COMMUNICATIONS TO THE EDITOR

PIPERAZINE DERIVATIVES FROM AMINO ALCOHOLS

Sir:

We have found that certain piperazine derivatives are conveniently prepared from β-hydroxyalkylamines by the catalytic alkylation method of Adkins [Paden and Adkins, THIS JOURNAL, **58,** 2487 (1936); Hill and Adkins, *ibid.*, **60,** 1033 (1938)].

Isopropanolamine in dioxane over copper chromite at 250–275° yields *trans*-2,5-dimethylpiperazine. It was identified as the 1,4-dinitroso derivative, m. p. 174° [Feist, *Ber.*, **30,** 1983 (1897)] and as the 1,4-dibenzoyl derivative, m. p. 228–229° [Bamberger and Einhorn, *ibid.*, **30,** 226 (1897)]. Similarly, diethanolamine yields piperazyl-1,4-*bis*-β-ethanol, m. p. 134–135°. This was identified as the dibenzoate, m. p. 104.5–105° [Pyman, *J. Chem. Soc.*, **93,** 1802 (1908)]. Good yields of diphenylpiperazine, m. p. 163.5–164.5°, were obtained from phenylethanolamine. When mixed with an authentic specimen of diphenylpiperazine the melting point was not changed.

The copper chromite catalyst was partially reduced in most runs. The yields ranged from 20 to 50%. This work is being continued.

CONTRIBUTION FROM THE ORGANIC LABORATORIES OF THE UNIVERSITY OF FLORIDA
GAINESVILLE, FLA.

J. P. BAIN
C. B. POLLARD

RECEIVED JANUARY 16, 1939

ULTRAVIOLET ABSORPTION SPECTRUM OF ANHYDROSARSASAPOGENOIC ACID

Sir:

Dr. F. S. Spring has kindly pointed out in a letter to one of us that the ultraviolet absorption curve for anhydrosarsasapogenoic acid reported in a recent paper with Jacobsen [Fieser and Jacobsen, THIS JOURNAL, **60,** 2753 (1938)] is not, as was stated, indicative of the presence of an α,β-unsaturated ketonic group because, according to the graph, the intensity of the band at 243 mμ is much too low for such a compound (log ε = 0.98). This inconsistency is due to an unfortunate error in reproducing Dr. Webb's curve. The figures for the intensity of absorption inadvertently were reported as log ε values, whereas they actually are values of $\epsilon \times 10^{-4}$. The absorption band thus has both the location and the high intensity characteristic of an α,β-unsaturated ketone. The evidence on this point has been confirmed and extended by an examination of the absorption spectrum of anhydrosarsasapogenoic acid methyl ether acetate in absolute ethyl alcohol. This shows an intense band at 243 mμ (log ε = 4.13) and a secondary band with a maximum at 303 mμ (log ε = 1.86).

CONVERSE MEMORIAL LABORATORY
HARVARD UNIVERSITY
CAMBRIDGE, MASSACHUSETTS

LOUIS F. FIESER
R. NORMAN JONES

RECEIVED JANUARY 21, 1939

THE USE OF PHOSPHOTUNGSTIC ACID IN THE PRELIMINARY REFINING OF EXTRACTS CONTAINING VITAMIN K

Sir:

In this Laboratory, one of the most successful preliminary treatments of hexane extracts of dried alfalfa for the preparation of vitamin K concentrates has been the removal of the green pigments by adsorption on activated magnesium oxide. This procedure, however, is laborious and time consuming, and involves some loss of the vitamin.

It has been found that removal of the green pigments can be accomplished more conveniently by the use of phosphotungstic acid. To one volume of alfalfa hexane extract representing about 5 g. of dried alfalfa per cc., is added one-half volume of redistilled ethyl ether and phosphotungstic acid as a fine powder in the proportion of 0.03 g. per gram of dried alfalfa. The mixture is shaken vigorously until two phases form on standing: an upper, light green layer containing hexane, ether, about one-half of the total solids of the extract, and all of the vitamin K; and a lower, dark, viscous layer of ether, phosphotungstic acid, and the remainder of the solids of the extract. The lower layer is discarded.

The upper layer is washed repeatedly with approximately 50% ethanol and then with water. The ethyl ether is removed by distilling under reduced pressure and the remaining solution chilled at 0° for at least twenty-four hours. Solids which precipitate out are removed by filtering at 0° and discarded. From this point, the

procedures formerly employed, namely, repeated chilling, filtering and concentrating in hexane solution and then in ethanol solution are continued. Finally, the concentrate may be fractionated by distillation under very low pressure and the vitamin isolated in the form of the colorless, highly active fraction as already described [*J. Biol. Chem.*, **120,** 635 (1937)].

In Table I are given assay results illustrating the relative vitamin K activities of crude extracts of alfalfa and of the same extracts after the preliminary treatment with phosphotungstic acid. Results with two distillates obtained under very low pressure subsequent to the phosphotungstic acid treatment are also given. The fact that the average blood clotting times of chicks were not greater after the crude extract had been treated with phosphotungstic acid is ample evidence that in this step the losses of vitamin K are very slight, if any. Further advantages of this step are speed and applicability to concentrated solutions. Assays were conducted according to a procedure given elsewhere [*Biochem. J.*, **32,** 1897 (1938)].

TABLE I

AVERAGE CLOTTING TIMES OF CHICKS FED CERTAIN SOURCES OF VITAMIN K

Source of vitamin K	Amount per kilogram of diet[a]	Average blood clotting time, minutes
Hexane extract	10 cc.	3.9
of alfalfa	12 cc.	4.0
Phosphotungstic	≃10 cc.	3.5
acid treated	≃12 cc.	2.4
extract	≃12 cc.	4.1
Molecular	20 mg.	5.4
distillate	20 mg.	4.0

[a] Standard solution representing 1 g. of dried alfalfa per cc.

We wish to acknowledge technical assistance obtained through the WPA under Project A. P. No. 465-03-3-209. We are also indebted to Philip J. Grant, who developed the use of phosphotungstic acid as a precipitant for the green pigment fraction.

DIVISION OF POULTRY HUSBANDRY
COLLEGE OF AGRICULTURE
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BERKELEY, CALIFORNIA
A. A. KLOSE
H. J. ALMQUIST

RECEIVED DECEMBER 19, 1938

THE MOLECULAR WEIGHT OF THE DIPHTHERIA TOXIN PROTEIN

Sir:

In connection with an investigation of the biological and chemical aspects of the flocculation reaction involving diphtheria toxin and antitoxin now under way in our respective laboratories, we have had occasion to study the sedimentation, diffusion and electrophoresis behavior of the purified toxin [method of preparation, Pappenheimer, *J. Biol. Chem.*, **120,** 543 (1937)]. By these criteria, the toxin is found to behave as a homogeneous substance.

Sedimentation velocity determinations have been made in the standard oil turbine ultracentrifuge of Svedberg by using the refractive index method to locate the position of the boundaries at finite times during the experiment. For a 1% solution of the diphtheria toxin in $M/15$ phosphate buffer at pH 6.9 and 0.17 M sodium chloride, the scale displacement–distance curves give as sedimentation constant, corrected to the basis of a process taking place in pure water at 20°, $s_{20} = 4.6 \times 10^{-13}$ cm./sec./dyne.

Diffusion experiments with a similar solution, except that the protein concentration was reduced to 0.4%, were made by observing, again by the refractive index method, the blurring of a boundary formed and held stationary in the "U" tube of a Tiselius electrophoresis apparatus. The boundary was moved to the middle of an upper section with the compensation mechanism. The diffusion constant was calculated from the area and maximum height of the usual scale displacement–distance diagram and corrected to a process occurring in water at 20° to give as provisional value $D_{20} = 6.2 \times 10^{-7}$ cm.^{2}/sec.

From the sedimentation constant, s, and diffusion constant, D, the molecular weight is obtained

$$M = \frac{RTs}{D(1 - V\rho)} \cong 72{,}000$$

In the absence of direct determination the toxin protein is assumed to have partial specific volume, $V = 0.75$.

When subjected to a potential gradient in the Tiselius electrophoresis apparatus, containing a 1% solution of the toxin in borate–phosphate buffer at pH = 7 and ionic strength = 0.02, the protein migrates essentially as a single component. However, when the schlieren diaphragm is nearly closed, a trace of faster moving material may be observed. The latter is probably identical with a small amount of inactive protein known to be present.

Electrodialyzed antitoxic pseudoglobulin in 1% solution in $M/15$ phosphate buffer at pH 6.9 and 0.17 M NaCl shows a sedimentation behavior not

unlike that of normal pseudoglobulin. The predominating constituent, about 95%, gives $s_{20} = 7.4 \times 10^{-13}$ cm./sec./dyne and there is present a trace of component with $s_{20} = 18 \times 10^{-13}$ cm./sec./dyne. The antitoxic preparation used was 35% specifically precipitable by diphtheria toxin.

LABORATORY OF PHYSICAL CHEMISTRY
UNIVERSITY OF WISCONSIN HAROLD P. LUNDGREN
MADISON, WISCONSIN ALWIN M. PAPPENHEIMER, JR.
ANTITOXIN AND VACCINE LABORATORY J. W. WILLIAMS
JAMAICA PLAIN, MASSACHUSETTS

RECEIVED JANUARY 13, 1939

OXYGEN EXCHANGE BETWEEN CARBON DIOXIDE, BICARBONATE ION, CARBONATE ION AND WATER

Sir:

The rate of oxygen exchange has been used to study the velocity of the reaction between carbon dioxide and water when the *p*H is less than 8. In this range the predominating reaction of carbon dioxide is with the solvent molecules rather than the hydroxide ions.[1] Assuming that the reaction proceeds through the formation of H_2CO_3, and the reversal of this reaction, the equation for the reaction velocity becomes

$$-\frac{d\alpha}{dt} = \frac{k(H_2O)(CO_2)(\alpha - \beta)}{4(CO_2)} \qquad (1)$$

where α is the mole fraction of O^{18} in carbon dioxide, and β is the mole fraction of O^{18} in water, which because of the large quantity of water remains constant, and k is the specific reaction rate constant for the reaction between carbon dioxide and water. The equation is of the first order as is usual in these cases.[2] Moreover, when the water is in excess as, of course, is true in this case, the rate will be independent of the concentration of the reactant.

In our experiments carbon dioxide containing heavy oxygen was dissolved rapidly in water and then samples of this solution were withdrawn from time to time into evacuated vessels in order to separate rapidly the dissolved carbon dioxide from the water. This carbon dioxide was analyzed for its O^{18} content with a mass spectrometer. We find that the velocity constant, $k[H_2O]$, of this reaction is equal to 0.0027 at 0°, the time being in seconds, in good agreement with Stadie and O'Brien.[3] There is no salt effect as shown by making the solution 0.045 molar in sodium chloride. Moreover, there is no hydrogen ion catalysis since 0.02 molar hydrochloric acid does not change the rate.

The velocity of exchange of O^{18} between bicarbonate ion and water has been investigated. It appears that the exchange takes place only through the formation of carbonic acid and carbon dioxide, for the reaction takes place much more slowly under these conditions. In this case the rate depends upon the ratio of the carbon dioxide to the bicarbonate concentrations. The kinetic equation is given by Equation (1) above if $4(CO_2)$ is replaced by $2[3(HCO_3^-) + 2(CO_2)]$.

Pure sodium carbonate containing 0.513% of O^{18} has been dissolved in ordinary water. At 25° the time of half exchange in the case of 0.02 molar solution of sodium carbonate is approximately twenty-eight hours, while no exchange was observed when the solution was 0.02 molar in sodium carbonate and 0.04 molar in sodium hydroxide.[4] The error in our analyses is perhaps less than one per cent. of the percentage of O^{18} present. These results indicate that the reaction proceeds through the formation of the bicarbonate ion.

At 30° we find that the exchange between CO_2 and H_2O is substantially complete in about seven minutes as compared to over an hour at 0°. Such a rapid exchange might account for the decreased O^{18} content found in the experiments by Day and Sheel,[5] on the oxidation process taking place when heavy oxygen is inhaled by rats. The exchange should be very much more rapid in this case because of the high temperature and the possible effect of carbonic anhydrase.

The results of these experiments will be reported in greater detail later.

(1) C. Faurholt, *J. Chim. Phys.*, **21**, 400 (1924).

(2) H. A. C. McKay, *Nature*, **142**, 997 (1938), has shown that this is generally true for exchange reactions.

(3) W. C. Stadie and H. O'Brien, *J. Biol. Chem.*, **103**, 521 (1933).

(4) T. Titani, N. Morita and K. Goto, *Bull. Chem. Soc. Japan*, **13**, 329 (1938). Our greater analytical precision probably accounts for the difference in results reported here.

(5) J. N. E. Day and P. Sheel, *Nature*, **142**, 917 (1938).

COLUMBIA UNIVERSITY G. ALEXANDER MILLS
NEW YORK, N. Y. HAROLD C. UREY

RECEIVED JANUARY 23, 1939

CRYSTALLINE β-METHYLMANNOFURANOSIDE AND MANNOSEDIMETHYLACETAL

Sir:

Application of the furanoside synthesis developed in this Laboratory [Pacsu and Green, THIS JOURNAL, **58,** 1823 (1936); Green and Pacsu, *ibid.*, **59,** 1205, 2569 (1937); **60,** 2056, 2288 (1938); Pacsu, *ibid.*, **60,** 2277 (1938)] to *d*-mannosediethylmercaptal resulted in a 60% yield of α-methylmannofuranoside (m. p. 118–119°; $[\alpha]^{20}D$

108°), previously prepared by Haworth and coworkers through the dicarbonate [Haworth and Porter, *J. Chem. Soc.*, 649 (1930)] and direct from mannose and methyl alcoholic hydrogen chloride [Haworth, Hirst and Webb, *ibid.*, 651 (1930)]. When the mother liquor of our preparation was treated with a saturated solution of calcium chloride, a non-reducing, crystalline substance with $[\alpha]^{20}D$ −58° in water solution separated. The analytical results and hydrolysis experiments indicated that this substance was a calcium chloride addition compound of β-methylmannofuranoside with the composition of $C_7H_8O_6 \cdot CaCl_2 \cdot 3H_2O$. Further investigations revealed also that the sirupy residue of the preparation of α-methylmannofuranoside from mannose and methyl alcoholic hydrogen chloride would combine with calcium chloride to produce the same addition compound with $[\alpha]^{20}D$ −58°. Removal of the calcium chloride from the double compound by silver oxalate yielded pure β-methylmannofuranoside, which was secured in crystalline condition from ethyl acetate; m. p. 47°; $[\alpha]^{20}D$ −107° in water solution. On the basis of the formula $C_7H_8O_5 \cdot CaCl_2 \cdot 3H_2O$, the rotation of −106° is calculated for the glycoside portion. The two methylmannofuranosides represent the first α,β-pair of methylglycofuranosides that has been obtained in crystalline condition. The half of the rotational difference (107°) of the two isomers is much closer to the value (97°) for the methylglucopyranosides, than to the abnormally low value (74°) for the methylmannopyranosides. It is suggested that the nonvalidity of Hudson's isorotation rules, in the case of the mannopyranosides, is largely due to unequal contents of differently puckered rings in the α- and β-isomers. Since a five-membered ring as it occurs in the furanosides is practically flat, both α- and β-mannofuranosides are considered as being derived from nearly identical rings. Then the small but real deviation (10°) from the "normal" value (97°) for the glucopyranosides can be attributed mainly to an effect produced by the *cis*-hydroxyl groups at carbon atoms 2 and 3 in mannose.

Incidental to this investigation, the crystalline dimethyl acetal of *d*-mannose was prepared from the pentaacetate of mannosediethylmercaptal by the above-mentioned method. After recrystallization from ethyl acetate, the acetal had m. p. 101° and $[\alpha]^{20}D$ 0.6° in water solution.

A detailed account of this work will be published shortly.

FRICK CHEMICAL LABORATORY
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EUGENE PACSU
A. SCATTERGOOD

RECEIVED JANUARY 3, 1939

THE SULFUR AND PHOSPHORUS CONTENTS OF TOBACCO MOSAIC VIRUS

Sir:

Tobacco mosaic virus protein isolated by chemical means has been reported to contain from 0.0 to 0.59% sulfur and from 0.0 to 0.55% phosphorus.[1] The amounts found vary with the chemical treatment and the manner in which the sample is prepared for analysis. It has now been found that virus protein isolated by the physical method of differential centrifugation and prepared for analysis by drying from the frozen state contains uniformly approximately 0.24% sulfur and 0.60% phosphorus. Neither element is removed by dialysis against water at *p*H 9.3, a result in accordance with that reported from this Laboratory by Loring.[2] It seems likely that in a previous experiment,[3] in which removal of these two elements was secured, the preparation was more alkaline than *p*H 9.3. Although the native protein does not give a color reaction with nitroprusside, the denatured protein gives positive tests for sulfhydryl groups. Since sulfur and phosphorus appear to be in organic combination, the nature of their distribution in the protein has been studied.

Sulfur occurs in the form of cysteine or cystine, methionine and sulfate sulfur, and phosphorus, in accordance with previous work, in the form of nucleic acid. Recent unpublished work of Loring indicates that the phosphorus in the virus protein can be accounted for by that isolated in the form of nucleic acid. Baernstein's procedure as modified by Kassell and Brand[4] gives values of 0.04% methionine sulfur, 0.0–0.04% sulfate sulfur, and 0.14% cystine plus cysteine sulfur, thus accounting for practically all of the sulfur. Application of Sullivan's method to hydrochloric–formic acid hydrolysates of the protein indicates that 0.11% sulfur is present as cystine or cysteine, while Lugg's modification[5] of the Folin–Marenzi

(1) Stanley, *Phytopathology*, **26**, 305 (1936); Bawden and Pirie, *Proc. Roy. Soc.* (London), **B123**, 274 (1937); Loring and Stanley, *J. Biol. Chem.*, **117**, 733 (1937).
(2) Loring, *ibid.*, **123**, 126 (1938).
(3) Stanley, *ibid.*, **117**, 325 (1937).
(4) Kassell and Brand, *ibid.*, **125**, 145 (1938).
(5) Lugg, *Biochem. J.*, **26**, 2160 (1932).

method gives still lower results, unless nucleic acid is removed from the protein. The lower results are probably due to the formation of much humin, for when cystine is added before hydrolysis it is incompletely recovered by the latter methods. Removal of nucleic acid from the protein by treatment with 5% sodium hydroxide for one minute at 0° does not eliminate humin formation and does not alter the cystine plus cysteine values obtained by the Baernstein and Sullivan procedures. Removal of nucleic acid results in a partial loss of methionine and complete elimination of sulfate sulfur. Virus nucleic acid gives only negligible or no volatile iodide, homocysteine, cysteine or sulfate titrations. In the methionine determinations on protein the volatile iodide and homocysteine titrations agree, yet the results following treatment with alkali make further work necessary to establish definitely the presence or absence of methionine as a part of the intact virus protein molecule.

Virus activity is unaffected following treatment of the protein with reducing agents,[6] hence labile disulfide groups either are not present or their reduction does not affect virus activity. Although free sulfhydryl groups have not been demonstrated in active protein, they appear following denaturation by even mild means. Furthermore, activity is lost following mild oxidation.[6] The possibility that sulfhydryl groups may be correlated with activity is being investigated.

(6) Stanley, *Phytopathology*, **25**, 899 (1935).

THE ROCKEFELLER INSTITUTE A. FRANK ROSS
PRINCETON, N. J. W. M. STANLEY

RECEIVED JANUARY 20, 1939

A VOLATILE COMPOUND OF ALUMINUM, BORON AND HYDROGEN

Sir:

As a result of successive treatments of aluminum methyl with excess of diborane at temperatures up to 80°, we have obtained a new compound AlB_3H_{12}. Vapor density measurements using 33.5, 94.6, and 55.5 mg. gave molecular weights of 71.5, 71.6, and 71.1, respectively; calculated, 71.5. Hydrolysis of two samples (4.50 and 4.35 cc. at S. C.) gave quantities of hydrogen corresponding, respectively, to 11.9 and 12.2 times the volumes of the samples; theory, 12.0 times. After hydrolysis, aluminum was precipitated as 8-hydroxyquinolinate, giving, respectively, 91.5 and 91.0 mg. of the salt (theory, 92.3 and 89.2 mg.). Another sample (3.17 cc.) was hydrolyzed; boron was removed from the residue as methyl borate and then titrated as boric acid: found, 27.9mg., calcd., 26.3.

For further confirmation of the composition, 5.30 cc. of the compound was treated with hydrogen chloride at −80°, yielding 17.2 cc. of hydrogen, aluminum chloride and a volatile mixture of diborane and chlorodiborane. This mixture was hydrolyzed, giving 43.6 mg. of boric acid (theory, 43.9 mg.) and 46.7 cc. of hydrogen. Total hydrogen thus is 63.9 cc.; calculated, 63.6 (12 volumes).

Physical properties determined are: m. p. $-64.5 \pm 0.5°$; vapor tension at 0°, 119 mm.; b. p. (extrapolated) 44°. The gaseous substance undergoes little change within a relatively long time and is not rapidly affected even at 100°; in the liquid phase, slow polymerization seems to occur at room temperature.

With an equimolecular quantity of methyl ether the substance gives a liquid of low vapor tension, having the composition $AlB_3H_{12}\cdot(CH_3)_2O$. From this, methane is evolved without formation of diborane. With ammonia, a series of products containing up to four moles of ammonia is obtained, but the individuals have not yet been isolated.

The reaction of AlB_3H_{12} with trimethylamine has been studied in more detail. At room temperature, four moles of trimethylamine are taken up, but the resulting product seems to be a mixture of solids which, though moderately volatile, are difficult to separate. That one of the products is borine trimethylamine, $BH_3\cdot N(CH_3)_3$, is indicated by the fact that this substance could be isolated when the reaction was carried out under somewhat different conditions. Furthermore, it seems probable that at lower temperatures trimethylamine removes borine groups stepwise from AlB_3H_{12}. We are continuing the study of the reaction of trimethylamine to determine whether an addition compound of the amine and aluminum hydride is present in the final product.

We have found that diborane reacts with alkyls of metals other than aluminum, giving in some cases alkyldiboranes and other as yet unidentified products. The investigation is being carried on along the lines herein indicated.

GEORGE HERBERT JONES LABORATORY H. I. SCHLESINGER
UNIVERSITY OF CHICAGO R. T. SANDERSON
CHICAGO, ILLINOIS A. B. BURG

RECEIVED JANUARY 16, 1939