loss during the addition there was an excess of hydrogen fluoride in the final mixture. Little heat was evolved. To make more certain that reaction did occur the loosely stoppered bottle with contents was warmed to 50–55° for about an hour.

Distillation at 11.5 to 12.0 mm. gave between 1 and 2 g. of distillate at 54.5 to 55.5° and 12 to 13 g. at 55.5 to 55.8°. After redistillation of the larger fraction the distillate was analyzed.

Anal. Calcd. for $(C_2H_5O)_2PSF$: F, 11.0; P, 18.0; S, 18.6. Found: F, 10.4; P, 18.2; S, 18.3.

Fluorine was determined by refluxing for two hours with alcoholic sodium hydroxide solution followed by distillation from perchloric acid and titration of the distillate with thorium nitrate in the presence of sodium alizarin sulfonate. The phosphorus and sulfur contents were determined by conventional methods following decomposition in a Parr peroxide bomb. Properties: d^{25}_4 1.1387, $n^{25}D$ 1.4188, b.p. 58.0–58.7° at 12.9 mm., 164.0–164.7° at 740 mm.; soluble in alcohol, acetone and ether; only slightly soluble in water; hydrolyzes only slowly, no effect on glass noticeable after two years storage. The compound has a sharp, nauseating odor but the toxicity is not particularly high; LD_{50} for rats is about 350 mg./kg. by intramuscular injection.[3] The chymotrypsin inhibitory potency is about one-tenth that shown by diisopropylmonofluorophosphate.[4]

(3) Private communication from Dr. Willy Lange, January 6, 1949.

(4) Private communication from Dr. Arnold Kent Balls, January 9, 1950.

OZARK-MAHONING COMPANY WAYNE E. WHITE
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RECEIVED SEPTEMBER 17, 1951

COMMUNICATIONS TO THE EDITOR

THE COMPOSITION OF COENZYME A[1]

Sir:

After the presence of a sulfhydryl group in coenzyme A (CoA) had been established,[2,3,4] the contamination of CoA preparations by disulfide formation with other mercaptans was recognized. Therewith, the high sulfur content in CoA, amounting in some preparations to nearly 2 atoms per mole of pantothenic acid,[3] was explained. It was found that the contaminating mercapto derivative could be removed through inclusion of a reduction step.[5] In this manner, preparations were obtained with close to 1 atom of sulfur per mole of pantothenic acid. We wish to report here on a compound assaying 384 units per mg. and approaching ultimate purity (413 units per mg., calculated for a pantothenic acid content of 0.7γ per unit, and a molecular weight of 767 for CoA). CoA was concentrated by adsorption on charcoal from a large-scale fermentation of *Streptomyces fradiae*. Elution with alkaline acetone, followed by a second acid adsorption and alkaline elution from charcoal, gave a preparation of 64 units per mg. in about 40% yield.[3]

This compound is reduced in 1% solution with zinc and 0.5 *N* hydrochloric acid for 30 minutes, then precipitated with excess mercuric acetate solution. The washed product is suspended, decomposed with hydrogen sulfide, and the supernatant passed through a column of Duolite CS-100 resin (100–200 mesh, acid form). Most of the impurities are removed by washing with 0.2 *N* hydrochloric acid, and the coenzyme is eluted with water and freeze-dried. This gives a compound of an average of 384 units per mg. in 20% yield, having the following analyses:

	Calcd. %[a]	% Found	Ratio
Pantothenic acid	28.6	26.8 (enzymatic assay) 25.6 (microbiological)	1
Adenine	17.6	17.0 (spectrophotometric)	1.05
Phosphorus (total)	12.12	10.6	2.83
Mono-ester phosphorus[b]	...	3.6	0.96
Sulfur	4.18	4.13	1.07

[a] Pantothenic acid, 2-mercaptoethylamine, 3 phosphoric acid, adenosine, $-5H_2O$; molecular weight 767. [b] Liberated by prostate phosphomonoesterase.

On paper chromatography of the acid hydrolysate, such a substance shows the presence of β-alanine and 2-mercaptoethylamine disulfide, but no other ninhydrin-reacting compound. By comparison with earlier data,[3,5] this indicates the removal by the reduction step of all cross-linked sulfur-containing amino acid.

Due to the danger of decomposition, the preparation was dried *in vacuo* over phosphorus pentoxide for one hour at 34°. Assuming this to be sufficient to remove all water, this preparation is at least 90 to 93% pure CoA.

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RECEIVED DECEMBER 15, 1951

(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, and from the Commonwealth Fund.

(2) F. Lipmann, N. O. Kaplan, G. D. Novelli and B. Guirard, *J. Biol. Chem.*, **167**, 869 (1947); **186**, 235 (1950).

(3) W. H. DeVries, W. M. Govier, J. S. Evans, J. D. Gregory, G. D. Novelli, M. Soodak and F. Lipmann, THIS JOURNAL, **72**, 4838 (1950).

(4) E. E. Snell, G. M. Brown, V. J. Peters, J. A. Craig, E. L. Wittle, J. A. Moore, V. M. McGlohon and O. D. Bird, *ibid.*, **72**, 5349 (1950).

(5) J.D. Gregory and F. Lipmann, *Abstracts*, 12th Interntl. Cong. of Pure and Applied Chem., p. 74 (1951).

A METHOD FOR PURIFICATION OF COENZYME A

Sir:

The following method for purification of coenzyme A (CoA), Lipmann's[1] acetylation coenzyme,

(1) F. Lipmann, N. O. Kaplan, G. D. Novelli, L. C. Tuttle and R. M. Guirard, *J. Biol. Chem.*, **167**, 869 (1947).

leads in one purification step from a crude concentrate to a product of high purity. This method is based on the observation[2] that CoA, owing to its character as a sulfhydryl compound,[3] can be precipitated in strong acid solution by Cu_2O in the presence of reduced glutathione (GSH). This step supplemented by available column procedures[4] for initial concentration and for elimination of carrier GSH permits preparation of CoA containing about 20% pantothenic acid from yeast in 10–15% yield.

CoA is adsorbed on a charcoal column from an aqueous extract (100°) of dried brewers' yeast and eluted with 5% pyridine (yield 80%). The eluate is shaken with chloroform, concentrated, and the coenzyme precipitated with 5 volumes of acetone (yield 60–70%). A solution of the acetone powder is again passed through a charcoal column and eluted as before after washing with dilute alkali (yield 80%). The eluate is freed from pyridine and concentrated to contain 5% solids.[5] GSH is added (10–20 mg./ml.) and the *p*H adjusted to 7. After a few minutes 0.05 volume of 10 *N* H_2SO_4 is added and Cu_2O is stirred in slowly as outlined by Hopkins.[6] The precipitate is washed with 0.5 *N* H_2SO_4, then with water until sulfate-free and finally decomposed with H_2S (yield 30–35%). After removal of CuS and H_2S the solution is passed through Dowex 50 (H^+) to remove GSH. The effluent is concentrated and lyophilized (yield 85%, over-all yield 10–15%). In a typical run from 8 kg. of dried yeast, 620 mg. of powder was obtained which analyzed as follows:

	%	Molar ratio
Pantothenic acid		
Microbiological[7]	22	1.0
Spectrophotometric, DPN reduction[8]	21	1.0
Transacetylase[9]	24	1.1
Sulfanilamide acetylation[10]	22	1.0
Adenine, from ultraviolet absorption at 260 mμ	13.7	1.0
Ribose	18.9	1.3
Glutathione, glyoxalase[11]		
GSH	3	0.1
GSSG	4	0.065
L-Glutamic acid, after acid hydrolysis[12]		
Microbiological, *L. arabinosus*	14	0.95
Decarboxylase, *E. Coli*[13]	11	0.75
Phosphorus, Fiske-SubbaRow[14]		
Inorganic	<0.1	
Total	10.3	3.3
Nitrogen, Kjeldahl	13.3	9.5
Sulfur[15]	4.68	1.45
Carbon[15]	32.3	27
Hydrogen[15]	4.83	48
Ash[15]	0	—

Electrophoresis and paper chromatography of such preparations revealed that the bulk of the material and CoA activity moved as a single component, although two to four minor components were detected.

(2) H. Beinert, R. W. Von Korff and D. E. Green, unpublished.

(3) W. H. Devries, W. M. Govier, J. S. Evans, J. D. Gregory, G. D. Novelli, M. Soodak and F. Lipmann, THIS JOURNAL, **72**, 4838 (1950); G. M. Brown, J. A. Craig and E. E. Snell, *Arch. Biochem.*, **27**, 473 (1950); F. Lynen and E. Reichert, *Angew. Chem.*, **63**, 47 (1951).

(4) D. A. Buyske, R. E. Handschumacher, Harvey Higgins, Tsoo E. King, F. M. Strong, V. H. Cheldelin, L. J. Teply and G. C. Mueller, *J. Biol. Chem.*, **193**, 307 (1951); D. A. Buyske, R. E. Handschumacher, Harvey Higgins, and F. M. Strong, unpublished.

(5) The second charcoal treatment may be omitted if a final pantothenic acid content of about 12–15% is satisfactory.

(6) F. G. Hopkins, *J. Biol. Chem.*, **84**, 269 (1929).

(7) J. B. Neilands and F. M. Strong, *Arch. Biochem.*, **19**, 287 (1948).

(8) Unpublished method of R. W. Von Korff.

(9) E. R. Stadman, G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **191**, 365 (1951).

(10) N. O. Kaplan and F. Lipmann, *ibid.*, **174**, 37 (1948).

(11) G. E. Woodward, *ibid.*, **109**, 1 (1935).

(12) This amount of glutamic acid obviously cannot be contained in the glutathione present, but may still have been derived in some manner from the glutathione used in the preparation. The data available do not permit the conclusion that glutamic acid is a component of the CoA molecule. In fact a similar subsequent preparation contained 24% P.A. but less than 2% glutamic acid.

(13) W. W. Umbriet and I. C. Gunsalus, *J. Biol. Chem.*, **159**, 333 (1945).

(14) B. L. Griswold, F. L. Humoller and A. R. McIntyre, *Anal. Chem.*, **23**, 192 (1951).

(15) Microanalyses by C. W. Beazley, Micro-Tech Laboratories, Skokie, Illinois.

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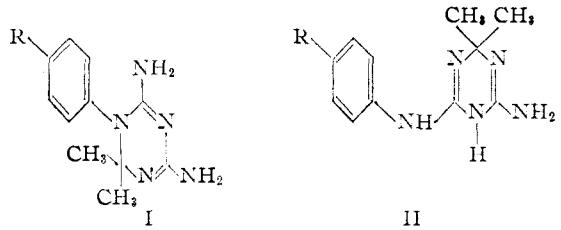
RECEIVED NOVEMBER 26, 1951

A SERIES OF NEW, BIOLOGICALLY SIGNIFICANT DIHYDROTRIAZINES

Sir:

We wish to report the synthesis (by E. J. M.) of a hitherto unreported class of compounds (I) with anti-vitamin and anti-malarial activity. In the course of a program of synthesis of arylbiguanides,[1] a new compound (m.p. 189–191°; $\lambda_{max.}^{H_2O}$ 236 mμ, log ε 4.24; *Anal.* Calcd. for $C_{14}H_{19}N_5O_2 \cdot HCl$: C, 51.61; H, 6.19; N, 21.50. Found: C, 51.97; H, 6.12; N, 21.22) was produced by the condensation of ethyl *p*-aminobenzoate, dicyandiamide and concentrated hydrochloric acid in acetone. The structure Ia·HCl, 4,6-diamino-1-(*p*-carbethoxyphenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride, is proposed for this substance. This reaction is general for a ring-substituted aniline hydrochloride, dicyandiamide or N^1-monosubstituted dicyandiamide and a number of ketones or aldehydes. Another synthesis of compounds with structure I has been developed through condensation of arylbiguanides with ketones or aldehydes under acid conditions.

(1) Synthesis of a group of arylbiguanides was undertaken originally in these laboratories at the suggestion of M. M. Pechet as one part of a broad program in the chemotherapy of cancer initiated by Sidney Farber.



I II

a, R = $COOC_2H_5$
b, R = Cl
c, R = H

The action of alkali or ammonia on Ib·HCl (m.p. 210–215°; $\lambda_{max.}^{H_2O}$ 241 mμ, log ε 4.16; *Anal.* Calcd. for $C_{11}H_{14}N_5Cl\cdot HCl$: C, 45.84; H, 5.25; N, 24.31. Found: C, 45.98; H, 5.17; N, 24.48) affords IIb[2,3] (m.p. 130–133°; $\lambda_{max.}^{H_2O}$ 255 mμ, log ε 4.25), 6-amino-2-(*p*-chloroanilino)-1,4-dihydro-4,4-dimethyl-*s*-triazine,[4] by an irreversible intramolecular rearrangement. IIb·HCl (m.p. 128–131°; $\lambda_{max.}^{H_2O}$ 255 mμ, log ε 4.24; *Anal.* Calcd. for $C_{11}H_{14}N_5Cl\cdot HCl$: C, 45.84; H, 5.25; N, 24.31; Cl, 24.61. Found: C, 45.67; H, 5.21; N, 24.53; Cl, 24.70), isomeric with Ib·HCl, results on treatment of IIb with hydrochloric acid.

Many compounds of structure I inhibit *Streptococcus faecalis 8043* and *Lactobacillus arabinosus 17-5* in standard pteroylglutamic acid and niacin assay systems,[5] whereas those of structure II are relatively inert. For example, Ib·HCl (0.05–0.20 gamma/ml.) inhibits 0.001–0.01 gamma/ml. of pteroylglutamic acid or niacin in corresponding microbiological systems, whereas IIb produces only partial inhibition versus 0.001 gamma/ml. of pteroylglutamic acid or niacin in concentrations of 100–1000 gamma/ml. Ib·HCl also inhibits *Lactobacillus casei 7469* and *Leuconostoc citrovorum 8081* in systems containing 0.001–0.01 gamma/ml. of riboflavin[5] and 0.6 unit/ml. of citrovorum factor[6] respectively, but only in concentrations of 100 gamma/ml. or more. Other derivatives of structure I exhibit similar activities, although the minimal inhibiting dose varies with substitution in the molecule while, in general, derivatives of structure II are inert.

Inhibition induced in pteroylglutamic acid systems by appropriate concentrations of compounds of structure I differs from that obtained with 4-aminopteroylglutamic acid (0.1 gamma/ml. or more) in that it is irreversible by 0.1–200 gamma/ml. of pteroylglutamic acid, adenine, or guanine. Reversal is obtained with citrovorum factor (0.1 unit/ml.), leucovorin (1.0 unit/ml.), dihydropteroylglutamic acid (0.01 gamma/ml.), N^{10}-formylpteroylglutamic acid (0.01 gamma/ml.), thymine (10 gamma/ml.), certain components of nucleic acid or high concentrations of ascorbic acid (200 gamma/ml.). When substituted for pteroylglutamic acid in *Streptococcus faecalis 8043* systems, citrovorum factor (0.000001 unit/ml.), leucovorin (0.001 unit/ml.), dihydropteroylglutamic acid (0.001 gamma/ml.) and N^{10}-formylpteroylglutamic acid (0.00001 gamma/ml.) are inhibited only by those concentrations required to inhibit *Leuconostoc citrovorum 8081* (*circa* 100 gamma/ml.). No significant inhibition was observed versus thymine.

Preliminary tests indicate that several compounds of structure I exhibit activity against experimental avian malaria, although compounds of structure II are comparatively inactive. For example, Ib·HCl is active against *Plasmodium lophurae* in the duck at oral dosages of approximately 3.13 mg./kg. and Ic·HCl at approximately 12.5 mg./kg. Ib·HCl is about six times as active as quinine, twice as active as atabrine or paludrine, and half as active as plasmochin in the duck.[7]

Further extensive studies on the scope and mechanism of the reaction and on the biological activity and mode of action of these compounds are in progress.

(2) S. Birtwell, F. H. S. Curd, J. A. Hendry and F. L. Rose, *J. Chem. Soc.*, 1645 (1948).

(3) N. N. Crounse, *J. Org. Chem.*, **16**, 492 (1951).

(4) Or a tautomer of this structure.

(5) Assoc. Vitamin Chemists, Inc., "Methods of Vitamin Assay," 2nd ed., Interscience Publishers, New York, N. Y., 1951.

(6) H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.*, **176**, 165 (1948).

(7) We are indebted to J. H. Williams and his colleagues of the Lederle Laboratories, Pearl River, New York, for the anti-malarial assays.

CHILDREN'S CANCER RESEARCH FOUNDATION
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RECEIVED NOVEMBER 20, 1951

QUANTITATIVE CALCULATION OF THE BEHAVIOR OF ELUTION BANDS IN THE SEPARATION OF RARE EARTHS ON ION-EXCHANGE COLUMNS

Sir:

This Laboratory, in a series of papers, has described procedures for separating rare earths on ion-exchange columns. In the accompanying letter a quantitative theory for describing this phenomenon in the *p*H range between 5.5 and 7.3 for 0.1% citric acid has been outlined. In the course of this work, some very interesting quantitative relationships concerning the movement of bands on the column have been observed. Since the high-capacity resins used have a constant equivalent capacity for the ions adsorbed, if the number of equivalents of rare earth on the column is known, then the length of the original rare earth band can be calculated. If this band is eluted down the column with a citrate solution of known *p*H, the band widens out until it reaches an equilibrium value and from then on the front edge of the band travels at the same rate as the rear edge. Within this band the rare earths separate into individual bands proportionate to the amount of rare earth present and in an order starting with the heavier rare earths and proceeding to the lighter rare earths. Under equilibrium conditions these bands are exceedingly sharp. However, in practice they tend to be blurred due to channeling and non-equilibrium conditions. Under very carefully controlled procedures they can be made very sharp. We expect to publish shortly detailed experimental results and calculations showing that it is possible to calculate to better than 5%, and in most cases to better than 1 to 2%, the length of the equilibrium band on the column, the rate at which the front and rear edges

of the band progress down the column, the length of the column necessary to reach the fully developed band and the number of liters of citric acid which will have to be passed through the column before the break-through of the rare earths at the bottom of the column is observed.

Experimental results will be presented correlating the shrinkage or expansion of the resins with the form of the resin present: for example, rare earth resin, ammonium resin and hydrogen resin. It has been possible to determine these band fronts much more precisely when neodymium was used since band edges become very clearly visible, due to the characteristic color of the neodymium ions, when the columns are illuminated in the dark with blue light.

Since the over-all change which occurs when a rare earth equilibrium band is being moved down the column is the conversion of the hydrogen resin which precedes the band into the ammonium resin which follows it, the break-through of the rare earth band at the bottom can be calculated with very high precision. While there is of course some hydrogen resin mixed with the ammonium resin which follows the band, the amount of this resin is extremely small as can be readily seen from the fact that the hydrogen ion in solution is so dilute compared to the ammonium ion. Accordingly, one needs only to divide the number of active points in the column occupied by hydrogen below the originally adsorbed rare earth band by the number of ammonium ions which are being put in the top of the column per liter to obtain the break-through volume. For example

$$V_B = \frac{A - B}{C_{NH_4^+}} = \frac{WQ - B}{C_{NH_4^+}}$$

V_B = volume of eluant required for break-through in liters
A = exchange capacity of the resin bed in milliequivalents
B = milliequivalents of rare earth adsorbed on bed
$C_{NH_4^+}$ = milliequivalents of ammonium ion per liter of eluant
W = weight of resin in the column in grams
Q = capacity of the resin in milliequivalents per gram

pH	Rare earth	A meq.	B meq.	$A - B$ meq.	$C_{NH_4^+}$ meq./l.	V_B (calcd.), l.	V_B (obs.), l.
6.4	Pr	852	44.0	808	13.87	58.2	58.0
	Nd	852	44.5	807	13.87	58.1	58.1
6.1	Pr	852	44.0	808	12.90	62.6	62.5
	Nd	852	44.5	807	12.90	62.5	62.4
5.8	Pr	852	44.0	808	11.78	68.6	68.5
	Nd	852	44.5	807	11.78	68.5	68.4
5.5	Pr	852	44.0	808	10.69	75.6	75.4
	Nd	852	44.5	807	10.69	75.5	75.4
7.3	Nd	852	178.0	674	15.38	43.8	43.7
7.6	Nd	852	178.0	674	15.70	42.9	43.0
8.2	Nd	852	178.0	674	16.88	39.9	39.8
5.6	Nd	852	27.0	825	10.99	75.1	75.0
5.9	Nd	852	53.5	798	12.10	65.9	65.5[a]
6.2	Nd	852	89.0	763	13.27	57.5	57.5
6.5	Nd	852	89.0	763	14.05	54.3	54.0
6.8	Nd	852	89.0	763	14.67	52.0	51.8[a]

[a] Extrapolated values obtained from band-front measurements.

CONTRIBUTION FROM THE
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(WORK WAS PERFORMED IN PART IN
THE AMES LABORATORY OF THE A.E.C.)

RECEIVED NOVEMBER 26, 1951

QUANTITATIVE THEORY OF RARE EARTH SEPARATIONS ON ION-EXCHANGE COLUMNS

Sir:

This Laboratory, in a series of papers (THIS JOURNAL, **69,** 2812 (1947); **72,** 2349, 2354 (1950); **73,** 4840 (1951)) has described how the rare earths can be separated from each other on ion-exchange columns. The method consists essentially of adsorbing a mixture of neutral rare earth chlorides on the top of a high-capacity ion-exchange column in the hydrogen cycle. The rare earth band which forms at the top is then moved down the column by eluting it with a solution of citric acid which has been adjusted to a known pH value by the addition of ammonium hydroxide. If the pH range of the eluant is between 5.5 and 7.3 for 0.1% citric acid, it is found that when the elution curves are plotted (concentration of rare earth in the eluate against liters of citric acid passed through the column), flat-topped elution curves are observed.

Shortly we expect to publish detailed experimental results and calculations showing that if the amount of rare earth adsorbed on the column, the concentration of citrate solution which goes in the top of the column and the pH of that solution, or its ammonium equivalent, are known, then it is possible to calculate to better than 5%, and in most cases to better than 1 or 2%, the composition of the eluate coming out the bottom. This includes the ammonium concentration, the rare earth concentration and the pH or hydrogen ion concentration. Some very interesting relations between the data and the activity coefficients of the resin can be observed. These can be deduced as a result of the constraints put upon the system by the fact that the resin has a fixed capacity. In order to make these calculations it is only necessary to use simple thermodynamics involving the ionization constants K_1, K_2, K_3 of citric acid, the equilibrium constant of the rare earth complex, the conservation conditions of the electrical neutrality of the solution and the fixed capacity of the resin.

It has been found that the predominating and only important rare earth complex formed in this pH range is (RE $cit_2^{\equiv}$). Experimentally, it has been found that if the ammonium ion of the eluant, the hydrogen total of the eluant H_T (a summation of the hydrogen combined with H_3cit, H_2cit^- and $Hcit^{\equiv}$ + the H^+ ion), the H_T of the eluate, the ammonium ion of the eluate, and the rare earth total of the eluate are plotted against the H^+ ion of the eluate, that linear curves result. Accordingly, if any pair of these are plotted against each other linear functions are also found. The slopes of these lines can be calculated from theoretical considerations. Furthermore, the instability constant K for the RE $cit_2^{\equiv}$ complex can be calculated for each of the rare earths.

CONTRIBUTION FROM THE
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(WORK WAS PERFORMED IN PART IN
THE AMES LABORATORY OF THE A.E.C.)

RECEIVED NOVEMBER 26, 1951