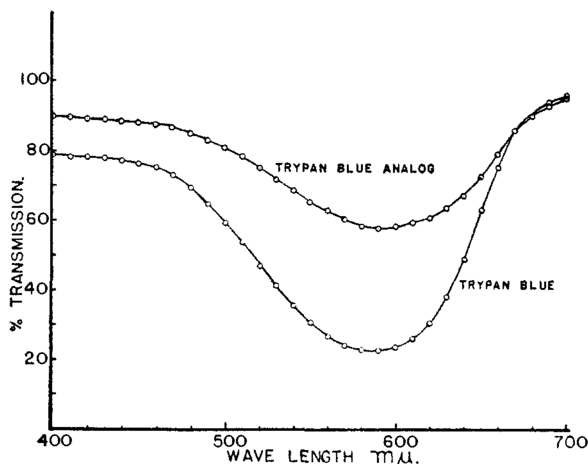

Fig. 1.

Since technical H-acid contains an impurity that forms a red dye with the tetrazonium chloride, it was purified by dissolving 27 g. of H-acid in 200 ml. of hot water and filtering. After reheating to 70°, it was salted out by the addition of 60 g. of sodium chloride. This procedure was repeated twice and the final product dried in a vacuum desiccator. This purification reduced the amount of the impurity but did not eliminate it entirely. It is therefore possible that it might be caused by mono-coupling.

Then 13.72 g. (0.04 mole) of H-acid was dissolved in 100 ml. of water containing 0.72 g. of sodium hydroxide (filtering if necessary). The solution was thus left faintly acid until just before coupling. This solution was cooled to 18°, 3.4 g. of sodium bicarbonate was added, and the tetrazonium chloride was run rather rapidly into the vigorously stirred alkaline solution of H-acid. It is important that the coupling mixture be kept alkaline, adding more sodium bicarbonate if necessary. Agitation was continued for 2 hours to ensure complete coupling. Tests were done for excess tetrazonium chloride and H-acid. The mixture was heated to 85°, 5 g. of decolorizing carbon was added, and the solution stirred for 15 minutes and filtered. The filtrate was reheated to 85° with agitation, and 27 g. of hydrated sodium acetate for each 100 ml. of solution was added slowly in four or five portions. The mixture, while still warm, was centrifuged, and the supernatant decanted. This procedure was repeated twice, and although it makes the filtration easier, it still was not possible to remove the red dye completely. For further purification, the dye was refluxed four times with 300-ml. portions of 95% ethyl alcohol which removes both the red dye and sodium acetate. To test for sodium acetate, a few drops of concentrated sulfuric acid were added to 10 ml. of filtrate until no turbidity was formed on cooling with ice. The absence of red dye was proven by the capillary test and by chromatography, using equal amounts of Hyflo-Super-cel and adsorptive magnesia. The dye was thus free of organic impurities; yield 42%.

Solubilities of the Dye.—Soluble in water, methyl alcohol, glacial acetic acid and methyl cellosolve; difficultly soluble in ethyl alcohol; insoluble in acetone, benzene, chloroform, dioxane, ether and petroleum ether.

Anal. Calcd. for $C_{33}H_{20}N_6O_{14}S_4Na_4$: N, 8.9; S, 13.6. Found: N, 8.9; S, 13.2.

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COMMUNICATIONS TO THE EDITOR

GERMBUDINE, ISOGERMIDINE AND VERATETRINE THREE NEW HYPOTENSIVE ALKALOIDS FROM VERATRUM VIRIDE

Sir:

Recent studies[1,2] have disclosed the isolation of the hypotensive ester alkaloids germitrine, neogermitrine and germidine from *Veratrum viride*. Fried, White and Wintersteiner have also shown that the triester germitrine can be partially hydrolyzed to the hypotensive diester germerine.

We wish to report that present investigations in our laboratories, on the alkaloids extractable from the ground roots and rhizomes of commercial *Veratrum viride*, have yielded germerine together with three new, highly potent, ester alkaloids for which we propose the names germbudine, isogermidine and veratetrine.

The benzene-extractable alkaloids, obtained by the procedure of Jacobs and Craig,[3] were separated into a crystalline non-ester alkaloid fraction and an amorphous fraction which contained the bulk of the hypotensive activity. This amorphous material was subjected to a 24-plate Craig counter-current distribution between benzene and 2 *M* acetate buffer at *p*H 5.5. The known triester neogermitrine was obtained by crystallizing the material in tubes 12–20 from acetone. Crystallization of the material in tubes 4–11 from benzene gave germerine (m.p. 203–205°; $[\alpha]^{26}D$ − 14.2° (*c*, 1 in pyridine), +6° (*c*, 1 in chloroform); the sample was identified further by comparison of its infrared spectrum, and by mixed melting point with authentic germerine kindly provided by Dr. J. Fried). In a personal communication, the latter disclosed that Dr. D. R. Walters of Squibb and Sons has also isolated germerine from *Veratrum viride*.

The alkaloids in tubes 0–3 were given a 72-plate Craig distribution between benzene and 2 *M* acetate at *p*H 6.5 and three fractions from this distribution, A, B and C, were crystallized from benzene to give three new alkaloids.

Fraction A (tubes 1–3), yielded germbudine (m.p. 158–160°, $[\alpha]^{27}D$ − 8.4° (*c*, 1 in pyridine), + 10.7° (*c*, 1 in chloroform)). Analytical data indicate the empirical formula $C_{37}H_{59}O_{12}N$ (calcd. C, 61.2; H, 8.20; eq. wt., 726; found: C, 61.0; H, 8.21; eq. wt., 732). Volatile acid determination, found: 0.91 equivalent of acid. Alkaline hydrolysis of germbudine afforded the alkamine germine and an acid fraction. The acids were converted to their *p*-phenylphenacyl esters which were separated chromatographically into the ester of α-meth-

(1) J. Fried, H. L. White and O. Wintersteiner, THIS JOURNAL, **71**, 3260 (1949); **72**, 4621–4630 (1950).

(2) J. Fried and P. Numerof, Abst. 119th Meeting A.C.S., Cleveland, Ohio, April, 1951.

(3) W. A. Jacobs and L. C. Craig, *J. Biol. Chem.*, **160**, 555 (1945).

ylbutyric acid and what appeared to be the ester of an unidentified acid (D). The presence of the latter was suggested by an examination of its infrared spectrum. It is being studied further.

The diester isogermidine crystallized from fraction B (tubes 12–18) (m.p. either 221–222° or 229–230°, depending on which form separated; $[\alpha]^{28}D$ − 63.2° (*c*, 1 in pyridine), −26.0° (*c*, 1 in chloroform)). Calcd. for $C_{34}H_{53}O_{10}N$: C, 64.2; H, 8.40; eq. wt., 635. Found: C, 64.0; H, 8.33; eq. wt., 646, 655. Hydrolysis of isogermidine yielded germine, α-methylbutyric acid and acetic acid. The acids were identified by conversion to their *p*-phenylphenacyl esters.

Fraction C (tubes 19–36) gave crystalline veratetrine (m.p. 269–270° (dec.); $[\alpha]^{26}D$ − 31° (*c*, 1 in pyridine), $[\alpha]^{28}D$ − 2° (*c*, 1 in chloroform)). The analytical data indicate the empirical formula $C_{43}H_{64}O_{16}N$ (calcd. C, 60.7; H, 7.58; N, 1.65; eq. wt., 850.5; found: C, 60.8; H, 7.56; N, 1.4; eq. wt., 840). We have also isolated the same alkaloidal ester from *Veratrum album*. Volatile acid determination, found 3.2 equivalents of acid.

Alkaline hydrolysis of veratetrine yielded the alkamine isoprotoverine (identified by melting point and by comparison of its infrared and ultraviolet absorption spectra with those of authentic isoprotoverine) and an acid fraction. The acids were converted to their *p*-phenylphenacyl esters which were separated chromatographically into the esters of α-methylbutyric acid, acetic acid and the unidentified acid "D," obtained above from germbudine. The weights of the phenylphenacyl esters suggest veratetrine gives on hydrolysis one equivalent each of α-methylbutyric acid and acid D, together with two equivalents of acetic acid.

The hypotensive activity of germbudine, isogermidine and veratetrine have been determined in the anesthetized dog by a modification[4] of the method of Maison and Stutzman.[5] In comparison with a mixed alkaloidal ester preparation from *Veratrum viride* ("Deravine"), which produced a 30% fall in the mean arterial blood pressure of the anesthetized dog at a dose level of 0.2 γ per kg. per min., their relative activities are 0.97, 0.12 and 0.87, respectively.

(4) F. R. Skelton, Marjorie Beck and G. A. Grant, *Fed. Proc.*, Vol. 11, No. 1, Part I, p. 390. March, 1952.

(5) C. L. Maison and J. W. Stutzman, *Arch. Int. Pharmacodyn.*, **85**, 357 (1951).

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RECEIVED MAY 14, 1952

THE ANALYSIS AND SEPARATION OF GLUCURONIC AND GALACTURONIC ACIDS BY ION EXCHANGE[1]

Sir:

Methods for the analysis of uronic acids in biological material have always been rather limited due to the interference caused by sugars. Recently Dische[2] has described a series of color tests, which depend upon the rate and difference in color formation to distinguish between glucuronic and galacturonic acids, polyuronides, and sugars. Roboz[3] has previously shown that galacturonic acid could be separated from other material by adsorption on an anion-exchange resin (IRA-400); then recovered by elution with 25–50% acetic acid. However, no methods reported so far have effected both a qualitative and quantitative determination of the uronic acids, with a complete recovery of the pure individual uronic acids as crystalline materials; such a method will be described here.

In these experiments, a dilute alkaline solution of galacturonic, glucuronic, and mannuronic acids was absorbed quantitatively on the acetate form of the strong base anion exchanger Dowex-1. The uronic acids were eluted with 0.15 *M* acetic acid and the fractions analyzed by a slight modification of the orcinol method as described by Brown[4] for pentose determination (samples were heated at 100° for at least one-half hour). This assay method, as applied here, obeys Beer's law through the concentration range of 2 to 60 μg./ml. of uronic acid.

The separation of galacturonic and glucuronic acids in the presence of arabinose and galactose is shown in Fig. 1. The same separation can be achieved with a formate system using 0.01 *M* formic acid as the eluting agent. Mannuronic acid was eluted from the column in the same fraction as the glucuronic acid and a separation of these two could not be effected in the systems reported here. The free sugars, which were not absorbed, were collected in the first fraction and determined colorimetrica ly (galactose was determined by the anthrone method of Dreywood[5] as developed by Morris[6];

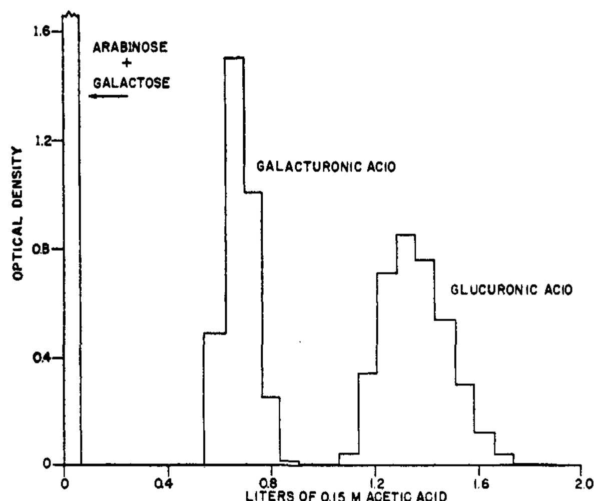

Fig. 1. The separation of galacturonic and glucuronic acids in the presence of sugar: exchanger, 0.85 sq. cm. × 12 cm. Dowex-1, *ca.* 300 mesh, acetate form; eluting agent, 0.15 *M* acetic acid at ~ 2.5 ml./min.; test material, 5.0 mg. each of arabinose and galactose, 10 mg. each of galacturonic and glucuronic acids in 10 ml. of 0.02 *M* sodium hydroxide [galactose was determined by anthrone method at 620 mμ; the other materials by orcinol method at 660 mμ].

(1) Work performed under Contract W-7405-eng-26 for the Atomic Energy Commission.

(2) Z. Dische. *J. Biol. Chem.*, **167**, 189 (1947); **171**, 725 (1947); **183**, 489 (1950); also *Arch. Biochem.*, **16**, 409 (1948).

(3) E. Roboz, Internat. Congr. of Pure and Applied Chem., Abstracts XII, 156 (1951).

(4) A. H. Brown, *Arch. Biochem.*, **11**, 269 (1946).

(5) R. Dreywood, *Ind. Eng. Chem.*, *Anal. Ed.*, **18**, 499 (1946).

(6) D. L. Morris, *Science*, **107**, 254 (1948).

arabinose, by the orcinol method of Brown[4]). If unknown sugars are present in this fraction they can be adsorbed directly on a borate column and analyzed individually by the method of Khym and Zill.[7] Essentially, quantitative recoveries of the sugars and the uronic acids were obtained.

The identity of the peaks was determined by column runs on the individual uronic acids and by isolation and characterization of the uronic acid in the peak. Eluate fractions were evaporated to dryness *in vacuo* and characterized as the benzimidazole derivative according to the procedure of Lohmar, Dimler, Moore, and Link.[8]

(7) J. X. Khym and L. P. Zill, THIS JOURNAL, **73**, 2399 (1951); **74**, 2090 (1952).

(8) R. Lohmar, R. J. Dimler, S. Moore and K. P. Link, *J. Biol. Chem.*, **143**, 551 (1942).

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RECEIVED MARCH 29, 1952

3,5-DIAMINO-*AS*-TRIAZINES AS INHIBITORS OF LACTIC ACID BACTERIA AND PLASMODIA

Sir:

The inhibitory activity of various 2,4-diaminopyrimidines on the growth of *Lactobacillus casei* was interpreted as a competitive antagonism between these substances and folic acid.[1] The formal structural analogy between certain 5-substituted diaminopyrimidines and the known antimalarial chlorguanide (I) led to the suggestions that the diaminopyrimidines might have antimalarial properties and that both the biguanide and pyrimidine might act on plasmodia as antagonists of folic acid and related compounds.[2] Further investigations of the antimalarial properties of the pyrimidines culminated in the synthesis of 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (II)[3] which has a minimum effective dose[4] of about 0.03 mg./kg. corresponding to activity of 60 times chloroguanide against *Plasmodium gallinaceum* and 200 times against *P. berghei*. Furthermore, evidence relating the antimalarial activity of both types of substance to the folic acid metabolism of the parasite was forthcoming.[5,6]

Recent investigations have shown that chlorguanide (I) is converted to 2,4-diamino-1-*p*-chlorophenyl-6,6-dimethyl-1,6-dihydro-1,3,5-triazine (III) *in vivo*,[7] and that this metabolite is an active antimalarial[7,8] and an antagonist of the folic acid group of vitamins in microbiological studies.[8] It is apparent that a real structural analogy between II and III exists and is determinative of the similarity of biological behavior of I and II.

(1) G. H. Hitchings, G. B. Elion, H. VanderWerff and E. A. Falco, *J. Biol. Chem.*, **174**, 765 (1948).

(2) E. A. Falco, G. H. Hitchings, P. B. Russell, and H. VanderWerff, *Nature*, **164**, 107 (1949).

(3) E. A. Falco, L. G. Goodwin, G. H. Hitchings, I. M. Rollo and P. B. Russell, *Brit. J. Pharm.*, **6**, 185 (1951).

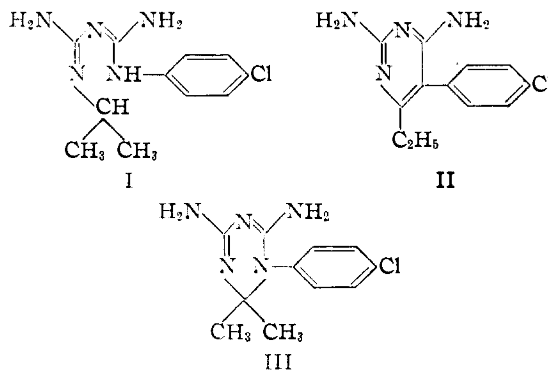
(4) I. M. Rollo, *Nature*, **168**, 332 (1951).

(5) J. Greenberg and E. M. Richeson, *J. Pharm. Exp. Therap.*, **99**, 444 (1950).

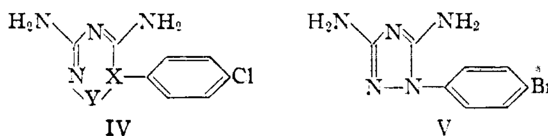
(6) J. Greenberg, *Proc. Soc. Exper. Biol. Med.*, **71**, 306 (1949).

(7) H. C. Carrington, A. F. Crowther, D. G. Davey, A. A. Levi and F. L. Rose, *Nature*, **168**, 1080 (1951).

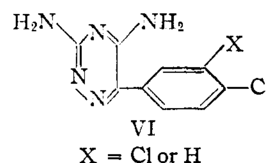
(8) E. J. Modest, G. E. Foley, M. M. Pechet and S. Farber, THIS JOURNAL, **74**, 855 (1952).



The structural feature common to II and III is the unit IV. The significance of this structural unit, in various heterocyclic systems, has been under investigation in these laboratories for some time. For example, 3,5-diamino-1-*p*-bromophenyl-1,2,4-triazole (*p*-bromophenylguanazole) (V) and several 3,5-diamino-6-phenyl-1,2,4-triazines (VI) have been synthesized, and their biological activities have been investigated. (**3,5-Diamino-1-*p*-bromophenyltriazole,** m.p. 210°. *Anal.* Calcd. for $C_8H_8N_5Br$: C, 36.8; H, 3.2. Found: C, 36.6; H, 3.3. **3,5-Diamino-6-(3′4′-dichlorophenyl)-1,2,-4-triazine,** m.p. 219–220°. *Anal.* Calcd. for $C_9H_7N_5Cl_2$: C, 42.2; H, 2.7. Found: C, 42.4; H, 3.0. **3,5-Diamino-6-(4′-chlorophenyl)-1,2,4-triazine,** m.p. 218–220°. Calcd. for $C_9H_8N_5Cl$: C, 48.9; H, 3.6. Found: C, 48.6; H, 3.6.)



X = N, C, C—CH$_2$, C—O
Y = C, N



X = Cl or H

Biologically the asymmetrical triazine (VI, X = H) closely resembles the pyrimidine (II) and the symmetrical dihydrotriazine (III). Thus it is a competitive antagonist of folic and folinic acids in the growth of *L. casei* and of folinic acid (synthetic "Leucovorin") in the growth of *Leuconostoc citrovorum*, with an inhibition index in the latter system of approximately 20,000 (at half-maximal growth). With VI, as with II and III, the minimum concentration for inhibition of *Streptococcus faecalis* is several hundred-fold greater when folinic acid is supplied than when the organisms are grown with folic acid. This is in contrast to the finding with *L. casei* where folic and folinic acids are essentially equivalent over a wide range of concentration of the inhibitor. The antimalarial activities of VI and its congeners are quite high. The activity of the 3,4-dichlorophenyl analog of VI against *P. berghei* is 230 times that of chlorguanide; it is, however, less active than chlorguanide in *P. gallinaceum* infections.

The biological activities of the guanazole (V) are

generally weaker than those of the triazines and perhaps qualitatively different. Thus V is only a weak inhibitor of *L. casei* and its activity is not clearly blocked by folic acid. Furthermore, its antimalarial activity is minimal.

The above observations lend support to the previously expressed views concerning the relationship between antifolic acid and antimalarial activity[2] and further delineate the chemical structures with which this activity is associated.

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RECEIVED APRIL 29, 1952

STEROIDAL SAPOGENINS. V.[1] ENZYMATIC HYDROLYSIS OF STEROIDAL SAPONINS

Sir:

It is well known that plants containing cardioactive glycosides have enzyme systems which can partially hydrolyze these substances.[2] We have recently found that leaves of some Agave and Yucca species which contain steroidal saponins have similar enzyme systems. In contrast to the enzymes acting on cardio-active glycosides, those found in the sapogenaceous plants tested cleave the saponin substrate to the sapogenin aglycone and component sugars or polysaccharides.

An enzyme preparation was obtained by grinding 2 kg. of frozen *Agave toumeyana*[3] leaves, followed by extraction with water at 10°. The filtered extract was shaken with benzene I and the aqueous layer containing the enzyme fraction held at 4°. The leaf residue remaining from the aqueous extraction was refluxed with 95% ethanol to extract residual saponins. The ethanol solution was concentrated, diluted with water, extracted with benzene II, and the aqueous saponin fraction heated on the steam bath to drive off residual alcohol.

The aqueous enzyme and saponin solutions were combined, adjusted to *p*H 5.25 and held at 37° for 90 hours. The resultant turbid suspension was extracted first with a mixture of 90% benzene–10% ethanol III to recover enzyme hydrolyzed sapogenins and then with butanol IV to obtain unhydrolyzed or partially hydrolyzed saponins.

Only a trace of sapogenin was found in the prehydrolysis benzene extracts I and II. The extract III was concentrated and chromatographed on activated alumina. Using methods presented in detail elsewhere[4] the following sapogenins were isolated: (1) hecogenin (isoallospirostan-12-one-3β-ol), m.p. Kofler 255–260°, on Wolf–Kishner reduction gave tigogenin (isoallospirostan-3β-ol) m.p. 203–205°, yield 3.9 g.; (2) manogenin (isoallospirostan-12-one-2α,3β-diol) m.p. 240–244°, yield 1.3 g., on Wolf-Kishner reduction gave gitogenin (isoallospirostan-2α-3β-diol) m.p. 268–270°. Infrared spectra of all sapogenins and their Wolf–Kishner reduction products were identical (with exception of manogenin which showed slight divergencies) with those of authentic specimens. Hecogenin and manogenin had a carbonyl peak in the infrared at 1708–1710 cm.$^{-1}$, absent in reduced products. Manogenin also showed conjugated carbonyl at 1678 cm.$^{-1}$ from which it is deduced that 10–20% of the $9^{(11)}$ dehydro component was present.

From IV after subsequent acid hydrolysis and chromatography were isolated 2.4 g. of sapogenin similar in composition to those found in III, indicating that the enzymatic hydrolysis was 78–80% complete under our experimental conditions.

In a duplicate experiment, the sapogenins were isolated entirely by means of acid hydrolysis. The yield of total sapogenin was similar and the sapogenins isolated identical with the enzymatic hydrolysis.

Using methods similar to those described above, acid or enzyme hydrolysis of *Agave serulata*[5] gave hecogenin, manogenin and small quantities of the $9^{(11)}$-dehydro analogs of both sapogenins; an unidentified Yucca species[6] yielded with both methods sarsasapogenin (spirostan-3β-ol) and an unidentified Dioscorea species gave diosgenin (Δ^5-isospirosten-3β-ol) in both cases.

In agreement with Marker, *et al.*,[7] we find that steroidal sapogenins occur in plants only as glycosides as evidenced by the fact that free sapogenin could not be found prior to hydrolysis. Our data are not in accordance with the views of Marker and Lopez[8] that the spiroketal side chain and the 12-keto group in sapogenins are artifacts produced by acid hydrolysis. The fact that the much milder enzyme hydrolysis gives the same products as acid cleavage tends to cast doubt on Marker's hypothesis. Our previous findings[9] based on infrared studies of saponins have also shown that the structure of the steroidal moiety of saponins and their sapogenins derived by acid hydrolysis are in all probability identical.

Because of the mild conditions used, tars, resins and pigments produced by acid hydrolysis are not found in the enzymatic method, thus simplifying the isolation of purified sapogenins.

The distribution, occurrence, and physico-chemical properties of the enzyme systems found in sapogenaceous plants are being studied and will be reported in detail at a later date.

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RECEIVED MAY 9, 1952

(1) Paper IV of this series submitted for publication in THIS JOURNAL. This work was done as part of a coöperative arrangement between the Bureau of Plant Industry, Soils, and Agricultural Engineering and the Bureau of Agricultural and Industrial Chemistry, United States Department of Agriculture, and the National Institutes of Health, Federal Security Administration.

(2) For pertinent references see A. Stoll and associates, *Helv. Chim. Acta*, 1933–1951, and W. A. Jacobs, *Physiol. Rev.*, **13**, 222 (1933).

(3) Collected by H. S. Gentry, #S 2046, Superior, Arizona, December, 1951.

(4) M. E. Wall, M. M. Krider, E. S. Rothman and C. R. Eddy, *J. Biol. Chem.*, in press.

(5) Collected by H. S. Gentry, #1865, San Ignacio, Baja California, November, 1951.

(6) Collected by H. S. Gentry, #2161, Jecabasco, Mexico, December, 1951.

(7) R. E. Marker, *et al.*, THIS JOURNAL, **69**, 2167 (1947).

(8) R. E. Marker and J. Lopez, *ibid.*, **69** 2390 (1947).

(9) E. S. Rothman, M. E. Wall and H. Walens, *ibid.*, in press.

ZYGADENUS ALKALOIDS. II. THE OCCURRENCE OF HYPOTENSIVE GERMINE ESTERS IN ZYGADENUS VENENOSUS

Sir:

A recent study[1] of the alkaloidal constituents of *Zygadenus venenosus* revealed the occurrence in the plant of the esters veratroylzygadenine and vanilloylzygadenine as well as the alkamines zygadenine and germine. We wish to report now the isolation from a batch of this plant collected in June, 1951 of the germine esters neogermitrine,[2] germidine,[3] protoveratridine,[4] and a new diester isomeric with germidine, for which we propose the name neogermidine.

Fractionation of the chloroform-extractable alkaloids of *Zygadenus venenosus* (WATS.)[5] by 8-plate countercurrent distribution using benzene and phosphate buffer at *p*H 7.1 yielded, in addition to the alkaloids obtained previously,[1] the germine monoester protoveratridine (from the O-plate fraction). Protoveratridine crystallized as rectangular plates upon reprecipitation from alcoholic acetic acid solution with aqueous ammonia; m.p. 272–273° dec.; $[\alpha]^{22}D$ −9° (*c* 0.76, pyr.). *Anal.* Calcd. $C_{32}H_{51}O_9N$: C, 64.73; H, 8.66; N, 2.36. Found: C, 64.79; H, 8.62; N, 2.62.

The filtrates after removal of the crystalline alkaloids from the plate-8 fraction were combined with the material from plates 4 to 7, and the amorphous mixture obtained upon lyophilization was designated the "organophilic" fraction. The filtrates after removal of the crystalline alkaloids from the plate-O fraction were combined with the material from plates 1 to 3, and the residue obtained upon lyophilization was designated the "hydrophilic" fraction.

Neogermitrine and germidine were obtained by 24-plate countercurrent distribution of the organophilic fraction using benzene and 2*M* acetate buffer at *p*H 5.5.[2] The identity of these substances was confirmed by mixed melting point and infrared spectral comparisons with authentic specimens from *Veratrum viride* kindly provided by Dr. J. Fried.

The hydrophilic fraction was subjected to 8-plate countercurrent distribution using chloroform and 2*M* acetate buffer at *p*H 5.5. Neogermidine was obtained by chromatography on alumina of the material recovered from plates 5 to 8. Neogermidine crystallized from benzene as heavy prisms; m.p. 221–223° dec.; $[\alpha]^{22}D$ −60° (*c* 2.00, pyr.); $[\alpha]^{22}D$ −25° (*c* 2.00, chf.). *Anal.* Calcd. $C_{34}H_{53}O_{10}N$: C, 64.22; H, 8.38. Found: C, 64.15; H, 8.70. Neogermidine thiocyanate crystallized from acetone as needles, m.p. 247–249° dec. *Anal.* Calcd. $C_{34}H_{53}O_{10}N \cdot HNCS$: C, 60.50; H, 7.82; S, 4.61. Found: C, 60.16; H, 7.87; S, 4.65. In a volatile acid determination, 17.52 mg. of neogermidine was equivalent to 5.77 ml. of 0.009126 *N* sodium thiosulfate; calcd. for germine monoacetate mono-α-methylbutyrate, 6.05 ml. Alkaline hydrolysis of neogermidine afforded germine, acetic acid and α-methylbutyric acid. The acids were identified by conversion to their *p*-phenylphenacyl esters which were characterized after chromatographic separation. Methanolysis of neogermidine afforded protoveratridine. The large change in rotation attending the methanolysis of neogermidine to protoveratridine suggests that the site of attachment of the acetyl group on the alkamine germine is the same as that of the labile acetyl group in both neogermitrine and germitrine.

Acetylation of neogermidine with acetic anhydride and pyridine yielded acetylneogermitrine, m.p. 248–249° dec., identical with a sample prepared by acetylation of germidine.[2] Acetylation of protoveratridine under the same conditions also gave acetylneogermitrine. These facts show that the site of attachment of the α-methylbutyryl group is the same in each of the four germine esters isolated from *Zygadenus venenosus.*

Pharmacological experiments carried out with neogermidine at the laboratory of Professor O. Krayer at Harvard Medical School indicate that the circulatory action in the cat and the veratrinic effect on the frog muscle are similar to those of germidine.

This work was supported (in part) by grants from the National Institutes of Health and Eli Lilly and Company. The assistance of Eli Lilly and Company in gathering and extracting *Zygadenus venenosus* is gratefully acknowledged.

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RECEIVED MAY 22, 1952

(1) S. M. Kupchan and C. V. Deliwala, THIS JOURNAL, **74**, 2382 (1952).

(2) J. Fried, P. Numerof and N. M. Coy, *ibid.*, **74**, 3041 (1952). We wish to thank Dr. J. Fried for private communication of these results prior to publication.

(3) J. Fried, H. L. White and O. Wintersteiner, *ibid.*, **72**, 4621 (1950).

(4) G. Salzberger, *Arch. Pharm.*, **228**, 462 (1890); W. Poethke, *ibid.*, **275**, 571 (1937).

(5) Plant gathered in northeastern Oregon in June, 1951. We are grateful to Dr. Reed Rollins, Gray Herbarium, Harvard University, for confirming the identity of the plant.

A NEW DISACCHARIDE PRODUCED BY LEUCONOSTOC MESENTEROIDES

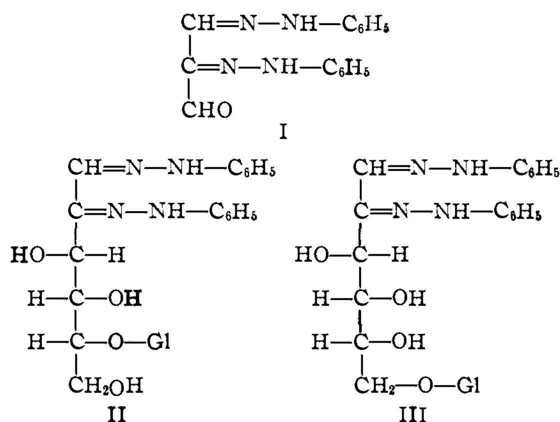
Sir:

In studies on the enzymatic synthesis of dextran from sucrose by *Leuconostoc mesenteroides* we have noted that under certain conditions as much as 3% of the sucrose is diverted to the production of a new disaccharide which has the following properties: Crystallizes in the form of bars from methanol–ethyl acetate; m.p. 161–163°; $[\alpha]^{25}D$ −8.8° after 3 minutes, −6.8° after 24 hours (*c*, 4; H_2O); analyzes for $C_{12}H_{22}O_{11}$; reducing power by Somogyi method 46% of that of fructose; low order of reaction with hypoiodite (9% by Willstätter–Schudel method; turanose gave 11%); positive Seliwanoff test for fructose; hydrolyzes to glucose and fructose as shown by paper chromatography; phenylosazone (analyzing for $C_{24}H_{32}N_4O_9$) as needles from wet ethyl acetate (m.p. 186–188°); yields an amorphous phenylosotriazole which hydrolyzes to D-glucose ($[\alpha]^{25}D$ +52°) and D-glucose phenylosotriazole (m.p. 197°; $[\alpha]^{25}D$ −81°).

From this preliminary work it would appear that the sugar is a D-glucosyl-D-fructose with the glucosyl unit probably in the pyranose form since

only 1% hydrolysis is observed in 24 hours at room temperature in 0.2 N HCl.

A tentative conclusion as to the point of attachment of the glucosyl group has been based on the following observations: Chargaff and Magasanik[1] noted that glucose phenylosazone was rapidly cleaved by sodium periodate to give a precipitate of compound I in 85% yield. We have extended this technique to the disaccharides and find it useful in the determination of the point of union of the component parts, since glycosidic linkages in the 3- and 4-positions block the reaction. The method should be applicable to any oligosaccharide giving an osazone.

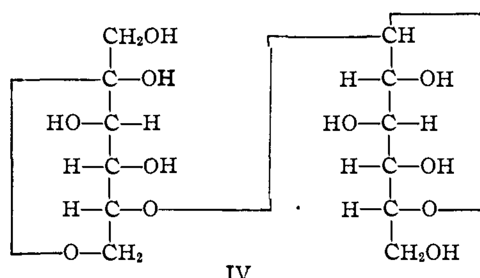


Under the conditions used by those workers we found that the phenylosazone of the new sugar as well as that of isomaltose (6-(α-D-glucopyranosyl)-D-glucose) and of gentiobiose (6-(β-D-glucopyranosyl)-D-glucose) gave rapid precipitation of compound I in yields of 70–80%. On the other hand, the phenylosazones of turanose (3-(α-D-glucopyranosyl)-D-fructose), laminaribiose (3-(β-D-glucopyranosyl)-D-glucose), maltose (4-(α-D-glucopyranosyl)-D-glucose), and cellobiose (4-(β-D-glucopyranosyl)-D-glucose) gave no precipitates (other than colorless inorganic salts) even on standing overnight. Such behavior is compatible only with structures II and III having the glucosidic linkage on positions 5 or 6, respectively. This conclusion is further supported by the fact that the phenylosazone of the new sugar gives an X-ray diffraction pattern readily distinguishable from that obtained with the phenylosazones of turanose, laminaribiose, maltose, or cellobiose.

The possibility of a 1,6 linkage was also rendered unlikely by the following considerations: The X-ray pattern of the phenylosazone of the new sugar was found to differ from that of gentiobiose phenylosazone; the pattern given by isomaltose phenylosazone, however, was so similar to that of the new phenylosazone that conclusive differentiation was not possible. Fortunately, identity could be ruled out by the melting point of isomaltose phenylosazone (205–207°) and the fact that it forms a crystalline phenylosotriazole (m.p. 179–180°).

Preliminary evidence, then, suggests that the new sugar may be a 5-(D-glucopyranosyl)-D-fructopyranose, a possible form of which is shown in formula IV.

(1) E. Chargaff and B. Magasanik, THIS JOURNAL, **69**, 1459 (1947).



Apparently no 1,5-phenylosazones of the proper configuration are available for comparison. Freudenberg and v. Oertzen[2] recently synthesized 5-(β-glucosido)-glucose but were unable to obtain pure osazones.

Our work on the mechanism of dextran formation indicates that the new sugar plays a role in the polymerization process; we believe it advisable, therefore, to assign it the common name of "leucrose" which is suggested by its particular microbial origin.

Further structure studies are in progress.

We are indebted to Prof. E. L. Hirst and Dr. V. C. Barry for laminaribiose samples, to Dr. Allene Jeanes for the isomaltose, to Drs. N. K. Richtmyer and C. S. Hudson for the turanose, and to Dr. N. Hellman and Mr. H. F. Zobel for the X-ray determinations.

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RECEIVED MAY 2, 1952

(2) K. Freudenberg and K. v. Oertzen, *Ann.*, **574**, 37 (1951).

(3) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

THE MOLECULAR WEIGHT AND SHAPE OF DESOXYPENTOSE NUCLEIC ACID

Sir:

The uncertainty which persists with regard to the molecular weight and shape of desoxypentose nucleic acid, DNA, from calf thymus appears to be due to the inadequacy of most macromolecular techniques in characterizing very large, charged polyelectrolytes and to the varying degrees of degradation inherent in the different methods of preparation. In a current paper[1] it is shown that the measurement of the angular distribution of scattered light from DNA solutions in the concentration range of 1 to 10 mg./100 cc. leads to a determination of the molecular weight as well as to some definite conclusions about the size and shape of several different samples. The sample having the highest molecular weight was that prepared by Schwander and Signer.[2] We wish to report measurements on a sample we have prepared by the Signer method which indicate the reproducibility of this preparative method and provide new information on the structure of the DNA molecule.

Light scattering measurements on the new preparation in 0.2 M NaCl show the molecular weight to be 7,700,000 in comparison with 6,700,000 found[1]

(1) P. Doty and B. H. Bunce, THIS JOURNAL, in press.

(2) H. Schwander and R. Signer, *Helv. Chim. Acta*, **33**, 1521 (1950).

for the Signer sample (VII). This difference is somewhat outside probable error, and the intrinsic viscosities at 1000 sec.$^{-1}$ of 22.2 and 19.9 (100 cc./g.) support this view, but the difference is marginal in comparison with the molecular weights of DNA prepared by other methods.[1] Further evidence of the reproducibility of the DNA samples prepared by the Signer method lies in the work of Katz[3] who prepared independently another sample and using the techniques previously worked out[1] found the molecular weight to be 8,000,000.

Information on the size and shape of the DNA molecule can be derived from the angular distribution of the reduced intensity extrapolated to zero concentration. Our results show that the molecule is not at all rod-like at *p*H 6.5 but that it has a three-dimensional structure only slightly more asymmetric than a random coil. The maximum dimension of the molecule is about 6500 Å.

Two other studies indicate that the molecule is not a linear, random coil but rather a lightly branched or cross-linked polynucleotide as suggested elsewhere.[1] One of these is the investigation of the changes produced upon lowering the *p*H of the 0.2 *M* NaCl solution of DNA to 2.6 by dialysis. It is found that the molecular weight does not change but that the molecule collapses into a rod 3000 Å. long, as shown by the radical change of the angular intensity distribution to that corresponding to a rod. These results are summarized in the accompanying figure which shows the intensity distribution before, during, and after the exposure to *p*H 2.6.

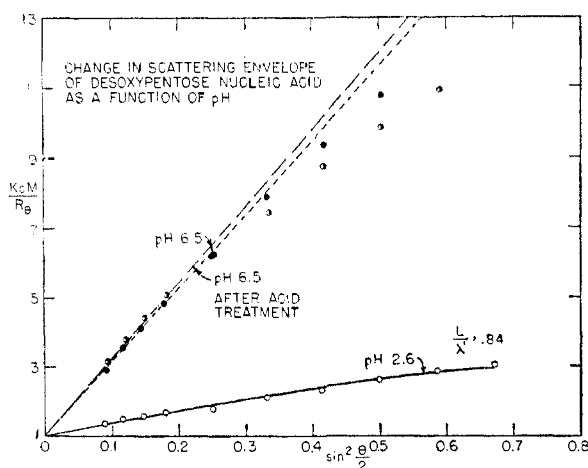

Fig. 1.

If this were a linear polynucleotide coil, it would, due to its charge, expand greatly upon removal of the salt from its solution and would exhibit an increase of the reduced specific viscosity upon dilution of its aqueous solution. In view of what has been found, for example with polymethacrylic acid,[4] this would for DNA involve about a ten-fold increase in its maximum dimension and at least a hundred-fold increase in the reduced specific viscosity. Instead it is found that at *p*H 6.5 the maximum dimension only increases about 60% when salt is removed[5] and that the reduced specific viscosity only increases from 55 at 16 mg./100 cc. to 85 at 1 mg./100 cc. Thus the polynucleotide chain is under considerable restraint probably due to a small number of branch or network points. On the other hand, even this limited expansibility of the molecule eliminates the possibility of it being rod-shaped at neutral *p*H.

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RECEIVED MAY 14, 1952

(3) S. Katz, THIS JOURNAL, **74**, 2238 (1952).

(4) A. Oth and P. Doty, *J. Phys. Chem.*, **56**, 43 (1951).

(5) E. P. Geiduschek, Ph.D. Thesis, Harvard University, 1951.

(6) U. S. Public Health Post-doctoral Fellow.

(7) A fellowship from the American Cancer Society Institutional Grant is gratefully acknowledged.

NATURE OF MONOESTER PHOSPHATE GROUP IN COENZYME A[1]

Sir:

The coenzyme A molecule has been reported to contain three phosphate groups.[2] Two of these phosphate groups are in a pyrophosphate linkage, whereas the third phosphate has been ascertained to be in the monoester form, esterified with the ribose moiety of the adenylic acid portion of the coenzyme.[3] Prostatic phosphatase, a monoesterase, has been found to inactivate coenzyme A by removal of one phosphate.[2] We have found that an enzyme from barley,[4] which splits only "b" nucleotides, also will remove the monoester phosphate group and inactivate the coenzyme.[5] Table I shows the action of the "b" nucleotidase on the coenzyme.[6]

TABLE I

SPLITTING OF COENZYME A BY "B" NUCLEOTIDASE

	Inorg. P, µM	Arsenolysis of acetyl-PO_4 units	Acetylation of sulfanil-amide units
CoA (0.05µM.)	0	14.8	14.8
CoA (0.05µM.) + "b"-Nucleotidase	0.052	0	13.9

The "b" nucleotidase has also been found to attack coenzyme A at about the same rate as it attacks adenylic acid "b," whereas the prostatic enzyme splits coenzyme A at a much slower rate than adenylic acid "b."

Kornberg and Pricer[7] have reported that the

(1) Contribution No. 28 of the McCollum-Pratt Institute. This work was supported in part by grants from the American Cancer Society as recommended by the Committee on Growth of the National Research Council, and the Williams-Waterman Fund.

(2) J. D. Gregory, G. D. Novelli, and F. Lipmann, THIS JOURNAL, **74**, 854 (1952).

(3) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 3421 (1951); G. D. Novelli, personal communication.

(4) L. Shuster and N. O. Kaplan, *Fed. Proc.*, **11**, 286 (1952).

(5) Coenzyme A was assayed by its activity in the arsenolysis of acetyl phosphate by transacetylase (E. R. Stadtman, G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **191**, 365 (1951)). Coenzyme A, inactivated by "b" nucleotidase or prostatic phosphatase, can be rephosphorylated to form active coenzyme with ATP and pigeon liver extract, so that no inactivation is apparent when the pigeon liver assay system (containing ATP, acetate and sulfanilamide) is used.

(6) The coenzyme A preparation used was approximately 90% pure (370 units per mg.), and was generously supplied by Drs. G. D. Novelli and Fritz Lipmann.

(7) A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **186**, 557 (1950).

monoester phosphate group in triphosphopyridine nucleotide (TPN) is esterified in the "a" position of the adenylic acid moiety of this coenzyme. The "b" nucleotidase *does not* cleave the monoester linkage of TPN. A diphosphoadenosine fragment obtained from coenzyme A, by treatment with snake venom nucleotide pyrophosphatase, was compared with the diphosphoadenosine fragment obtained from TPN under identical conditions. It was found that the fragment from coenzyme A was chromatographically different from TPN, when analysed under the same conditions and under conditions where there was no opportunity for migration of ·phosphate groupings. Treatment of the two fragments with the "b" nucleotidase yields "5" adenylic acid only with the coenzyme A product and not with the TPN derivative. This and other evidence indicates that the monoester phosphate in coenzyme A is in the "b" form, as contrasted to TPN, which is an adenylic acid "a" derivative.

We wish to thank Drs. Fritz Lipmann, G. D. Novelli, and S. P. Colowick for their interest in this work.

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RECEIVED MAY 1, 1952

PREPARATION OF ACETYL COENZYME A[1]

Sir:

The separation of acetyl coenzyme A (CoA) from respiring yeast and its characterization by Lynen and Reichert as a thioester[2] has been a most important contribution to the study of the acetylations and condensations in which this compound occupies the key position of being a common intermediate. It would be of considerable advantage if acetyl CoA were readily available for study in itself and as a substrate rather than an intermediate in the numerous important reactions in which it plays so prominent a role.

Stadtman has developed a method of preparing acetyl CoA enzymatically from acetylphosphate using transacetylase.[3]

This note describes a simple and efficient means of acetylating CoA from preparations of either high or low purity and containing the CoA in either the oxidized or reduced forms.

The acetylating reagent is a 5.8 *M* solution of sodium thiolacetate prepared from distilled thiolacetic acid and 10 *M* NaOH solution. The CoA is dissolved in the reagent and kept at room temperature for one hour or more. The thiolacetate is removed by adding an equivalent amount of 4 *N* HCl. Thiolacetic acid separates and may be almost completely removed by aeration with an aspirator for 5–10 minutes or by ether extraction. This procedure introduces an equivalent quantity of NaCl.

If the CoA is in reduced form a saturated solution of thioacetic acid may be used. This reagent is much slower but does not introduce salt.

The acetyl CoA was assayed by conversion to acetylcholine with partially purified choline acetylase from the head ganglion of Squid. A typical result is shown in Fig. 1. The experiment was performed as follows:

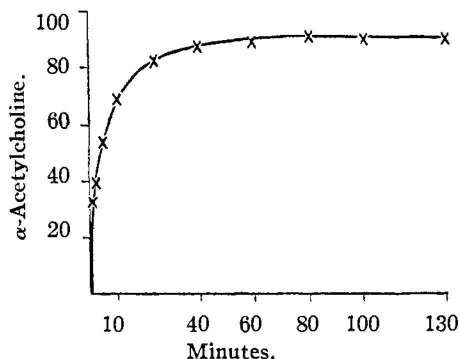

Fig. 1.

650 ug. of CoA assaying 360 units/mg. (kindly supplied and evaluated by Dr. S. Ochoa) were dissolved in 0.05 ml. of sodium thiolacetate reagent at *p*H about 7. After 3 hours at room temperature 0.07 ml. of 4 *N* HCl was added and the mixture aerated for 10 minutes. The assay was then carried out as follows: 0.5 ml. of water, 0.2 ml. of 0.7 *M* sodium phosphate buffer *p*H 7, 0.1 ml. of choline chloride (0.9 *M*) and $MgCl_2$ (0.01 *M*) solution, and finally 0.75 ml. of choline acetylase solution containing 120 μg./ml. of tetraethylpyrophosphate were added. The volume was adjusted to 2.30 ml. and samples were withdrawn at various time intervals, diluted 400 to 1200 times and tested for acetylcholine by bio-assay.[4]

No acetylcholine is obtained if enzyme, choline, or CoA is omitted or if CoA is added last after aeration.

The concentration of thiolacetate in the incubation mixture is about 3 μmol. per ml. corresponding to about 98% removal by aeration. If untreated CoA is made up as above and sodium thiolacetate is added to even 70 μmol. per ml., acetylcholine is not formed in measurable amounts. Some enzyme preparations can catalyze the formation of acetyl CoA from thiolacetate and CoA, but this squid ganglion preparation cannot.[5] These considerations show that there is no reformation of acetyl CoA during the above acetylation of choline. The method is, therefore, valid for assaying the acetyl CoA formed by the method described in this communication.

The curve shows a formation of 90 ug. of acetylcholine. Based upon the CoA assay in terms of units and assuming complete conversion to acetyl CoA and thence to acetylcholine, we should have expected 130 μg. Our yield of acetyl CoA is, therefore, at least 70%. The same yield was obtained in two cases with highly purified CoA, one in which the incubation with sodium thiolacetate extended over 1 hour and the other 3 hours. Higher yields

(1) This work was supported by a grant from the National Institute of Health, Public Health Service, National Heart Institute, Bethesda 14, Md.

(2) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951).

(3) Personal communication.

(4) H. C. Chang and J. H. Paddum, *J. Physiol.*, **79**, 225 (1933).

(5) D. Nachmansohn, I. B. Wilson, S. R. Korey and R. Berman, *J. Biol. Chem.*, **195**, 25 (1952)

were obtained with very impure CoA, but the CoA assay in terms of units is in this case much less reliable.

Other acetylating agents, such as acetic anhydride and isopropenyl acetate, also acetylate CoA, but the yields were markedly lower.

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BOOK REVIEWS

Absorptions-Spektralanalyse. By FRANZ X. MAYER, Dr.-Ing., Priv.-Doz. fur gerichtliche Chemie am Institut fur Gerichtliche Medezin der Universitate Wien and ALFRED LUSZCAK, Dr.-Ing., Mitglied des österr. Patentamtes und titl. a. o. Professor an der Technischen Hochschule Wien. Walter de Gruyter and Co., Berlin W 35, Genthiner Strasse 13, Germany. 1951. xiv + 238 pp. 14.5 × 19 cm. Price, DM 14, —.

According to the authors, this book was intended primarily for students. The main part of the text is devoted to a description of apparatus and methods for spectrophotometric analyses in the ultraviolet and visible regions of the spectrum. The authors rely only on their twenty years experience with analyses of materials bordering on chemistry and medicine, and hence do not describe recent developments in chemical spectrometry which make use of photoelectric cells and thermoelements. Eighty-five per cent. of the references are to German and French literature. Only three English texts, by Lothian, Snell and Mellon, are among the thirty-seven titles listed.

The book is divided into three divisions. In the theoretical part (60 pages) there are three chapters devoted to: A. Fundamentals of light absorption, B. Light absorption as a basis for quantitative analyses, and C. as a basis for quantitative analyses. The second division (123 pages) covers a description of: A. Condensed spark units, B. Prism spectrographs of Steinheil, Zeiss-Jena, Hilger and König-Martens, C. Cuvettes, D. Solvents, and E. Elementary photographic theory. F. Methods of measurement are described with titles such as: Hartley-Baly, Henri, Winther, Judd Lewiss Hilger, Scheibe and Pool. The three visual photometer, described in this section are the Pulfrich, Leifo and Zeiss-Ikon.

In the third division (40 pages) twelve examples of actual analyses are given with experimental details of concentrations, wave length at which absorbancies were determined and a sample calculation. Typical examples described are the determination of (1) the solubility of benzene in water, (2) benzene in ethanol, (3) strychnine and brucine in mixtures, (4) the concentration of carbon monoxide in blood, and (5) benzines in aromatics.

There are amongst the 74 figures a few typical absorption curves in which the ordinate is given in absorbancy and the abscissa in ångströms, frequencies and wave numbers.

Within the space limitations of the book, the subjects are thoroughly covered and give an excellent view of European equipment and methods. The text is free from typographical errors, it is well printed on a good quality paper. The binding conforms to the standards of good German bookbinding.

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Vitamins and Hormones—Advances in Research and Applications. Volume IX. By ROBERT S. HARRIS, Professor of Biochemistry of Nutrition, Massachusetts Institute of Technology, Cambridge, Mass., and KENNETH V. THIMANN, Professor of Plant Physiology, Harvard University, Cambridge, Mass. (Editors). Academic Press Inc., 125 East 23rd Street, New York 10, N. Y. 1951. xi + 395 pp. 16 × 23.5 cm. Price, $8.00.

This volume contains nine reviews; four of these are concerned with vitamins, four with hormone action, and one with vitamins and hormones.

T. H. Jukes and E. L. R. Stokstad deal in an expert fashion with the recent studies on the action of B_{12} vitamins and the folic acids. Their discussion of the effects of these vitamins on microörganisms is particularly enlightening. There is a very interesting discussion by Wm. Shive relative to the function of B vitamins in the biosynthesis of purines and pyrimidines. The author interprets many of the older results in the literature on the interrelationship of purines and pyrimidines with vitamin B_{12} and the folic acids in the light of recent discoveries linking the function of these growth factors with the metabolism of one carbon compound. The relationship between the metabolic effect of certain B vitamins, *e.g.*, folic acid and biotin, and the purines is also apparent from studies on the antimetabolites of nucleic acid metabolism which are reviewed by L. D. Wright.

The functions of biotin in enzyme systems are discussed by H. C. Lichstein. Unfortunately, up to the present date, there are very few studies with isolated enzyme systems in which a direct effect of biotin has been demonstrated. Most of the experiments reported were done with whole cells. The author suggests that the lack of correlation in biotin content and activity in the case of certain isolated enzyme systems, *e.g.*, oxalacetate decarboxylase and the malic enzyme, can be ascribed to inadequate liberation of bound biotin from the protein prior to assay. A new theory of biotin action is presented in which it is assumed that biotin exerts its effect by acting as an electron carrier.

The relationship between certain vitamins and adrenal hormones is very thoroughly reviewed by Agnes Fay Morgan. W. H. Fishman discusses the effect of estrogens on enzyme activity. A detailed account of the influence of various sex hormones on the β-glucuronidase of different tissues is given.

A brief summary of methods available for the synthesis of isotope labeled steroids is presented by G. H. Twombly. The remainder of this chapter deals with experiments on the distribution in the tissues and pathways of excretion of administered isotopically labeled steroids.

Clinical and physiological observations of the effects of cortisone and ACTH are reviewed by R. G. Sprague. The purification of urogastrone and the depression of gastric secretion by this material are discussed by M. H. F. Friedman.

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