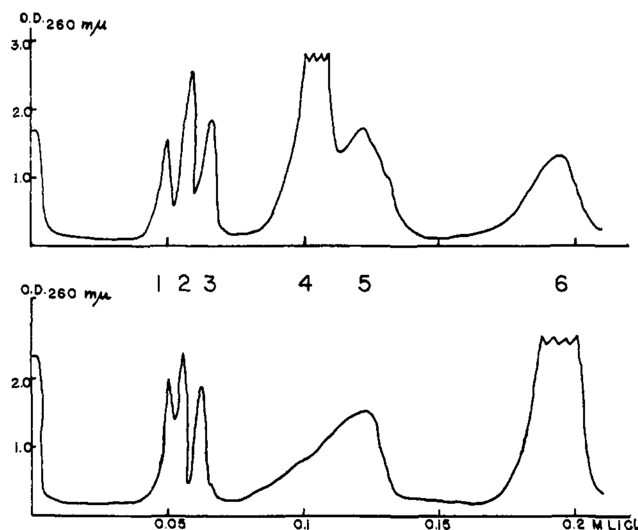


Figure 2 Comparative elution patterns in DEAE Cl^- chromatography of condensation products in the synthesis of coenzyme A (top) and selenocoenzyme A (bottom). Peak 4: coenzyme A–SH, note the absence of a corresponding selenocoenzyme A–SeH peak; peak 6: coenzyme A disulfide and selenocoenzyme A diselenide. For details see text.

Condensation of 4′-phosphoselenopantethine with the adenosine 5′-phosphoromorpholidate (X) in anhydrous pyridine at room temperature was carried out in a fashion analogous to the preparation of dephosphocoenzyme A.[23] The crude reaction products were chromatographed on DEAE–cellulose ion-exchange resin in the chloride form, elution being effected with a linear gradient of lithium chloride. The elution pattern is shown in Figure 1. Compounds eluted before a 0.05 *M* salt concentration was achieved either contained no selenium (peaks 1 and 2) or contained selenium and phosphorus but showed no ultraviolet absorption peaks at 260 mμ (peak 3 and its trailings). Thin layer chromatography of material from peak 4 separated small amounts of 4′-phosphoselenopantethine and dephosphoselenocoenzyme A (XI_{DL}) from a major component shown by elemental analysis to be the mixed diselenide of IX_{DL} and XI_{DL}. On standing in aqueous solution the mixed diselenide slowly rearranged to yield the diselenides of IX_{DL} and XI_{DL}. Thin layer chromatography showed the material in peak 5 to be homogeneous; elemental analysis proved it to be the diselenide of dephosphoselenocoenzyme A which was isolated as the lithium salt.

The synthesis of selenocoenzyme A involved the condensation of 4′-phosphoselenopantethine with the 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (XII) in anhydrous pyridine. Mild acid hydrolysis following removal of solvent pyridine cleaved the 2′,3′-cyclic phosphate of the condensation product to yield a mixture of the 2′- and 3′-phosphates as in the synthesis of the sulfur analog.[23] Figure 2 shows the chromatography on a DEAE–cellulose column of reaction mixtures obtained in carrying out the above reaction either with 4′-phosphopantetheine or with 4′-phosphoselenopantethine. It can be seen that both compounds show a similar elution pattern except that for the selenocoenzyme A isomers the major peak corresponded to the disulfide peak 6 rather than the thiol peak 4. Material in the major selenium peak 6 proved to be an analytically pure mixture of selenocoenzyme A and its isomer isoselenocoenzyme A.

The high oxidizability of selenols presented a major problem. The Ecteola–cellulose column used by Moffatt and Khorana[23] is capable of resolving coenzyme A and isocoenzyme A fairly efficiently when these compounds are present in the thiol form; however, when applied to the disulfides of the 2′-phospho and 3′-phospho isomers, use of this resin resulted in a single broad peak, only the later fractions of which had activity in the pigeon liver transacetylation system,[33] showing that clean separation of the isomers took place only when these were kept reduced. Similarly, Ecteola gave only a partial separation of the isomeric diselenides.

2-Mercaptoethanol, addition of which to the chromatographic system had been used to keep coenzyme A and isocoenzyme A reduced during their separation,[23] was found to be incapable of reducing diselenides. To obtain a more effective reducing agent 2-selenoethanol was synthesized.[34] Addition of this compound to the mixture of the diselenides of XIV and XV resulted in the evolution of hydrogen selenide and the deposition of elemental selenium from the reagent. Chromatography of the mixture under an atmosphere of nitrogen using deaerated solvents on an Ecteola column pretreated with 2-selenoethanol failed to yield any fractions in the region where the selenols would have been expected. The coenzyme A analogs were eluted nearly quantitatively as the isomeric diselenides, although use of the pigeon liver transacetylation system[33] showed that partial resolution had been achieved. As can be seen in Figure 2, the sulfur and selenium analogs gave similar elution patterns on ion exchange columns, an observation which proved to be very helpful in preliminary structure assignment. Similarly, with the use of sodium borohydride, reoxidation to the diselenides could not be avoided whatever precautionary measures were taken.

(32) J. M. Osbond, British Patent 749,715; *Chem. Abstr.*, **51**, 2853 (1957).

(33) N. O. Kaplan and F. Lipmann, *J. Biol. Chem.*, **174**, 37 (1948).
(34) W. H. H. Günther, unpublished data.

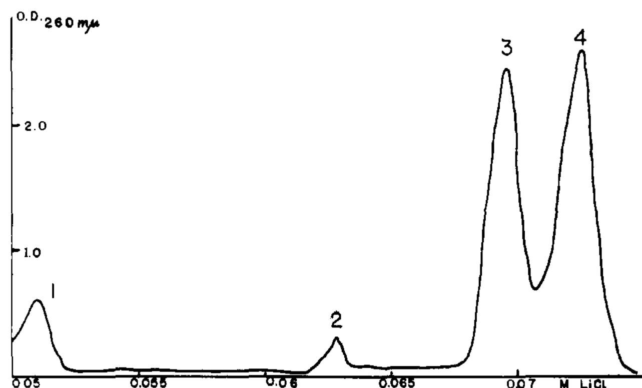


Figure 3. Separation of Se-benzoylisoselenocoenzyme A (peak 3) and Se-benzoylselenocoenzyme A (peak 4) on Ecteola Cl^- by elution with a shallow gradient of lithium chloride. For details see text.

In view of these difficulties, we instituted a search for selenol derivatives which would be stable for a period of several days under the conditions of ion exchange chromatography on Ecteola columns, but which could be cleaved under conditions too mild to damage selenocoenzyme A or isoselenocoenzyme A. The recent observation that thiol esters and selenol esters undergo hydrolysis at comparable rates,[2,19] while selenol esters undergo aminolysis very much more rapidly than their thio isologs,[17,18] suggested that selenol esters might have the desired qualifications. Model experiments with acetylselenocholine and benzoylselenocholine[35] showed that aqueous solutions of these compounds were stable on Ecteola columns but that subsequent to elution the acyl groups could be removed readily with *n*-butylamine. Dimethylaminoethyl selenobenozate,[35] an extremely potent acylating agent,[36] converted the diselenides of selenocoenzyme A and isoselenocoenzyme A to the corresponding selenobenzoyl derivatives in aqueous solution near neutral pH and at room temperature. These were applied to an Ecteola column which had been washed with a dilute solution of hydrochloric acid to maintain the pH below 4. Gradient elution with lithium chloride now yielded well-separated peaks of benzoylselenocoenzyme A and benzoylisoselenocoenzyme A (Figure 3). Treatment with crude rattlesnake venom yielded adenosine 2′,5′-diphosphate and adenosine 3′,5′-diphosphate from material in peaks 3 and 4, respectively. Benzoylselenocoenzyme A and benzoylisoselenocoenzyme A underwent aminolysis at identical rates, as followed by observing the decrease of the selenobenzoyl band at 285 mμ.

Phosphotransacetylase, an enzyme widely used in coenzyme A assays,[37-39] has a specific requirement for reduced coenzyme A and cannot utilize coenzyme A in the disulfide form, dephosphocoenzyme A, or isocoenzyme A.[23] Selenocoenzyme A had neither catalytic nor inhibitory activity in this system, possibly because of difficulties in preventing oxidation to the diselenide. Attempts to obviate this problem by reducing selenocoenzyme A with sodium borohydride and carrying out the enzyme incubation in an atmosphere of nitrogen or hydrogen were unsuccessful. Se-Benzoylselenocoenzyme A was inactive in this system, although S-benzoylation did not reduce the activity of coenzyme A.

On the other hand, the ATP-dependent acetylation of 4-aminoazobenzene by a bicarbonate extract of pigeon liver acetone powder[33,40,41] was markedly influenced by some of the selenium compounds.[2,42,43] In contrast to phosphotransacetylase, the pigeon liver system can utilize either reduced or oxidized coenzyme A, dephosphocoenzyme A, or 4′-phosphopantetheine. Pantetheine has slight activity,[44a] as does isocoenzyme A. Selenocoenzyme A, dephosphoselenocoenzyme A, and 4′-phosphoselenopantethine were all catalytically active in this preparation; isoselenocoenzyme A and selenopantethine were not. The product of the reaction, 4-acetamidoazobenzene, was isolated in quantities greatly exceeding that of the catalyst in all cases in which the selenium compounds were active. While the pH optimum of the reaction, when catalyzed by the sulfur compounds, was 7.6, replacement of these by selenium analogs lowered the pH optimum to 6.7.

In the pigeon liver system the selenium compounds exhibited catalytic activity which was less than that of the corresponding thiols, while at the same time they were capable of reducing the catalytic activity of their sulfur analogs to their own level, when sulfur and selenium isologs were added simultaneously. Selenocoenzyme A and related selenium compounds thus act as "partial agonists"[44b] of their sulfur analogs in this system. In contrast to the above observations, it is interesting to note that selenopantethine completely replaced the natural substrate on a mole for mole basis[45] in cultures of *Lactobacillus helveticus*, an organism requiring preformed pantethine.

Experimental

2-Benzylselenoethylamine Hydrochloride (*I*). Sodium borohydride (7.5 g., 0.2 mole) was added under an atmosphere of nitrogen to a well-stirred solution of dibenzyldiselenide[31] (34.0 g., 0.1 mole) in a mixture of methanol (200 ml.) and tetrahydrofuran (200 ml.) at a rate which kept the evolution of hydrogen from becoming too vigorous. When all the reducing agent had been added and the yellow color of the diselenide had largely disappeared, the mixture was heated to reflux and a solution of 2-bromoethylamine hydrobromide (40.8 g., 0.2 mole) in methanol (150 ml.) was added dropwise over a period of 15 min. The clear solution was heated under reflux for 2 hr., followed by removal of the solvent under reduced pressure. The residue was shaken with 2 *N* sodium hydroxide solution

(35) W. H. H. Günther and H. G. Mautner, *J. Med. Chem.*, **7**, 229 (1964).
(36) W. H. H. Günther and H. G. Mautner, unpublished data.
(37) E. R. Stadtman, *J. Biol. Chem.*, **196**, 527 (1952).
(38) H. U. Bergmeyer, G. Holz, H. Klotzsch, and G. Lang, *Biochem. Z.*, **338**, 114 (1963).
(39) We are indebted to Dr. H. U. Bergmeyer of C. F. Boehringer Söhne, GmbH, Mannheim, Germany, for generously supplying a sample of the crystalline enzyme.
(40) R. E. Handschumacher, G. C. Mueller, and F. M. Strong, *J. Biol. Chem.*, **189**, 335 (1951).
(41) Pigeon liver acetone powder was purchased from the Worthington Biochemical Corp., Freehold, N. J.
(42) H. G. Mautner and W. H. H. Günther, *Federation Proc.*, **21**, 240 (1962).
(43) J. K. Krackov, W. H. H. Günther, and H. G. Mautner, Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., July 1964, p. V-G-174.
(44) (a) O. Brenner-Holzach and F. Leuthardt, *Helv. Chim. Acta*, **39**, 1796 (1956); R. P. Stephenson, *Brit. J. Pharmacol.*, 11, 379 (1956).
(45) H. G. Mautner and W. H. H. Günther, *Biochim. Biophys. Acta*, **36**, 561 (1959).

(150 ml.) and ether (200 ml.), and the aqueous phase was re-extracted twice with fresh portions (100 ml.) of ether. The combined ether extracts were washed with small amounts of 2 *N* sodium hydroxide solution and water, followed by extraction with 3 *N* hydrochloric acid (three 100-ml. portions). The combined acid extracts were evaporated to 50 ml. and cooled in ice to yield a first crop of colorless crystals. The mother liquors were evaporated to dryness and the combined solids were recrystallized from absolute ethanol to yield 2-benzylselenoethylamine hydrochloride (39 g., 78%) as colorless wedges, m.p.[46] 133°.

Anal.[47] Calcd. for $C_9H_{13}NSe \cdot HCl$: C, 43.13; H, 5.63; N, 5.59; Se, 31.50. Found: C, 43.32; H, 5.70; N, 5.78; Se, 31.65.

N-(2'-Benzylselenoethyl)3-phthalimidopropionamide (II). A mixture of I (25 g., 0.1 mole) and 3-phthalimidopropionyl chloride (24 g., 0.1 mole) in benzene (300 ml.) was heated under reflux until the evolution of hydrogen chloride ceased (6–12 hr.). The resulting clear solution was cooled to give II in 90% yield (37.5 g.) as colorless needles, m.p. 123–124°.

Anal. Calcd. for $C_{20}H_{20}N_2O_3Se$: C, 57.83; H, 4.85; N, 6.75; Se, 18.54. Found: C, 58.18; H, 4.88; N, 6.74; Se, 18.54.

Benzylselenoalletheine Hydrochloride (III). A mixture of II (21 g., 0.05 mole) and hydrazine hydrate (3.0 g. of 85% solution, 0.05 mole) in ethanol (300 ml.) was heated under reflux overnight. The solution was then evaporated to near dryness, warm 3 *N* hydrochloric acid (100 ml.) was added to the residue, and the mixture was filtered. The insoluble material was extracted once more with 3 *N* hydrochloric acid (100 ml.) and twice with hot water (100 ml.). The combined extracts, from which crystals started to separate, were evaporated to dryness under reduced pressure and the residue was recrystallized from absolute ethanol to give III (13.1 g., 82%) as colorless flakes, m.p. 180–183°.

Anal. Calcd. for $C_{12}H_{18}N_2OSe \cdot HCl$: C, 44.81; H, 5.95; N, 8.71. Found: C, 44.98; H, 6.01; N, 8.61.

Se-Benzyl-2'-benzyloxyselenopantetheine (IV_{DL}). Benzylselenoalletheine hydrochloride (48.2 g., 0.15 mole) was added to a solution of sodium (3.55 g., 0.15 g.-atom) in absolute ethanol (300 ml.). The mixture was stirred at room temperature for 10 min. until no more sodium chloride precipitated, then mixed with DL-benzyloxypantolactone (33.0 g., 0.15 mole) and heated under reflux for 6 hr. The solution was then filtered and evaporated to dryness under reduced pressure, and the residue was heated under vacuum on a steam bath for 3 more hr. Residual ethanol was removed by one evaporation with benzene (150 ml.), and the very viscous oil was dissolved in fresh benzene (300 ml.). The solution was passed through a column of neutral alumina (Woelm, Activity Grade I, 700 g. in a tube of 54 mm. diameter) and washed with anhydrous benzene until an initial yellow band had been eluted (fraction I: 450 ml. containing 23 g. of yellow viscous oil). Further washing with benzene (fraction II: 800 ml., containing 12 g. of slightly turbid colorless oil) and with benzene containing 5% methanol (fraction III: 1000 ml., containing 36 g. of colorless sirup) completed the chromatography. The evaporated fractions were separately dissolved in benzene (1.5 ml./g. of oil) at room temperature and anhydrous ether was added until crystallization set in. The solutions were then chilled to −20° to give IV_{DL} (61 g., 82%) as fine needle clusters, m.p. 64°.

Anal. Calcd. for $C_{25}H_{34}N_2O_4Se$: C, 59.40; H, 6.78; N, 5.54. Found: C, 59.23; H, 6.85; N, 5.65.

DL-Selenopantetheine (V_{DL}). Se-Benzyl-2'-benzyloxyselenopantetheine (10.1 g., 0.02 mole) was dissolved in 50 ml. of liquid ammonia and small pieces of sodium were added until the solution showed a stable blue color. Excess sodium was discharged by addition of a few drops of absolute methanol and the solvent was allowed to evaporate at room temperature. After the last traces of ammonia had been removed under vacuum, a slurry of Amberlite IR-120, H^+ form (30 g., 150 mequiv.) in water was added quickly; the acidic supernate was passed through a column of Amberlite IR-120 (20 g.) and washed with water until the effluent did not give the bright yellow color with silver nitrate solution, which is indicative of the selenol group. Air oxidation of selenopantetheine was prevented throughout the experiment by keeping the solutions under an atmosphere of nitrogen or carbon dioxide; small pieces of Dry Ice added to all flasks were found to be a convenient method of accomplishing this. Evaporation of water under reduced pressure and drying of the residue below 40° at a pressure of 0.05 mm. yielded DL-selenopantetheine as a very hygroscopic colorless glass (5.9 g., 91%). Exposure to atmospheric oxygen yielded DL-selenopantethine (the diselenide (VII_{DL})), as a yellow glass, which was indistinguishable from the product obtained by condensation of pantothenic acid and selenocystamine.[27]

Se-Benzoyl-DL-selenopantetheine (VI_{DL}). A mixture of DL-selenopantetheine (5.0 g., 0.015 mole), sodium bicarbonate (15 g., 0.18 mole), and benzoyl chloride (2.8 g., 0.02 mole) in water (50 ml.) was shaken in an atmosphere of carbon dioxide until the smell of benzoyl chloride was no longer noticeable. The reaction mixture was extracted with ethyl acetate (three 100-ml. portions), and the combined organic layers were washed with saturated sodium sulfate solution, dried over anhydrous sodium sulfate, evaporated to a small volume, and passed through a short column of alumina (Woelm Activity Grade III, 10 g.). The alumina was washed with ethyl acetate until a test drop of effluent did not leave any residue (150 ml.), and the combined effluent was evaporated to 20 ml. and cooled in ice to yield Se-benzoyl-DL-selenopantetheine (5.2 g., 78%) as colorless needles melting at 82–85°; ultraviolet spectrum (absolute ethanol) ϵ_{242}^{max} 11,900; ϵ_{286}^{max} 5600.

Anal. Calcd. for $C_{18}H_{26}N_2O_5Se$: C, 50.35; H, 6.10; N, 6.52; Se, 18.39. Found: C, 50.57; H, 6.30; N, 6.76; Se, 18.60.

DL-Selenopantethine 4'-Phosphate ($IX_{D,L}$). Se-Benzyl-2'-benzyloxyselenopantetheine (4.0 g., 0.008 mole) was dried carefully by three evaporations with anhydrous pyridine leaving 40 ml. of final solution. This was frozen in Dry Ice and acetone and a solution of dibenzyl phosphorochloridate[30] (from 2.3 g. of dibenzyl-phosphite and 1.13 g. of N-chlorosuccinimide

(46) All melting points are uncorrected.
(47) Microanalyses were carried out at the Schwarzkopf Microanalytical Laboratories, Woodside, N. Y.

in 10 ml. of benzene) was added. The mixture was thawed and immediately refrozen, then kept at $-18°$ overnight. Water (2 ml.) was added, and the solution was concentrated under reduced pressure and dried by several evaporations with ethyl acetate. Finally a solution of the residue in ethyl acetate (120 ml.) was extracted with small portions of 2 *N* sulfuric acid, sodium bicarbonate solution, and saturated aqueous sodium sulfate. The organic layer was dried with anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The residue was dissolved in glacial acetic acid (30 ml.), placed on a steam bath, gradually diluted with water (30 ml.), and kept at 100° for 1 hr. The clear solution was evaporated under reduced pressure and residual acetic acid removed by several evaporations with methanol. The final residue was dissolved in liquid ammonia (50 ml.) and a solution of sodium in liquid ammonia[23] was added until the solution showed a transient blue color throughout. The excess of sodium was destroyed quickly by addition of a few drops of methanol; ammonia was removed by evaporation at room temperature under nitrogen, followed by evacuation of the flask to leave a white powder. A slurry of Amberlite IR-120 (H^+) was added quickly until the supernate was strongly acidic. The mixture was then applied to the top of a fresh 3 × 15 cm. Amberlite column. After all acidic material had been washed from the column with water the effluent was brought to pH 7.5 with barium hydroxide solution, and the filtered solution was evaporated to a small volume under reduced pressure at a bath temperature of 35°. The gummy residue was dissolved in a minimum amount of methanol and the turbid solution centrifuged. The clear supernate was again evaporated to dryness and dried to a clear glass (2.9 g.) on an oil pump. The barium salt of DL-4'-phosphoselenopantethine (2.0 g.) was obtained analytically pure by precipitation from a methanol solution with ten volumes of dry ether. An iodine–thiosulfate titration[48] of the yellow powder showed that all selenium was present as the diselenide (calcd. 3.00 iodine equivalents/Se; found 3.02) and not as the selenol (calcd. 4.00). The barium salt of the diselenide was isolated as the dihydrate.

Anal. Calcd. for $C_{22}H_{40}N_4O_{14}P_2Se_2Ba_2 \cdot 2H_2O$: C, 23.70; H, 3.98; N, 5.02; Se, 14.16; Ba, 24.63. Found: C, 24.18; H, 4.27; N, 4.60; Se, 14.18; Ba, 24.34.

D-*Selenopantethine 4'-Phosphate* (IX_D). D-Calcium pantothenate (25 g., 0.105 mole) was dissolved in water (150 ml.) and stirred with a suspension of Dowex 50W-X8 in the triethylammonium form until the supernate was calcium free. The solution was filtered and the resin was washed in a chromatographic column with several portions of water to yield, after evaporation of the aqueous solvent under reduced pressure at 40°, the triethylammonium salt of D-pantothenic acid as a colorless sirup. This was dried on an oil pump for 2 days, then dissolved in anhydrous dimethylformamide (150 ml.), and the mixed carbonic ester anhydride was formed by the dropwise addition of ethyl chloroformate (10.8 g., 0.1 mole) with stirring at $-10°$. Stirring was continued for 30 min. after the addition had been completed and then an azeotropically dried solution of I (0.1 mole, from 25 g. of the hydrochloride) in chloroform (100 ml.) was added. The resulting mixture was stirred for 1 hr. at room temperature, filtered from precipitated salts, and the filtrate evaporated to dryness under reduced pressure. The oily residue was taken up in ethyl acetate (500 ml.) and washed in turn with water, 2 *N* sulfuric acid, saturated sodium bicarbonate solution, and saturated sodium sulfate solution, dried over anhydrous sodium sulfate, and evaporated to a clear sirup of D-benzylselenopantethine ($VIII_D$, 23.0 g., 55%). This product was not purified further since all attempts to obtain an analytical sample by chromatography on alumina led to extensive decomposition.

The crude sirup was dissolved in anhydrous pyridine and dried by two evaporations with fresh pyridine under reduced pressure. The final solution (200 ml.) was then frozen at $-80°$, a solution of dibenzyl phosphorochloridate (from 17 g. of dibenzyl phosphite and 11 g. of N-chlorosuccinimide) in benzene (100 ml.) was added, and the mixture was thawed and refrozen quickly. The reaction mixture was allowed to stand at $-20°$ overnight, then water (25 ml.) was added and, after a few minutes, all solvents were removed *in vacuo* at a temperature below 35°. The residue was evaporated with portions of water until there was only a negligible smell of pyridine, then the gum was dissolved in glacial acetic acid (100 ml.) and heated on a steam bath with gradual addition over the first 15 min. of an equal volume (100 ml.) of distilled water. The heating was continued for 1 hr. and the solvent again was removed under vacuum, followed by several evaporations with anhydrous methanol until all smell of acetic acid had disappeared. The residue was dried on an oil pump and dissolved in liquid ammonia, and a solution of sodium in liquid ammonia was added until a blue color persisted throughout the solution for a few seconds. It is important to avoid an excess of sodium or a long reduction time at this stage since the carbon–selenium bond appears to be unstable under these conditions. After evaporation of the ammonia under reduced pressure, the residue was worked up in the same manner as described for DL-4'-phosphoselenopantethine, yielding the barium salt of IX_D (10.5 g., 20% over-all yield) as a pale yellow powder, $[\alpha]^{29}_D$ 8.4° (c = 2, H_2O), which was chromatographically identical with the optically inactive material.

DL-*3'-Dephosphoselenocoenzyme A* (XI_{DL}). An aqueous solution of the barium salt of IX_{DL} (1.5 g., 2.5 mmoles) was stirred with the pyridine salt of Amberlite IR-120 until the supernate was free of barium. The mixture was filtered and the resin washed twice with small amounts of water. The combined filtrates were then evaporated under reduced pressure with a bath temperature below 35° and dried by three evaporations with anhydrous pyridine to a final pressure of 0.05 mm. 4-Morpholine-N,N'-dicyclohexylcarboxamidinium adenosine 5'-phosphoromorpholidate[49] (760 mg., 1.0 mmole) was dried separately by three evaporations with anhydrous pyridine. Both residues were redissolved in dry pyridine, combined, evaporated twice more with readmission of dry air, and kept in pyridine (20 ml.) overnight at room tem-

(48) A. Fredga, *J. prakt. Chem.*, 123, 141 (1929).

(49) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 649 (1961).

perature. The solution, which contained a small amount of oily precipitate, was evaporated under reduced pressure, traces of pyridine were removed by several evaporations with methanol, and the residue was dried at room temperature at 0.05 mm.

The residue was dissolved in 300 ml. of water. The solution (16,500 optical density [O.D.] units at 257 mμ) was passed through a column of DEAE–cellulose (34 × 500 mm.). The column was washed with 0.003 *N* HCl until no more material absorbing at 257 mμ was eluted and the effluent had pH 3. Approximately 5800 O.D. units were thus eluted and discarded. Linear gradient elution with 1 l. each of 0.003 *N* HCl and 0.05 *N* LiCl in 0.003 *N* HCl (see Figure 1) yielded three ultraviolet-absorbing fractions which were discarded. Further elution with a linear gradient from 0.05 to 0.15 *M* LiCl in 0.003 *N* HCl yielded two ultraviolet-absorbing fractions at 0.109 and 0.135 *M* LiCl concentration, respectively. The solutions were adjusted to pH 4.5 with lithium hydroxide and evaporated to dryness under reduced pressure below 40°. Consecutive addition of methanol (one part) and acetone (ten parts) resulted in the separation of pale yellow precipitates, which were treated with methanol and acetone until the supernate was chloride free.

The first of the above fractions (peak 4, Figure 1, 119.1 mg.) gave an analysis corresponding to the mixed diselenide of dephosphoselenocoenzyme A and 4′-phosphoselenopantetheine.

Anal. Calcd. for $C_{32}H_{53}O_{20}N_9P_3Se_2Li_3$: C, 33.26; H, 4.62; N, 10.91; P, 8.04; Se, 13.67. Found: C, 33.33; H, 4.87; N, 10.60; P, 7.00; Se, 13.58.

The second main fraction (peak 5, Figure 1, 120.1 mg.) eluted by 0.135 *M* LiCl gave an analysis corresponding to that of the diselenide of dephosphoselenocoenzyme A.

Anal. Calcd. for $C_{42}H_{64}N_{14}O_{30}P_4Se_2Li_4 \cdot 4H_2O$: C, 32.27; H, 4.64; N, 12.54; P, 7.90. Found: C, 32.59; H, 5.06; N, 12.48; P, 6.99.

DL-*Selenocoenzyme A and* DL-*Isoselenocoenzyme A* (*XIV*$_{DL}$ *and XV*$_{DL}$). A solution in 20 ml. of pyridine of IX_{DL} (from 810 mg., 1.5 mequiv. of the barium salt), which had been dehydrated by three evaporations with anhydrous pyridine, was added to a similarly dehydrated solution of bis(4-morpholine-N,N′-dicyclohexylcarboxamidinium) adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate[23] (XII, 440 mg., 0.4 mmole) in 10 ml. of pyridine. The mixture was evaporated twice more with anhydrous pyridine, dissolved in pyridine (20 ml.), and kept at room temperature overnight. The solvent was removed under reduced pressure followed by several evaporations with water. The residue was taken up in 0.1 *N* HCl (20 ml.), kept at room temperature for 1 hr., evaporated to dryness again, and taken up in water (100 ml.). This solution was applied to the top of a DEAE Cl^- column (25 × 400 mm.) which was then washed with 0.003 *N* HCl until the effluent had pH 3 and until no more material absorbing at 257 mμ could be eluted. Linear gradient elution with 0.003 *N* HCl (4000 ml.) in the mixing flask and 0.40 *M* LiCl in 0.003 *N* HCl (4000 ml.) in the reservoir flask was used to separate the products. Fractions of 13.5 ml. were collected, the drop rate was maintained at 1.5 ml./min., and the ultraviolet density of the effluent was monitored continuously at 257 mμ or at higher wave lengths (270 or 285 mμ) if the optical density at the adenine peak became too great. Selenocoenzyme A and the 2′-phospho isomer, isoselenocoenzyme A, were eluted together as the last peak of the chromatogram at a 0.2 *M* LiCl concentration (Figure 2, peak 6, 1740 O.D. units, 29% of theoretical). After neutralization with lithium hydroxide, the combined fractions were freeze dried and the residual white powder was treated with methanol (one part) and acetone (ten parts) until the supernate was chloride free. After this isolation and washing procedure the total yield was 84 mg. of white powder.

A microanalysis suggested the presence of 3 moles of methanol and 2 moles of water per mole of the selenocoenzyme A–isoselenocoenzyme A mixture.

Anal. Calcd. for $C_{42}H_{64}N_{14}O_{32}P_6Se_2Li_6 \cdot 3CH_3OH \cdot 2H_2O$: C, 30.11; H, 4.49; N, 10.92; P, 10.36; Se, 8.79. Found: C, 30.2; H, 4.55; N, 10.66; P, 10.40; Se, 8.05.

The product was dissolved in a small amount of water and reprecipitated by addition of acetone (20 volumes). An analysis now showed the presence of 6 molecules of water of hydration per selenocoenzyme A unit, which was not removed by drying at 100° under vacuum.

Anal. Calcd. for $C_{42}H_{64}N_{14}O_{32}P_6Se_2Li_6 \cdot 12H_2O$: C, 26.85; H, 4.72; N, 10.44; P, 9.89; Se, 8.41. Found: C, 27.04; H, 4.42; N, 10.21; P, 9.44; Se, 8.41.

D-*Selenocoenzyme A and* D-*Isoselenocoenzyme A Diselenides.* The preparation of optically uniform selenocoenzyme A isomers was carried out in the same manner as described for the racemic material, starting with the adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (XII, 2.2 g., 2 mmoles) and barium D-selenopantethine 4′-phosphate (IX_D, 4.0 g., 7 mequiv.). The product from this reaction was chromatographed on a column of DEAE Cl^- (45 × 600 mm.) with a linear salt gradient of 4 l. each of 0.003 *N* HCl and of 0.25 *N* LiCl in 0.003 *N* HCl, with a drop rate of 5 ml./min. An elution pattern similar to the one described in Figure 2 was obtained, except that the peak containing D-selenocoenzyme A and D-isoselenocoenzyme A diselenides (peak 6, 428.7 mg. of lithium salt; 6080 O.D. units; 25.6% of total column recovery) was preceded by a large peak (487.4 mg. of lithium salt; 6950 O.D. units; 29.3%) which consisted to a large part of the mixed diselenides of D-4′-phosphoselenopantetheine and the selenocoenzyme A mixture. This peak, when converted into a mixture of Se-benzoyl derivatives, as described below, and rechromatographed on Ecteola Cl^-, yielded another crop of selenocoenzyme A and isoselenocoenzyme A (combined yield 192 mg., 11.8%), which raised the total yield of selenocoenzyme A and isoselenocoenzyme A to 41.1%. The mixed product was chromatographically uniform and indistinguishable from the previously described DL-selenocoenzyme A mixture.

Se-Benzoylselenocoenzyme A (XVI$_D$*) and Se-Benzoylisoselenocoenzyme A (XVII*$_D$*).* A sample of selenocoenzyme A–isoselenocoenzyme A mixture in the diselenide form (107.7 mg. of the lithium salt, 0.114 mequiv.) was dissolved in water (50 ml.), 2-dimethylaminoethyl selenobenzoate hydrochloride[35] (300 mg., 1 mequiv.) and sodium bicarbonate (200 mg.) were

added, and the mixture was brought to about pH 8.5 by addition of saturated sodium carbonate solution until the solution was slightly turbid. The reaction was allowed to proceed for 1 hr. at room temperature with stirring and dropwise addition of more sodium carbonate to maintain the turbidity. Sufficient Amberlite IR-120 ion-exchange resin in the H^+ form was then added to make the solution strongly acidic, and the resin was removed by filtration and washed several times with water until the eluate was free of ultraviolet-absorbing material. The combined filtrates were then diluted to about 350 ml., an aliquot was extracted with ether, and the ultraviolet absorption was determined at λ_{max} 250 mμ. All 1700 O.D. units were then applied to the top of a column (15 × 600 mm.) of Ecteola-cellulose Cl^-, which had been packed with a 200-cm. liquid head and washed in turn with 1 N LiCl in 0.003 N HCl and with 0.003 N HCl. The benzoylated products were followed by 1 l. of 0.05 N LiCl in 0.003 N HCl; material eluted at this stage consisted entirely of benzoic acid (peak 1, Figure 3). A linear gradient was now applied, consisting of 2 l. of 0.05 N LiCl in 0.003 N HCl in the mixing flask and 2 l. of 0.09 N LiCl in 0.003 N HCl in the reservoir flask, the drop rate was adjusted to about 1.5 ml. min., and fractions of 13.5 ml. were collected while the effluent was monitored continuously at 250 mμ. Benzoylisoselenocoenzyme A (406 O.D. units) and benzoylselenocoenzyme A (475 O.D. units) were eluted in this order in two clearly separated peaks (Figure 3) between fractions 56 and 95, with only a small overlapping fraction (69 O.D. units). Further elution of the column with 0.25 N LiCl yielded a crop of nonacylated selenocoenzyme A mixture diselenides (655 O.D. units) which had either not been acylated in the first instance or had been hydrolyzed on the column. Total recovery was 1605 O.D. units (94%). Aminolysis of both benzoylated selenocoenzyme A fractions, which proceded at identical rates, yielded compounds which could not be differentiated from the starting mixture on paper or thin layer chromatography. Incubation at 37° with crude rattle snake (*Crotalus adamanteus*) venom (1 mg./ml.) in 0.2 N Tris buffer, 0.004 N in magnesium ion at pH 9.0, yielded exclusively adenosine 2′,5′-diphosphate from the peak first eluted from the Ecteola column and adenosine 3′,5′-diphosphate from the second of the peaks, while the intermediate tubes gave a mixture of both diphosphates. The diphosphates were identified by comparison on paper chromatograms[23] with authentic material.

Thin Layer Chromatography of Compounds Related to Coenzyme A. Slurries were prepared from cellulose powder (MN300, Macherey, Nagel & Co., Dueren, Switzerland; 10 g.) and distilled water (70 ml.), and the mixtures were homogenized for 1 min. in a Waring Blendor and applied to glass plates with a variable thickness applicator (Desaga, Heidelberg, Germany) set to 0.35 mm. The plates were dried at room temperature in air with an average relative humidity of 40–60%. Chromatograms were run until the solvent front had moved 10–15 cm.; this yielded spots with an average diameter of 3–4 mm. The relative mobilities were determined on the same plate for each group of compounds and are shown in Table I.

Table I. Thin Layer Chromatography[a]

Solvent: 1-Butanol–Acetic Acid–Water, 5:2:1	Sulfur compd. R_f	Selenium compd. R_f
Pantetheine	SH 0.63	SeH 0.61
Pantethine	S_2 0.72	Se_2 0.86
4′-Phosphopantetheine	SH 0.67	SeH 0.72
4′-Phosphopantethine	S_2 0.50	Se_2 0.50

Solvent: Ethanol–0.5 N ammonium acetate, 3:2	Sulfur compd. R_f pH 4	Sulfur compd. R_f pH 8	Selenium compd. R_f pH 4	Selenium compd. R_f pH 8
Dephosphocoenzyme A	SH 0.48	...	SeH ...	...
	S_2 0.23	0.34	Se_2 0.25	0.36
Coenzyme A	SH 0.36	0.29	SeH (0.34[a])	...
(or isocoenzyme A)	S_2 0.10	0.06	Se_2 0.10	0.06
Benzoylcoenzyme A (or isobenzoyl CoA)	0.63[b]	...	0.59[b]	...

[a] The value quoted for selenocoenzyme A–SeH is based on the spot produced when Se-benzoylselenocoenzyme A (or Se-benzoylisoselenocoenzyme A) was chromatographed in the ammonium acetate solvent. Benzoylcoenzyme A was also subject to aminolysis in this solvent and yielded a single spot of coenzyme A–SH. [b] The compounds were chromatographed in a solvent containing sodium acetate instead of ammonium acetate; even so there was some hydrolysis of the selenol ester resulting in a trail.

Compounds containing sulfur and adenine were detected by a nitroprusside spray and by observation under ultraviolet light, respectively. Selenium compounds were detected by a spray reagent containing soluble starch (1 g.), sodium bicarbonate (1 g.), and 0.1 N iodine solution (3–5 ml.) in water (100 ml.). The solution is unstable and has to be prepared freshly every day. Selenium compounds (selenols, diselenides, monoselenides, but not selenoacyl esters) appeared as white spots on a blue background. The background color changed to brown on drying the plates, which decreased the contrast considerably. The limits of detection by this method varied somewhat with the amount of spray used and with individual compounds, but 10 mμmoles of selenium were detected regularly without difficulty. To detect white spots caused by the oxidation of other materials (*e.g.*, thiols), the dried plates were sprayed with a dilute nonoxidizing acid such as phosphoric acid. In acidic medium only white spots caused by selenium compounds return to the blue color of the background, due to reduction of selenoxides and seleninic acids by hydrogen iodide. A convenient quantitative determination of selenium compounds on inorganic thin layer materials, such as silica gel or alumina, is based on a variation of the above technique. Oxidation by starch and iodine in the presence of the thin layer support and extraction of the selenoxides and seleninic acids into aqueous solution, followed by acidification and colorimetric measurement of the starch–iodine complex, gave reproducible results at concentrations as low as 10^{-8} mole/ml.

Acknowledgments. We are indebted to Dr. Shih-Hsi Chu for the synthesis of a batch of adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate, to Mr. Joseph Sizensky for capable assistance in the chemical work, and to Mrs. Julia Krackov and Mrs. Barbara Ahern for the biochemical assays.