COMMUNICATIONS TO THE EDITOR

THE CONSTITUTION OF CEREBROSTEROL, A HYDROXYCHOLESTEROL ISOLATED FROM HORSE BRAIN

Sir:

We recently reported[1] isolation from horse brain of a substance, m.p. 175–176°, α_D −48.3° Chf, diacetate, m.p. 96–97°, α_D −27.6° Chf, isomeric with all known cholestenediols and hence designated cerebrosterol[2]; in later work the sterol was also isolated from human brain. The steroidal nature of the substance was established by hydrogenation to the saturated diol, cerebrostanediol, oxidation of this to cerebrostandione, and Wolff–Kishner reduction to cholestane.

Observation that cerebrosterol is precipitated by digitonin, gives a positive Liebermann–Burchard test, is levorotatory, is converted on Oppenauer oxidation to an α,β-unsaturated diketone, and forms a diacetate indicated that it is a secondary alcoholic derivative of cholesterol. Some of the possible locations for the extra secondary hydroxyl group can be eliminated: C_2, C_4, C_7 (known alcohols or ketones); C_{11} (cerebrostenedione forms a disemicarbazone); C_1 (cerebrostenedione undergoes Wolff–Kishner reduction without alkaline cleavage and hence is not a 1,3-diketone); C_{15} or C_{16} (absence of a band at 5.77μ in the infrared spectra of cerebrostanedione and cerebrostenedione). Of the remaining positions: C_{12}, C_{22}, C_{23}, and C_{24}, the location C_{24} at first seemed excluded since our cerebrostenedione melts at 118–119°, whereas Hey, Honeyman and Peal[3] report the m.p. 90–91° for Δ^4-cholestene-3,24-dione obtained by ozonization of fucostadienone. However, in an Oppenauer oxidation of 24-ketocholesterol[4] we obtained Δ^4-cholestene-3,24-dione melting at 118–119° (λ^{EtOH} 242 mμ, log E = 4.22, α_D + 83.5° Chf, λ^{Chf} 5.86, 6.01, 6.18μ; calcd.: C, 81.35; H, 10.62; found: C, 81.32; H, 10.64) and identical with cerebrostenedione (mixed m.p., infrared spectrum).

Previous workers[4,5] have reported that 24-ketocholesterol on reduction according to Meerwein–Ponndorf or with lithium aluminum hydride affords a single diol, m.p. 166–169°; diacetate, m.p. 93–95°. We repeated the Meerwein–Ponndorf reduction and on fractional crystallization as dibenzoate isolated two products, m.p. 179–181°, α_D −15.5° Chf (calcd.: C, 80.61; H, 8.91; found: C, 80.65; H, 8.83), and m.p. 141–142°, α_D −11.8° Chf (found: C, 80.68; H, 8.90). Hydrolysis of the first dibenzoate yielded a product identical with cerebrosterol, which is thus assigned the constitution of Δ^5-cholestene-3β,24ξ^1-diol. Saponification of the second dibenzoate gave the 24ξ^2-epimer, m.p. 182–183°, α_D −26.8° (calcd.: C, 80.54; H, 11.52; found: C, 80.52; H, 11.57); diacetate, m.p. 100–102°, α_D −37.2° Chf.

On mild Oppenauer oxidation the synthetic 24ξ^1- and 24ξ^2-diols afforded, along with Δ^4-cholestene-3,24-dione, Δ^4-cholestene-24ξ^1-ol-3-one, m.p. 138°, α_D +79.5° Chf, λ^{EtOH} 242 mμ (log E 4.22), λ^{Chf} 2.8, 6.01, 6.18μ (calcd.: C, 80.94; H, 11.07; found: C, 80.98; H, 11.00), acetate, m.p. 97°, α_D + 86.5° (calcd.: C, 78.68; H, 10.47; found: C, 78.70; H, 10.38), and Δ^4-cholestene-24ξ^2-ol-3-one, m.p. 143°, α_D + 93.5° (found: C, 80.90; H, 11.02), acetate, m.p. 91°, α_D + 70° (found: 78.70, H, 10.45). Both products on chromic acid oxidation afforded Δ^4-cholestene-3,24-dione.

Catalytic hydrogenation of Δ^5-cholestene-3β,24ξ^1-diol gave cholestane-3β,24ξ^1-diol, m.p. 202–203°, α_D + 24° Di (calcd.: C, 80.14; H, 11.96; found: C, 80.09; H, 11.92), diacetate, m.p. 119°, α_D +22.5° Chf (calcd.: C, 76.18; H, 10.72; found: C, 76.19; H, 10.62). These substances were not depressed in m.p. on admixture with cerebrosterol and its diacetate, respectively. Chromic acid oxidation of cholestane-3β,24ξ^1-diol afforded a dione, m.p. 122°, α_D +40° Chf, λ^{Chf} 5.87μ (calcd.: C, 80.94; H, 11.07; found: C, 80.80; H, 11.03) that showed no depression in m.p. on admixture with cerebrostanedione.

We are indebted to Professor L. F. Fieser for his helpful advice and for the infrared absorption spectra.

VISTER RESEARCH LABORATORIES
CASATENOVO (COMO), ITALY
ALBERTO ERCOLI
PIETRO DE RUGGIERI

RECEIVED JUNE 3, 1953

(1) A. Ercoli, S. Di Frisco and P. de Ruggieri, *Gazz. Chim. Ital.*, **83**, 78 (1953).

(2) Previously[1] named cerebrostenediol.

(3) D. H. Hey, J. Honeyman and W. J. Peal, *J. Chem. Soc.*, 2881 (1950).

(4) B. Riegel and I. A. Kaye, THIS JOURNAL, **66**, 723 (1944).

(5) D. H. Hey, J. Honeyman and W. J. Peal, *J. Chem. Soc.*, 4836 (1952).

XYLOSE ISOMERASE[1]

Sir:

Cohen[2] has recently described an enzyme in *E. coli* which catalyzes the equilibrium reaction: D-ribulose ⇄ D-arabinose and named it "pentose isomerase."

We now wish to report the finding of an enzyme which catalyzes the reaction: D-xylose ⇄ D-xylulose. This enzyme obtained from *Pseudomonas hydrophila* is specific for xylose and does not catalyze the formation of ketopentoses from D-ribose, D- or L-arabinose, D-lyxose or L-rhamnose. It will therefore be referred to as "xylose isomerase." A similar enzyme from *Lactobacillus pentosus* has been briefly reported by Lampen.[3]

The enzyme preparation used was an acetone-dried powder obtained, as previously described,[4] from a cell-free extract of *P. hydrophila* grown on D-xylose, except that the cells were ruptured by sonic vibration (10 kc., 1.0 amp., 10 min.).

The xylose isomerase was found to be a soluble

(1) Issued as N.R.C. No. 3011.

(2) S. S. Cohen, *J. Biol. Chem.*, **201**, 71 (1953).

(3) J. O. Lampen, "Symposium on Phosphorus Metabolism," Vol. II, Johns Hopkins Press, Baltimore, Md., 1952, p. 363.

(4) R. M. Hochster and R. W. Watson, *Nature* (*Lond.*), **170**, 357 (1952).

enzyme, heat sensitive and stable for more than one year when stored as the acetone powder at 4° over calcium chloride. Up to 80% enzyme inactivation was obtained by prolonged dialysis at 0–4°. Addition of 0.01 *M* Mn^{++} or Mg^{++} restored 92 and 70% of the lost activity, respectively. The optimum *p*H was 7.5.

Ketopentose was determined by the cysteine–carbazole method,[5] by the use of $FeCl_3$–orcinol spectra, and by paper chromatography in several solvent systems. An equilibrium of 16% D-xylulose and 84% D-xylose was reached in 3 hr. at 27° under the conditions used. However, this equilibrium was shifted to 81.5% D-xylulose and 18.5% D-xylose by the addition of borate (0.062 *M*) to trap the ketopentose. The isomerase reaction did not require phosphate and could be carried out aerobically or anaerobically. The enzyme was not inhibited by iodoacetate, fluoride, azide or β-methyl-D-xyloside. A typical example of the isomerase reaction showing the rate of ketopentose formation anaerobically (without added borate) is given in Table I.

TABLE I

RATE OF CONVERSION OF D-XYLOSE TO D-XYLULOSE BY XYLOSE ISOMERASE

Reaction components per 3-ml. aliquot were: acetone powder 30 mg., D-xylose 80 mg., NaF 0.02 *M*, $MgCl_2$ 0.0033 *M*, $NaHCO_3$ 0.02 *M* in 95% N_2 + 5% CO_2 at 27°. Proteins and nucleic acids were precipitated with trichloroacetic acid and protamine sulfate. Supernatant containing the reaction product was treated with cysteine-carbazole and after 1 hr. at room temperature the resulting colors were read spectrophotometrically.

Enzymatic reaction time, min.	% D-xylose converted to D-xylulose
0	0.00
10	4.25
30	9.00
60	12.00
90	14.85
120	15.50
180	16.25
240	16.00

Paper chromatograms run with phenol–water (4:1),[6] benzyl alcohol–acetic acid–water (3:1:3)[7] and toluene–dioxane–water (4.17:12.5:1) all resulted in good separations of control D-xylulose,[8] D-ribulose,[9] D-xylose and D-ribose. With each solvent system, the product of the enzymatic reaction, freed from D-xylose by bromine oxidation followed by removal of the resultant xylonic acid with Amberlite IRA 400 resin, had an R_f value identical with authentic D-xylulose. The reaction product gave no ribulose spot and did not exhibit the characteristic spectrophotometric peak for ribulose at 540 mμ in the $FeCl_3$–orcinol reaction.

Positive identification of the product of the isomerase reaction as D-xylulose was effected as follows: the reaction product was freed of protein,[10] adjusted to *p*H 7.5, concentrated *in vacuo* (at 30–40°)[11] to a small volume which was then transferred to a cellulose column[12,13] and the components separated using water-saturated butanol as the mobile phase. A modified cysteine–carbazole spot test was used to identify the effluent ketopentose. When combined, the ketopentose fractions consisted of pure sirupy D-xylulose having $[\alpha]^{25}D$ −32.2° (*c* 2.7% in H_2O)[14] and gave a crystalline *p*-bromophenylhydrazone, melting at 128–129° (uncor.) which was unchanged on admixture with an authentic sample.[14]

In view of our previous report[4] on the enzymatic phosphorylation of D-xylose, the existence of xylose isomerase raises the question whether the substrate in the phosphorylation is D-xylose, D-xylulose or both sugars. Conclusive evidence is not yet available and this phase of the problem is under investigation.

(5) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).
(6) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).
(7) A. Benvenue and K. T. Williams, *Arch. Biochem. Biophys.*, **34**, 225 (1951).
(8) R. M. Hann, E. B. Tilden and C. S. Hudson, THIS JOURNAL, **60**, 1201 (1938).
(9) C. Glatthaar and T. Reichstein, *Helv. Chim. Acta*, **18**, 80 (1935).
(10) M. Somogyi, *J. Biol. Chem.*, **117**, 771 (1937).
(11) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).
(12) L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.*, 2511 (1949).
(13) C. T. Bishop, *Chemistry in Canada*, **5**, 39 (1953).
(14) O. Th. Schmidt and R. Treiber, *Ber.*, **66**, 1765 (1933).

DIVISION OF APPLIED BIOLOGY
NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA
ROLF M. HOCHSTER
R. WADE WATSON

RECEIVED MAY 18, 1953

ON THE ENZYMATIC MECHANISM OF COENZYME A ACETYLATION WITH ADENOSINE TRIPHOSPHATE AND ACETATE[1]

Sir:

The activation of acetate was recently partially clarified when it was found that this reaction involved a pyrophosphate split of ATP, indicating the over-all reaction

$$ATP + HS\cdot CoA + acetate \rightleftharpoons AMP + PP + acetyl\sim S\cdot CoA \quad (1)$$

As intermediary, an S-phosphoryl derivative had been suggested as initial product of a reaction ATP and CoA.[2,3] More recent work in our laboratories, however, made such a mechanism more and more unlikely, suggesting, rather, mechanisms involving enzyme-bound intermediaries.[4,5] A considerable clarification of a mechanism of this type appeared to be possible by the use of isotopes. Through testing for exchange with labelled pyrophosphate as well as labelled acetate, it appeared possible to obtain a rather precise information on the intermediate course of the reaction.

Radioactive pyrophosphate was prepared by

(1) The following abbreviations are used in this communication: ATP = adenosine triphosphate; HS·CoA = sulfhydryl-coenzyme A; CoA = coenzyme A; AMP = adenosine monophosphate; PP = pyrophosphate; ex = enzyme, whereby "x" may represent the group which takes part in the chemical reactions; and Ad = adenosine. This investigation was supported in part by research grants from the U. S. Public Health Service, from the Rockefeller Foundation, and from the Atomic Energy Commission.
(2) F. Lipmann, M. E. Jones, S. Black and R. M. Flynn, THIS JOURNAL, **74**, 2384 (1952).
(3) F. Lynen, E. Reichert and L. Rueff, *Ann. Chem.*, **574**, 1 (1951).
(4) F. Lipmann, M. E. Jones, S. Black and R. M. Flynn, *J. Cell. and Comp. Physiol.*, **41**, Supplem. 1, 109 (1953).
(5) F. Lynen and H. Hilz, unpublished experiments.

heating to red heat radioactive K_2HPO_4; purified yeast enzyme was used for the exchange experiments. As shown in Table I, pyrophosphate exchanged with the pyrophosphoryl group in ATP very rapidly and, significantly, in the absence of CoA. This observation excluded immediately a CoA pyrophosphate as an intermediary. Instead, it indicated an initial reaction between ATP and enzyme, resulting in an AMP∼enzyme link with liberation of inorganic pyrophosphate.

TABLE I

PYROPHOSPHATE EXCHANGE BETWEEN RADIOACTIVE INORGANIC PYROPHOSPHATE AND ATP WITH YEAST ENZYME

Enzyme, units	CoA, μM	Pyrophosphate cts./min./ml.	Pyrophosphate cts./min./μM	ATP cts./min./ml.	ATP cts./min./μM	Exchange, %
...	..	97,100	29,400	140	60	0.3
10	..	68,600	21,100	28,270	13,400	74.3
10	1	79,700	23,100	16,350	7,680	44.7
10	2	80,700	24,700	13,920	6,360	36.8

The vessels were incubated at 37° for 20 minutes. Each vessel contained in 1 ml.: 3.4 μM P^{32}-potassium pyrophosphate buffered at *p*H 7.5; 2.2 μM ATP; 50 μM KF; 10 μM $MgCl_2$; 20 μM H_2S; 200 μM tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.5 in addition to the enzyme and CoA as noted above. The enzyme fraction used was purified 11-fold over the original extracts from quick-frozen baker's yeast. The separation of ATP and pyrophosphate was carried out by charcoal adsorption of ATP in the manner described by Crane and Lipmann.[6]

As shown in the last two lines of Table I, CoA inhibits the ATP ⇄ PP exchange proportional to concentration. This indicates that AMP∼enzyme subsequently exchanges AMP for CoA. Therefore, the presence of CoA decreases the concentration of AMP∼enzyme and thereby slows down the rate of exchange with pyrophosphate. The correctness of this mechanism is further suggested by the results of the exchange of isotopic acetate with acetyl CoA. The data of Table II demonstrate the last step of the sequence to be an exchange of enzyme∼S·CoA with acetate to form acetyl ∼ S·COA and free enzyme. The combined results

TABLE II

EXCHANGE OF ACETATE BETWEEN $CH_3C^{14}OOH$ AND ACETYL CoA WITH YEAST ENZYME

Enzyme, units	AMP μM	Acetate cts./min./ml.	Acetate cts./min./μM	Acetyl CoA cts./min./ml.	Acetyl CoA cts./min./μM	Exchange, %
...	..	77,900	39,000	108	0.6	0.003
20	..	58,100	26,800	16,830	10,650	53.3
20	5	56,400	26,000	16,900	10,700	54.6

The vessels were incubated at 37° for 40 minutes. Each vessel contained in 1 ml.: 1.58 μM acetyl CoA; 2.17 μM C^{14}-acetate; 10 μM $MgCl_2$; 200 μM tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.5 in addition to the enzyme and AMP as noted above. The enzyme fraction used was the same as in Table I. *Note that, compared with the experiment of Table I, twice as much enzyme and double the incubation time was used here.* Reaction 4, therefore, is considerably slower than reaction 2. The separation of acetyl CoA and acetate was carried out by charcoal adsorption of acetyl CoA in a manner similar to that used for separation of ATP and pyrophosphate.[6] Acetate activity was determined in the supernatant of the charcoal adsorbate. Acetyl CoA was decomposed on the charcoal with hydroxylamine and the hydroxamic acid activity measured in the supernatant.

(6) R. K. Crane and F. Lipmann, *J. Biol. Chem.*, **201**, 235 (1953).

prompt us to propose the following sequence of reactions:

$$\text{Ex} + \text{Ad-P}\sim\text{PP} \rightleftharpoons \text{Ex}\sim\text{P-Ad} + \text{PP} \quad (2)$$

$$\text{Ex}\sim\text{P-Ad} + \text{HSCoA} \rightleftharpoons \text{Ex}\sim\text{SCoA} + \text{Ad—P} \quad (3)$$

$$\text{Ex}\sim\text{SCoA} + \text{CH}_3\text{COOH} \rightleftharpoons \text{Ex} + \text{CH}_3\text{CO}\sim\text{ScOA} \quad (4)$$

It is noteworthy that, as shown in the last two lines of Table II, addition of AMP does not appreciably influence the rate of acetyl∼CoA ⇄ acetate exchange. It therefore seems that the bond between enzyme and CoA is less energy-rich than the AMP∼enzyme bond. This is confirmed further through the inhibition by CoA of the ATP ⇄ PP exchange which indicates that CoA favorably competes with AMP for the enzyme. In other words, a fall in free energy occurs in the direction from AMP∼enzyme to CoA·S∼enzyme. The enzyme used in these experiments was practically free of Mg. Through exchange experiments with and without magnesium we were able to show that magnesium is involved, in the ATP-enzyme reaction, but is not involved in the enzyme∼CoA-acetate reaction.

Although no definite suggestions can be made at the present time with regard to the grouping on the enzyme which binds AMP and CoA·SH, it is attractive to presume the group is enzyme-bound phosphate. In such a case, the primary reaction between enzyme and ATP would closely resemble the type of reaction described by Kornberg,[7] namely, a pyrophosphate split of ATP with simultaneous formation of a pyrophosphate bridge from the residual AMP to another molecule. Furthermore, the interchange of AMP and sulfhydryl-CoA then would lead to enzyme-phosphoryl∼S·CoA. It seems rather attractive, on the other hand, to speculate that the here-observed formation of an enzyme-mononucleotide may well foreshadow this as a rather general biosynthetic mechanism involved, for instance, possibly in nucleic acid synthesis.

BIOCHEMICAL RESEARCH LABORATORY, MARY ELLEN JONES[8]
MASSACHUSETTS GENERAL HOSPITAL AND THE
DEPARTMENT OF BIOLOGICAL CHEMISTRY FRITZ LIPMANN
HARVARD MEDICAL SCHOOL
BOSTON, MASSACHUSETTS

CHEMISCHES LABORATORIUM DER UNIVERSITÄT MÜNCHEN
BIOCHEMISCHE ABTEILUNG HELMUT HILZ
GERMANY FEODOR LYNEN

RECEIVED JUNE 4, 1953

(7) A. Kornberg, in McElroy and Glass, "Phosphorus Metabolism," Vol. I, Johns Hopkins Press, Baltimore, Md., 1951, p. 392.

(8) Postdoctoral fellow of the Atomic Energy Commission.

A THEORY OF A CERTAIN TYPE OF IRREVERSIBLE POLAROGRAPHIC WAVE

Sir:

The present study was undertaken in an attempt to elucidate polarographic processes in which, regardless of the fact that the electron transfer reaction is faster than the diffusion rates, "irreversible" slopes of the plot of log $(i_d - i)/i$ against the potential[1] are obtained. This type of polarographic process belongs to a group in which the product of

(1) J. Tomeš, *Coll. Czechoslov. Chem. Communs.*, **9**, 12, 81, 150 (1937).

the electron transfer reaction is involved in a chemical reaction.

The theoretical part of this investigation is based upon the use of steady state[2,3] and thermodynamic treatments. Consider the simplest possible case represented by the reaction scheme:

$$X \underset{}{\overset{k_x}{\rightleftharpoons}} X_0 + ne^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Y_0 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Z_0 \xrightarrow{k_z} \quad (1)$$

$$\downarrow k_y$$

in which the concentrations of the species involved are denoted by X, Y and Z. The subscript $_0$ refers to surface concentrations. The various processes occurring at the electrode are considered as unimolecular rate processes, the rate constants are defined as heterogeneous constants. Application of the steady state treatment to the above mentioned scheme, on the assumptions that $i = nAF(X_0k_1 - Y_0k_2)$ and that $i_d = nAFXk_x$, leads to the following result

$$\frac{i_d - i}{i} = \frac{k_x}{k_1}\left[\frac{k_2k_4 + k_2k_z + k_3k_z + k_4k_y + k_yk_z}{k_4k_y + k_3k_z + k_yk_z}\right] \quad (2)$$

This expression is valid whether or not the process is reversible. Depending upon the relative magnitude of the various rate constants (*e.g.*, for the reversible case $k_1, k_2 \gg k_3, k_4, k_x, k_y, k_z$) and invoking the potential dependence of the electron transfer rate constants,[4] equations of polarographic waves can be derived, which show log plots with varying degree of deviation from the reversible behavior. A suitable reaction for testing some of the implications of the theory was found in the system

$$\mathrm{Co\,en_3^{+++}} + e^- \rightleftharpoons \mathrm{Co\,en_3^{++}} \quad (3)$$

The trisethylenediaminecobalt(III, II) couple in excess of ethylenediamine has been shown to be reversible potentiometrically by Bjerrum[5] and polarographically by Grieb.[6] The couple was found to show irreversible log plot slopes in the absence of the complexing agent in spite of the fact that the process was diffusion controlled, and that the electron transfer reaction was reversible, which was shown using alternating current polarography.[7] Using the successive complex constants for the divalent form

$$\mathrm{Co\,en_3^{++}} \overset{K_1}{\rightleftharpoons} \mathrm{Co\,en_2^{++}} + \mathrm{en} \overset{K_2}{\rightleftharpoons}$$

$$\mathrm{Co\,en^{++}} + \mathrm{en} \overset{K_3}{\rightleftharpoons} \mathrm{Co^{++}} + \mathrm{en} \quad (4)$$

as determined by Bjerrum[5] and assuming that establishment of the various equilibria is instantaneous an expression for the current-voltage curve was derived

$$E_{d.e.} = E_0' - \frac{RT}{F} \ln \alpha \cdot \frac{[\mathrm{Co\,en^{++}}]_0}{[\mathrm{Co\,en^{+++}}]_0} \quad (5)$$

where α is a function of the concentration of divalent complex. The predicted shift of the half-wave potential and change of the log plot slope were confirmed using a 50-fold concentration variation. It is believed that this type of polarographic wave is found in inorganic systems although it might be more numerous in organic polarography as pointed out by Kolthoff and Lingane.[8]

At a later date the details of this investigation, which is in progress, will be published.

(2) K. B. Oldham, Ph.D. Thesis, Univ. of Manchester, England, 1952.

(3) M. G. Evans and N. S. Hush, *J. chim. phys.*, **49**, C 159 (1952).

(4) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, N. Y., 1941, p. 584.

(5) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, 1941, p. 223.

(6) M. W. Grieb, Ph.D. Thesis, Univ. of Illinois, 1953.

(7) H. A. Laitinen and Pekka Kivalo, THIS JOURNAL, **75**, 2108 (1953).

(8) I. M. Kolthoff and J. J. Lingane, "Polarography," 2nd Ed., Interscience Publishers, Inc., New York, N. Y., 1952, p. 266.

DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING
UNIVERSITY OF ILLINOIS PEKKA KIVALO
URBANA, ILLINOIS

RECEIVED APRIL 20, 1953

THE VIBRATIONAL SPECTRUM OF TETRACHLORODIBORINE

Sir:

While structural investigations in the field of boron chemistry have not been so numerous as might be desired there is now abundant evidence that the presence of two or more boron atoms in a molecule may result in its having an uncommon or even unique structure.[1a,b,c,d] An interesting example is afforded by tetrachlorodiborine (B_2Cl_4) in which boron exhibits a normal tricovalence while being singly bonded to another boron atom. We have determined its infrared and Raman spectra and achieved a satisfactory assignment. The spectral data suggest, in agreement with the results of a recent electron diffraction study,[2] that B_2Cl_4 has the symmetry (V_d) of a non-planar ethylene model.

Approximately 4 ml. of liquid B_2Cl_4 was prepared by the method of Wartik, Moore and Schlesinger.[3] Raman exposures were made with the liquid sample held at $-35°$. The infrared spectra were obtained with a Perkin–Elmer spectrometer (equipped with NaCl and KBr prisms) on about 5 mm. pressure of gaseous B_2Cl_4 at room temperature. The small amount of BCl_3 formed as a result of the instability of the compound at room temperature was corrected for by running blanks on pure BCl_3.

The observed frequencies and their present interpretation are given in Table I. The frequency to be associated with the b_1 torsional mode cannot yet be estimated. The b_2 deformation is assigned the value 445 cm.$^{-1}$ as deduced from combination bands. The a_1 B-B stretch appears as a polarized doublet in the Raman spectrum as a result of the B^{10}-B^{11}, B^{11}-B^{11} isotopic shift. The observed intensity ratio accords with the natural isotopic distribution of boron. A fuller discussion of the assignment, together with the results of a normal-coördinate, force-constant treatment, will be given elsewhere.

(1) (a) R. C. Lord and E. Nielsen, *J. Chem. Phys.*, **19**, 1 (1951); (b) K. Hedberg, M. E. Jones and V. Schomaker, *Proc. N. A. S.*, **38**, 678 (1952); (c) J. S. Kasper, C. M. Lucht and D. Harker, THIS JOURNAL, **70**, 881 (1948); (d) W. J. Dulmage and W. N. Lipscomb, *Acta Cryst.*, **5**, 260 (1952).

(2) Private communication from Dr. K. Hedberg.

(3) T. Wartik, R. Moore and H. I. Schlesinger, THIS JOURNAL, **71**, 3265 (1949).

TABLE I

THE INFRARED AND RAMAN SPECTRA AND THEIR INTERPRETATION FOR TETRACHLORODIBORINE

Raman (cm.$^{-1}$)	Infrared (cm.$^{-1}$)	Intensity and polarization	Interpretation
107	..	m	ν_9(e; β-BCl_2)
177	..	m	ν_8(e; β-BCl_2)
291	..	w,p	ν_3(a_1; δ BCl_2)
347	..	vw	2 × 177 (e) = 354(A_1 + B_1 + B_2)
401	..	vs,p	ν_2(a_1; ν-BCl)
550	..	vvw	107 (e) + 445 (b_2) = 552 (E); 2 × 291(a_1) = 582 (A_1)
..	622	w	177(e) + 445 (b_2) = 622 (E); 730(b_2) − 107(e) = 623 (E)
..	689	w	107(e) + 2 × 291(a_1) = 689 (E)
..	720	s	
..	729	vs	ν_6(b_2; ν-BCl)
..	739	vs	
..	746	s	291(a_1) + 445(b_2) = 736(B_2)*
750	..	vvw	291(a_1) + 445(b_2) = 736(B_2)*
..	760	vw	177(e) + 2 × 291(a_1) = 759 (E)
..	821	ms	107(e) + 729(b_2) = 836(E)
900	..	w	2 × 445(b_2) = 890 (A_1); 177 (e) + 729(b_2) = 906 (E)
..	920	vvs	ν_7(e; ν-BCl)
927	..	w	ν_7(e; ν-BCl)
948	..	w	107(e) + 401(a_1) + 445(b_2) = 953 (E); 2 × 107(e) + 729 (b_2) = 943 (A_1 + A_2 + B_1 + B_2)
..	1029	w	291(a_1) + 729(b_2) = 1020(B_2); 107(e) + 920(e) = 1027(A_1 + A_2 + B_1 + B_2)
..	1057	w	177(e) + 2 × 445(b_2) = 1067 (E)
..	1090	w	177(e) + 920(e) = 1097 (A_1 + A_2 + B_1 + B_2)
1123	..	w,p	ν_1(a_1; ν-$B^{11}B^{11}$)
1150	..	w,p	ν_1(a_1; ν-$B^{11}B^{10}$)
..	1232	w	107(e) + 1123(a_1) = 1230 (E)
1259	1259	vw	107(e) + 1150(a_1) = 1257 (E)
..	1324	w	401(a_1) + 920(e) = 1321 (E)
..	1362	vw	445(b_2) + 920(e) = 1365 (E)
..	2058	w	920(e) + 1123(a_1) = 2043 (E); 920(e) + 1150(a_1) = 2070 (E)

* Shifted by Fermi resonance with ν_5 fundamental.

We should like to thank Professor D. H. Rank for making available the spectroscopic equipment used in this study, and the Department of the Air Force and the Navy Bureau of Aeronautics for sponsorship of the investigation.

DEPARTMENT OF CHEMISTRY
THE PENNSYLVANIA STATE COLLEGE
STATE COLLEGE PENNSYLVANIA, AND
THE NATIONAL BUREAU OF STANDARDS
WASHINGTON 25, D. C.

M. J. LINEVSKY
E. R. SHULL
D. E. MANN
THOMAS WARTIK

RECEIVED JUNE 5, 1953

BUTYRYL CoA-DEHYDROGENASE, A CUPRO-FLAVOPROTEIN

Sir:

Butyryl CoA dehydrogenase, previously shown to be a flavoprotein of vivid green color,[1] contains copper as part of its prosthetic group. This identification rests on the following observations: (1) a solution of the enzyme of highest purity isolated from a preparative electrophoresis run, and shown to be homogeneous in the analytical ultracentrifuge, was dialyzed exhaustively against 10^{-4} M tris-hydroxymethyl-aminomethane buffer of pH 8.0. Part of the preparation was then denatured by boiling for three minutes and the supernatant flavin solution, the denatured protein and the original preparation were examined by arc and spark spectroscopy.[2] The results summarized in Table I clearly indicate the presence of copper tightly bound to the enzyme protein. (2) Aliquots of two preparations shown to be approximately 22% and 98% pure by analytical electrophoresis (in 0.1 M tris-hydroxymethyl-aminomethane buffer of pH 8.0), and containing 4.7×10^{-5} and 2.6×10^{-5} M flavin,[3] respectively, were washed in platinum crucibles for 1 hour at 1000° F. Manometric copper determination with internal standards[4] showed the presence of 6.9 γ and 3.2 γ of cupric ion per ml. in the two preparations. Thus the mole ratios Cu^{++}/flavin were 2.23/1 and 1.90/1 or approximately equal to 2, at two purity levels of the dehydrogenase.

TABLE I

SPECTROSCOPIC METAL DETERMINATION

	Cu[a]	Mg[a]
Buffer before dialysis	−	−
Buffer after dialysis	−	−
Enzyme after dialysis	++	+
Supernatant of boiled enzyme	−	+
Residue of boiled enzyme	++	−

[a] Only metals found present, all others, specifically Fe, Zn, Mn, Mo, Co, Ca, absent in all samples.

(3) The four-banded spectrum of the enzyme (λ_{max} at 265, 355, 432.5 and 685 mμ) undergoes the following transformations: (a) on treatment with butyryl CoA the peaks at 355, 432.5 and 685 mμ all disappear and the extinctions at these wave lengths are reduced to 0.86, 0.47 and 0.45 times their original value; (b) after dialysis against 5×10^{-3} M KCN,[5] buffered at pH 7.5, for 36 hours, the flavin peaks at 265, 355 and 432.5 are unchanged in both position and extinction while the peak at 685 mμ disappears.

(4) The cyanide-treated enzyme can still be reduced by butyryl CoA as shown by changes in the flavin region (390–480 mμ) of the spectrum, and is capable of catalyzing the interaction between butyryl CoA and dyes such as 2,6-dichlorophenolindophenol, a two-electron change. However, when the enzyme is tested with oxidizing agents mediating one-electron changes such as ferricytochrome c

(1) H. Beinert, R. Bock, D. S. Goldman, D. E. Green, H. R. Mahler, S. Mii, P. G. Stansly and S. J. Wakil, unpublished.

(2) We are indebted to Mr. Rubin Shapiro, Department of Chemistry, University of Wisconsin for performing the spectroscopic analyses.

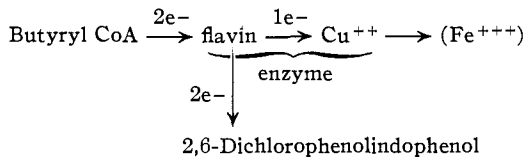
(3) Flavin was determined by assuming a molecular extinction coefficient $\epsilon = 10.6 \times 10^3$ for flavoproteins at their visible maximum (O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150 (1938)). The value so found, was in good agreement with the amount of flavin liberated by boiling the enzyme preparations and calculated assuming an extinction coefficient at 450 mμ $\epsilon = 11.3 \times 10^3$ (E. Dimant, D. R. Sanadi and F. M. Huennekens, THIS JOURNAL, **74**, 5440 (1952)).

(4) O. Warburg and H. A. Krebs, *Biochem. Z.*, **190**, 143 (1927); O. Warburg, *ibid.*, **187**, 255 (1927).

(5) F. Kubowitz, *Biochem. Z.*, **292**, 221 (1937); **299**, 32 (1939).

or ferricyanide, the rates of reaction catalyzed by the CN^--treated enzyme are markedly (50–70%) lower when compared to those of an untreated preparation. Full activity can be restored by preincubating the enzyme in 5×10^{-3} *M* $CuSO_4$.

The above experiments indicate that the intra- and intermolecular oxidoreductions mediated by the enzyme may be represented as follows:



The identification of cupric ion as part of the prosthetic group of this flavoprotein dehydrogenase, together with the preliminary report on the role of molybdenum in xanthine oxidase,[6] possibly suggest a more general involvement of metals in flavoprotein catalyses.

(6) E. C. DeRenzo, E. Kaleita, P. Heytler, J. J. Oleson, B. L. Hutchings and J. H. Williams, THIS JOURNAL, **75**, 753 (1953).

INSTITUTE FOR ENZYME RESEARCH
UNIVERSITY OF WISCONSIN H. R. MAHLER
MADISON, WISCONSIN

RECEIVED MAY 27, 1953

3,4-DIHYDROXYPHENYLACETIC ACID—A METABOLITE OF QUERCETIN

Sir:

Rutin, the rhamno-glucoside of quercetin, is being used extensively for therapeutic purposes, alone and in a variety of pharmaceutical formulations. Investigations on the fate of orally administered rutin have yielded contradictory results. Unfortunately, these investigations were concerned with the urinary excretion of rutin instead of the metabolic products of the aglycone quercetin. Ozawa[1] gave the closely related compound, 3',4'-dihydroxyflavonol, to animals orally and found less than one-tenth of the material excreted in the urine. However, Ozawa found by chromatography three substances of different R_f values in the urine and concluded these substances were metabolites of the compound administered. Because of the unfavorable report of Clark and MacKay[2] on the absorption of orally administered rutin, Haley and Bassin[3] injected rats with rutin subcutaneously. They found the urine contained rutin and unidentified breakdown products conjugated with sulfate and glucuronic acid. The results of Haley and Bassin showed that any rutin or quercetin which might enter the blood stream after oral administration of rutin would be metabolized in part at least.

Evidence obtained in this laboratory during the last four years has shown that oral administration of rutin or its aglycone quercetin to rabbits results in the urinary excretion of appreciable amounts of metabolites of quercetin. One of these breakdown products of quercetin has been isolated recently from rabbit urine in crystalline form, m.p. 127°, and identified as 3,4-dihydroxyphenylacetic acid, (Calcd. for $C_8H_8O_4$: C, 57.54; H, 4.80; neut equiv., 168.1. Found: C, 57.3; H, 4.86; neut equiv. 167.7). Its mixed melting point with an authentic sample was unchanged. The X-ray diffraction pattern of its dimethyl ether was identical with that of a sample of synthetic dimethoxyphenylacetic acid. Crystallographic examination of the compound was confirmatory.

(1) H. Ozawa, *J. Pharm. Soc. Japan*, **71**, 1191 (1951).
(2) W. J. Clark and E. M. MacKay, *J.A.M.A.*, **143**, 1411 (1950).
(3) T. J. Haley and M. Bassin, *Proc. Soc. Expll. Biol. Med.*, **81**, 298 (1952).

WESTERN REGIONAL RESEARCH LABORATORY
CHARLES W. MURRAY
BUREAU OF AGRICULTURAL AND INDUSTRIAL CHEMISTRY
ALBERT N. BOOTH
U. S. DEPARTMENT OF AGRICULTURE FLOYD DEEDS
ALBANY 6, CALIF. ROBERT H. WILSON

RECEIVED MAY 4, 1953

PREPARATION OF CRYSTALLINE 2,3,5-TRI-*O*-BENZOYL-D-RIBOSE FROM D-RIBOSE

Sir:

The procedure developed for the synthesis of benzoylated D-xylofuranose derivatives from D-xylose[1] has now been applied to the D-ribose series. D-Ribose was dissolved in methanol containing 1% hydrogen chloride and the solution left at room temperature until its reducing power had nearly vanished. Pyridine was then added and, after removal of the solvents, the product was benzoylated. The resulting amorphous benzoate, freed of excess reactants, was treated with hydrogen bromide in glacial acetic acid and the crude tri-*O*-benzoyl-D-ribofuranosyl bromide then hydrolyzed in aqueous acetone in the presence of silver carbonate. From aqueous pyridine there was obtained 2,3,5-tri-*O*-benzoyl-D-ribose containing an indefinite amount of pyridine of crystallization. Most of the pyridine was removed by brief drying *in vacuo* over sulfuric acid and the tribenzoate then recrystallized in pure form from alcohol-pentane or ether-pentane. The over-all yield of crystalline solvent-free 2,3,5-tri-*O*-benzoyl-D-ribose varied from 70–81%. The substance melts at 112–113° (cor.) and rotates $[\alpha]^{20}D$ +68.4° in chloroform (*c* 2.65). *Anal.* Calcd. for $C_{26}H_{22}O_8$: C, 67.52; H, 4.80. Found: C, 67.31; H, 4.91.

The structure of the 2,3,5-tri-*O*-benzoyl-D-ribose was confirmed by the following unequivocal synthesis. D-Ribose was dissolved in benzyl alcohol containing 1% hydrogen chloride and, after the reducing power of the solution had nearly disappeared, the acid was removed with silver carbonate. Concentration of the solution *in vacuo* afforded a crystalline benzyl pentoside [m.p. 95–96° (cor.); $[\alpha]^{20}D$ −60.5° (H_2O)] which consumed one mole of periodate to give a solution which showed the same rotation as an equivalent quantity of benzyl β-D-glucopyranoside which had been similarly oxidized. These facts showed the substance to be benzyl β-D-ribofuranoside. The corresponding tribenzoate [m.p. 87–88° (cor.); $[\alpha]^{20}D$ +14.9° ($CHCl_3$)] gave, on hydrogenation over palladium-charcoal, 2,3,5-tri-*O*-benzoyl-D-ribose identical with that prepared directly from D-ribose.

(1) H. G. Fletcher, Jr., THIS JOURNAL, **75**, 2624 (1953).

While 2,3,5-tri-*O*-benzoyl-D-ribose shows little if any mutarotation in chloroform or aqueous dioxane, methylation studies and comparisons between its rotation and those of some closely related substances (both to be published in the near future) appear to justify the tentative conclusion that it belongs to the β-D-series.

Acetylation of 2,3,5-tri-*O*-benzoyl-D-ribose in pyridine at a low temperature afforded in 88% yield crystalline 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribose [m.p. 130–131° (cor.); $[\alpha]^{20}D$ +44.2° ($CHCl_3$)]. Similarly, benzoylation at low temperature gave in 89% yield D-ribofuranose tetrabenzoate [m.p. 121–122° (cor.); $[\alpha]^{20}D$ +17.0 ($CHCl_3$)]; the same substance was also obtained through the benzoylation of D-ribose in pyridine at 100° although the yield in this case (11%) was low owing to the difficulty of separating the product from β-D-ribopyranose tetrabenzoate which is formed simultaneously.

2,3,5-Tri-*O*-benzoyl-D-ribose has been used for the synthesis of benzyl β-D-ribofuranoside tribenzoate; it is possible that it may prove of general utility for the synthesis of ribofuranosides.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES
NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE
DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
BETHESDA 14, MARYLAND
ROBERT K. NESS
HEWITT G. FLETCHER, JR.

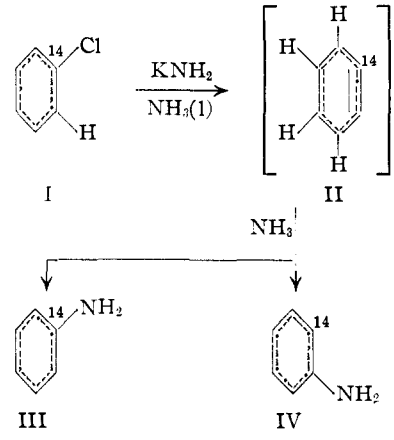
RECEIVED MAY 21, 1953

REARRANGEMENT IN THE REACTION OF CHLOROBENZENE-1-C^{14} WITH POTASSIUM AMIDE[1]

Sir:

No satisfactory explanation has been published for the rearrangements which often occur in the amination of "non-activated" aryl halides with alkali-metal amides.[2] The pattern of the rearrangements shows a considerable disregard for the influences governing the usual aromatic substitutions and is well illustrated by the products obtained from the amination of the methoxy- and trifluoromethylhalobenzenes. Although the methoxy and trifluoromethyl groups orient oppositely in aromatic nitration, *o*- and *m*-methoxy- and trifluoromethylhalobenzenes with alkali-metal amides yield exclusively *m*-substituted anilines, while the *p*-isomers yield mixtures containing roughly equal amounts of *m*- and *p*-substituted anilines.[3]

Besides the seemingly anomalous influence of substituents any mechanism proposed for the reaction must be in accord with the following observations: (1) the reactions are very rapid, even with chlorobenzene, in liquid ammonia at −33°; (2) the entering amino group has never been found farther than one carbon away from the position occupied by the leaving halogen[3,4]; (3) the starting halides and resulting anilines are not isomerized under the reaction conditions[3d,4]; (4) no reaction occurs in the benzene series with halides (*i.e.*, bromomesitylene,[3d] bromodurene[5] and 2-bromo-3-methylanisole[4]), where a hydrogen is not attached to the position adjacent to that occupied by the leaving halogen. These facts as well as the orientation data for various substituents can be accommodated by an elimination-addition mechanism involving at least transitory existence of an electrically neutral "benzyne" intermediate (II).



As is evident from the above reaction sequence, a critical test of the proposed mechanism would be afforded by the reaction of chlorobenzene-1-C^{14} with potassium amide. If a symmetrical intermediate such as II were involved equal amounts of aniline-1-C^{14} (III) and aniline-2-C^{14} would be formed since C-1 and C-2 become equivalent in II.

We have carried out the reaction of I[6] with potassium amide in liquid ammonia and obtained a 43% yield of C^{14}-labeled aniline. The C^{14} in the product was found to be distributed almost exactly as predicted for intermediate formation of II. While this experiment is not considered to "prove" the "benzyne" mechanism, it strongly indicates formation of an intermediate in which the 1- and 2-positions of the ring are, or can become, equivalent.[7] The only alternative is the occurrence of simultaneous rearranging and non-rearranging displacements in a ratio of almost exactly one to one. The utility of intermediates like II in accounting for the pattern of rearrangements with substituted halobenzenes will be demonstrated in a later paper.

An outline of the tracer experiments follows. The last steps were those developed by Loftfield.[8]

(1) Supported in part by the program of research of the U. S. Atomic Energy Commission.

(2) The scope of this type of reaction has been investigated principally by Gilman and Bergstrom and their co-workers. For a review, see J. F. Bunnett and R. E. Zahler, *Chem. Revs.*, **49**, 273 (1951).

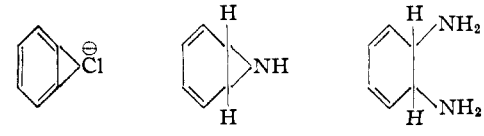
(3) (a) H. Gilman and S. Avakian, THIS JOURNAL, **67**, 349 (1945); (b) H. Gilman and R. H. Kyle, *ibid.*, **70**, 3945 (1948); **74**, 3027 (1952); (c) R. A. Benkeser and R. G. Severson, *ibid.*, **71**, 3838 (1949); (d) C. W. Vaughan, B.S. Thesis, M.I.T., 1951; (e) L. A. Carlsmith, M.S. Thesis, M.I.T., 1953.

(4) R. A. Benkeser and W. E. Buting, THIS JOURNAL, **74**, 3011 (1952).

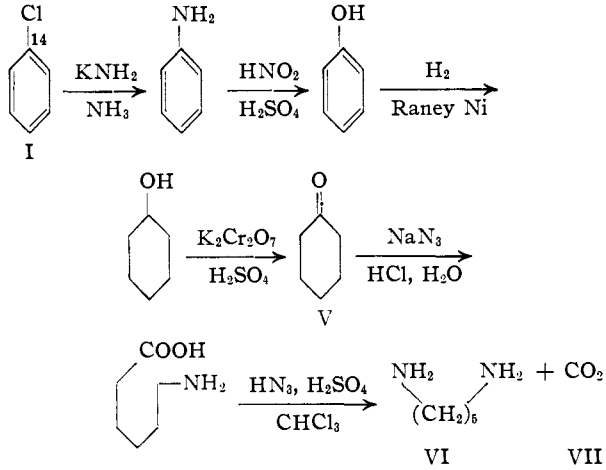
(5) Unpublished experiments by Mr. R. L. Harris.

(6) Obtained from Tracerlab, Inc., on allocation from the U. S. Atomic Energy Commission.

(7) Other possible symmetrical intermediates which would accommodate the C^{14}-tracer experiment and fit the general character of the reaction to a more or less satisfactory degree are:



(8) R. B. Loftfield, THIS JOURNAL, **73**, 4707 (1951).



VI (as the dibenzenesulfonamide) and VII (as barium carbonate) had respectively 51.8 ± 1% and 43.1 ± 1% of the radioactivity of V (as the semicarbazone or 2,4-dinitrophenylhydrazone). In a blank degradation of aniline-1-C^{14} (III),[8,9] the corresponding percentage activity figures for VI and VII were 0.2 ± 1 and 96.7 ± 1%, respectively. Since in the blank degradation the barium carbonate activity was consistently low, we consider the activity of VI to be the most reliable index of the amount of rearrangement in the amination of I.

DEPARTMENT OF CHEMISTRY AND LABORATORY FOR NUCLEAR SCIENCE AND ENGINEERING
MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE 39, MASS.
JOHN D. ROBERTS[10]
HOWARD E. SIMMONS, JR.
L. A. CARLSMITH
C. WHEATON VAUGHAN

RECEIVED MARCH 12, 1953

(9) Since I was prepared from III by Tracerlab, Inc., using the Sandmeyer reaction, this degradation constitutes a proof of the isotope position assignment for I.

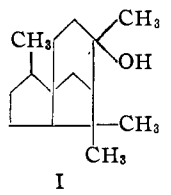
(10) Crellin Laboratory, California Institute of Technology, Pasadena 4, Calif.

THE STRUCTURE OF CEDRENE

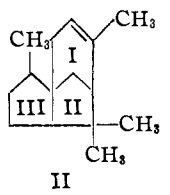
Sir:

The tricyclic sesquiterpenes cedrene, $C_{15}H_{24}$, and cedrol, $C_{15}H_{26}O$, have been the object, since their isolation by Walter in 1841,[1] of a large number of chemical investigations[2] which have led to the proposal of no fewer than thirteen different structures for the tertiary alcohol cedrol and the related anhydro compound, cedrene.[3]

We have elucidated the structures of these two sesquiterpenes which can now be represented by I (cedrol) and II (cedrene).



I

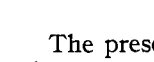


II

(1) Ph. Walter, *Ann.*, **39**, 247 (1841).

(2) Reviews of the work in this field up to 1947 are found in J. Simonsen and D. H. R. Barton, The Terpenes, Vol. III, Cambridge University Press, London (1952); see also S. H. Harper, *Ann. Repts. Chem. Soc.*, **44**, 143 (1948).

(3) The last published proposals are by Pl. A. Plattner, *Chimia*, **2**, 248 (1948).

The presence of the grouping  in the unsaturated ring of cedrene is well established.[2] The size of that unsaturated ring (ring I, fig. II) has incorrectly been deduced to be five-membered by previous investigators[4,4a] on the basis of observations which appeared to us to point clearly to a *six-membered* ring. We have confirmed our deduction by examination of the infrared spectrum of the anhydride of the bicyclic C_{13} diacid, norcedrenedicarboxylic acid (NCDA),[5] which proved to be that of a *glutaric* anhydride, with peaks at 5.57 and 5.67 μ, while a succinic anhydride, derived from a five-membered ring I, would have absorption maxima at 5.40 and 5.63 μ.[6] We have further prepared by the action of phenylmagnesium bromide on dimethyl norcedrenedicarboxylate a *diphenyl lactone*, m.p. 173–173.5°, which had its infrared band at 5.75 μ, in confirmation of the six-membered lactone structure corresponding to a glutaric acid.

The two carboxyls of NCDA are attached to a ring, the size of which we have proved by examination of the infrared spectrum of the anhydride of the monocyclic C_{11} dibasic acid obtained by further degradation of NCDA.[4] Again this proved to be the anhydride of a glutaric acid and ring II is thus established to be five-membered. The position of the *gem* dimethyl group shown in fig. II is considered elsewhere.[7] With respect to ring III two facts pointed strongly to the arrangement indicated in structure II: The isolation in high yield of *p*-cymene from the catalytic dehydrogenation of cedrene,[8] indicating the probable presence of a potential isopropyl group *para* to the methyl group of ring I; and the obvious biogenetic relationship to β-curcumene, of which cedrol is a formal cyclization product. In any event, this surmise is confirmed by the fact that the dehydroacid obtained from the C_{11} dibasic acid[4,5] is oxidized to α,α-dimethylhomotricarballylic acid,[3] an important result, a possible structural implication of which was incorporated, together with previous erroneous structural deductions, into the last published proposals for the structure of cedrene.[3] The full development of the necessarily sketchy outline presented here will be given in a subsequent paper.[9]

CHEMICAL LABORATORIES
HARVARD UNIVERSITY
CAMBRIDGE 38, MASSACHUSETTS
GILBERT STORK

CHANDLER LABORATORY
COLUMBIA UNIVERSITY
NEW YORK 27, NEW YORK
RONALD BRESLOW

RECEIVED JUNE 10, 1953

(4) Pl. A. Plattner, G. W. Kusserow and H. Kläui, *Helv. Chim. Acta*, **25**, 1345 (1942).

(4a) L. Ruzicka, Pl. A. Plattner and G. W. Kusserow, *ibid.*, **25**, 85 (1942).

(5) Pl. A. Plattner and H. Kläui, *Helv. Chim. Acta*, **26**, 1553 (1943).

(6) We have established that the position of the diagnostically more important lower wave length band is not affected by the degree of substitution.

(7) G. Stork and R. Breslow, THIS JOURNAL, **75**, 3292 (1953).

(8) W. Treibs, *Ber.*, **68**, 1041 (1935).

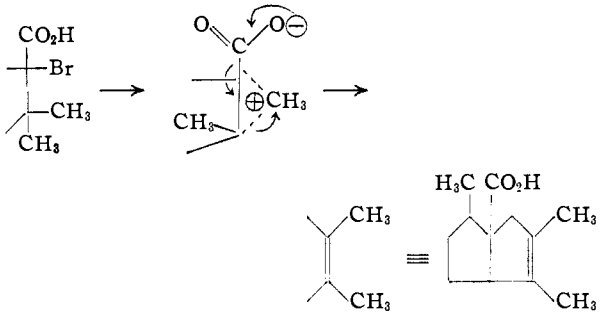
(9) Structures I and II for cedrol and cedrene were first presented formally in a lecture given by one of us at Harvard on January 27, 1953.

THE REARRANGEMENT OF BROMONORCEDRENEDICARBOXYLIC ACID

Sir:

It was first noted by Ruzicka and van Melsen that treatment of bromonorcedrenedicarboxylic acid (bromoNCDA) with base leads to the loss of the elements of hydrobromic acid and carbon dioxide and the formation of an unsaturated bicyclic monobasic acid, $C_{12}H_{18}O_2$.[1] On the (incorrect) assumption that bromoNCDA is a bromosuccinic acid[2] the reaction is unexceptional and has many precedents.[3] It was eventually recognized however that the C_{12} acid is not an α,β unsaturated acid and that apparently bromide ion and carbon dioxide are lost from the same carbon atom.[1,2] Little further progress was made in the elucidation of the structure of the C_{12} acid, and the rearrangement which leads to it has remained one of the arcana of cedrene chemistry.[4]

An entirely new light was shed on the reaction when it was demonstrated that NCDA is a glutaric acid derivative,[5] and the problem of the base decomposition of bromoNCDA was re-examined. It seemed to us likely that the reaction was essentially one of solvolysis of a neopentyl bromide type, undoubtedly facilitated in the present case by the cancellation of the positive charge on the relevant carboxyl group by formation of a carboxylate anion:



The correctness of this assumption was proved in the following manner: The monomethyl ester of NCDA, m.p. 131°,[2] was converted into the methyl ketone, dinitrophenylhydrazone m.p. 138°, by reaction of the acid chloride with dimethyl cadmium. Perbenzoic acid cleavage, followed by base hydrolysis of the resulting acetate gave the anticipated *hydroxy acid* $C_{12}H_{20}O_3$, m.p. 195–196°; which was then heated with phosphorus tribromide. After hydrolysis with water a crystalline acid was obtained which infrared comparison showed to be *identical* with the C_{12} acid of Ruzicka and van Melsen.

An attractive hypothesis was that the quaternary grouping involved in the rearrangement of bromoNCDA included the *gem* dimethyl group of cedrene.[6] This hypothesis received support from our observation that the characteristic *gem* dimethyl split peak at about 7.3 μ,[7] which is clearly evident in the infrared spectra of practically all the cedrene degradation products which we have examined, is changed to the usual C-methyl band in the rearranged C_{12} acid. Conclusive proof of the involvement of the *gem* dimethyl group was obtained by taking advantage of the fact that a rearranged acid in which the original *gem* dimethyl grouping had changed to two separate methyl groups must show between one and two more C-methyls than the parent compound. Experimental results were in agreement with predictions: NCDA monomethyl ester showed C-methyl: Calcd. for one C-methyl, 6.4. Found: 5.9%. The C_{12} acid showed C-methyl: Calcd. for three C-methyls, 23.2. Found: 22.6%.

The rearrangement of bromonorcedrenedicarboxylic acid, which incidentally finds a striking parallel in the transformation of bromocamphoric acid into laurolenic acid,[8] thus serves to locate the *gem* dimethyl group in cedrene.

(1) L. Ruzicka and J. A. van Melsen, *Ann.*, **471**, 40 (1929).

(2) L. Ruzicka, Pl. A. Plattner and G. W. Kusserow, *Helv. Chim. Acta*, **25**, 85 (1942); Pl. A. Plattner, G. W. Kusserow and H. Kläui, *ibid.*, **25**, 1345 (1942).

(3) See for instance R. Fittig and A. Landolt, *Ann.*, **188**, 71 (1877); S. J. Cristol and W. P. Norris, THIS JOURNAL, **75**, 632 (1953).

(4) The last published investigation of the C_{12} acid is by W. Treibs, *Ber.*, **76**, 160 (1943).

(5) G. Stork and R. Breslow, THIS JOURNAL, **75**, 3291 (1953).

(6) J. Simonsen and D. H. R. Barton, The Terpenes, Vol. III, Cambridge University Press, London, 1952.

(7) A. W. Thompson and P. Torkington, *Trans. Faraday Soc.*, **41**, 246 (1945).

(8) O. Aschan, *Ber.*, **27**, 2112 (1894); A. Lapworth and W. H. Lenton, *J. Chem. Soc.*, **79**, 1284 (1901).

CHEMICAL LABORATORIES
HARVARD UNIVERSITY
CAMBRIDGE 38, MASSACHUSETTS
GILBERT STORK

CHANDLER LABORATORY
COLUMBIA UNIVERSITY
NEW YORK 27, NEW YORK
RONALD BRESLOW

RECEIVED JUNE 10, 1953

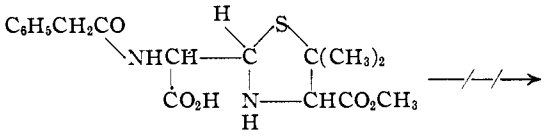
THE SYNTHESIS OF SUBSTITUTED PENICILLINS AND SIMPLER STRUCTURAL ANALOGS. VII. THE CYCLIZATION OF A PENICILLOATE DERIVATIVE TO METHYL PHTHALIMIDOPENICILLANATE

Sir:

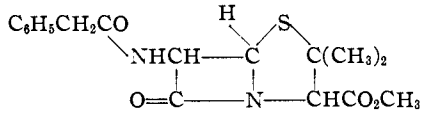
In the very intensive efforts made to cyclize β-methyl penicilloates (I) and related compounds to penicillin derivatives (II), the typical reaction products definitely identified were penicillenates (III), in which azlactonization has occurred and the thiazolidine ring has been disrupted.[1] It is not surprising that the five-membered oxazolone (azlactone) ring is formed in preference to a fused four-membered β-lactam ring whenever that possibility exists. However, of the very large number of recorded attempts[1] to effect a ring-closure of a penicilloate, none was conducted on a structure which could not azlactonize.

By a cyclization procedure we have synthesized a β-lactamthiazolidine (VI), which has the complete structure (configuration unassigned) of the natural penicillins, except for the substitution of a phthalimido group for the acylamino side chain. We have chosen to call this compound methyl phthal-

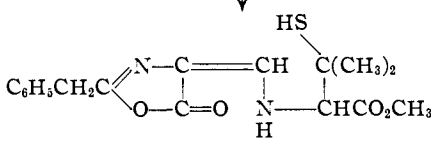
(1) H. T. Clarke, J. R. Johnson and R. Robinson, editors, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 851.



β-Methyl benzylpenicilloate (I)

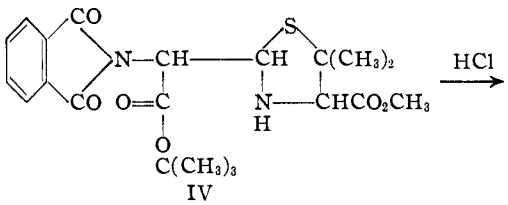


Methyl benzylpenicillinate (II)

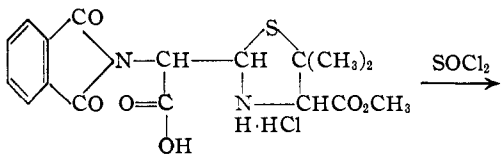


Methyl benzylpenicillenate (III)

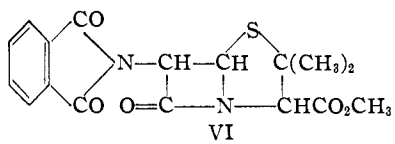
imidopenicillanate.[2] The key intermediate is V, a penicilloic acid derivative structurally incapable of azlactonization.



IV



V



VI

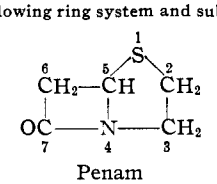
Condensation of *t*-butyl phthalimidoacetate with *t*-butyl formate in the presence of sodium hydride led to 31% of *t*-butyl α-phthalimidomalonaldehydrate, m.p. 155–156° (dec.).[3] *Anal.* Calcd. for $C_{15}H_{15}NO_5$: C, 62.27; H, 5.23; N, 4.84. Found: C, 62.48; H, 5.29; N, 4.70. A crystalline, stereoisomeric mixture of *t*-butyl 4-carboxy-5,5-dimethyl-α-phthalimido-2-thiazolidineacetates was obtained in 84% yield by condensation of this aldehyde-ester with DL-penicillamine. Several recrystallizations from acetone-water afforded a homogeneous sample, m.p. 179.5–180.5° (dec.). *Anal.* Calcd. for $C_{20}H_{24}N_2O_6S$: C, 57.13; H, 5.75; N, 6.66. Found: C, 57.20; H, 5.79; N, 6.35. Treatment with diazomethane generated the corresponding methyl ester IV (90% yield), m.p. 121–122°. *Anal.* Calcd. for $C_{21}H_{26}N_2O_6S$: C, 58.05; H, 6.03; N, 6.45. Found: C, 58.02; H, 6.09; N, 6.52.

By cleavage of the *t*-butyl ester with dry hydrogen chloride, an 85% yield of 4-carbomethoxy-5,5-dimethyl-α-phthalimido-2-thiozalidineacetic acid hydrochloride (V) was formed, m.p. 160–161° (dec.). *Anal.* Calcd. for $C_{17}H_{19}N_2O_6SCl$: C, 49.21; H, 4.62; N, 6.75. Found: C, 48.99; H, 4.86; N, 7.07. Treatment of V with thionyl chloride, followed by oxidation with potassium permanganate in acetic acid solution gave the sulfone of VI in 13% yield; m.p. 200–201° (dec.). *Anal.* Calcd. for $C_{17}H_{16}N_2O_7S$: C, 52.03; H, 4.11; N, 7.14. Found: C, 52.18; H, 4.05; N, 7.27. From a similar reaction mixture before oxidation there was isolated by chromatography over alumina the pure β-lactam-thiazolidine VI,[4] m.p. 171–172° (dec.). *Anal.* Calcd. for $C_{17}H_{16}O_5N_2S$: C, 56.67; H, 4.47; N, 7.78. Found: C, 56.64; H, 4.56; N, 8.04.

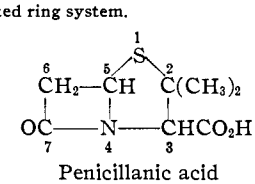
The infrared spectrum of methyl phthalimidopencillanate (VI) has the intense band at 5.62 μ which is associated with the β-lactam carbonyl in natural pencillins[5] and in synthetic β-lactam-thiazolidines. Conversion to the sulfone causes the expected shift[6] of this band to about 5.57 μ; in the spectrum of both VI and VI sulfone the characteristic phthalimido bands at 5.65 μ and 5.82 μ are observed.

We are indebted to Bristol Laboratories of Syracuse, N. Y., for generous financial support of this work.

(2) As a convenience in naming VI and similar analogs of the penicillins we suggest the terms "penam" and "penicillanic acid" for the following ring system and substituted ring system.



Penam



Penicillanic acid

As in the case of the penicilloic acids, these terms carry no stereochemical implications. Thus methyl benzylpenicillinate (penicillin G methyl ester) is one of the stereoisomers of methyl phenylacetamidopenicillanate. The numbering is that generally accepted for the penicillins, and the point of attachment of the side chain is understood to be 6 unless otherwise stated.

(3) All melting points are corrected.

(4) This lactam is inactive when tested by routine penicillin assay procedures (Bristol Laboratories, Syracuse, N. Y.).

(5) Ref. 1, p. 404.

(6) J. C. Sheehan, H. W. Hill, Jr., and E. L. Buhle, THIS JOURNAL, **73**, 4374 (1951); Ref. 1, p. 411.

DEPARTMENT OF CHEMISTRY
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
CAMBRIDGE 39, MASSACHUSETTS
JOHN C. SHEEHAN
K. R. HENERY-LOGAN
D. A. JOHNSON

RECEIVED MAY 16, 1953

FORMATION OF THE ISONICOTINIC ACID HYDRAZIDE ANALOG OF DPN[1]

Sirs:

In a previous paper from this laboratory[2], diphosphopyridine nucleotidase (DPNase) of beef spleen was shown to catalyse the exchange of added C^{14}-labeled nicotinamide with the nicotinamide moiety of DPN resulting in the isolation of C^{14}-labeled DPN. The speculation that structural analogs of nicotinamide might take part in a similar

(1) Contribution No. 53 of the McCollum-Pratt Institute. Aided by grants from the American Tuberculosis Association, the American Cancer Society as recommended by the Committee on Growth of the National Research Council, the Williams-Waterman Fund and the Rockefeller Foundation.

(2) L. J. Zatman, N. O. Kaplan and S. P. Colowick, *J. Biol. Chem.*, **200**, 197 (1953).

exchange reaction to yield a DPN analog,[2,3] has now been tested experimentally with the recently described antituberculous drug isonicotinic acid hydrazide (INH). The over-all reaction can be summarized as $ARPPRN^+ + INH \rightarrow ARPPR(INH)^+ + N$, where the symbol $ARPPRN^+$ represents the DPN structure adenine-ribose-phosphate-phosphate-ribose-nicotinamide, and the symbol $ARPPR(INH)^+$ similarly represents the DPN analog in which the nicotinamide moiety (N) has been replaced by isonicotinic acid hydrazide (INH).

The early studies with INH indicated a surprising species specificity as regards its inhibitory action on the tissue DPNases[3] and recent experiments have shown that the enzyme of human spleen and prostate is remarkably insensitive; thus even at 4×10^{-2} *M* INH the rate of DPN cleavage is unaffected. Upon incubation of DPN with INH in the presence of these "INH-insensitive" DPNases, the development of a yellow color was observed on making the incubation mixture alkaline (*p*H 9.5) prior to addition of the alcohol dehydrogenase system for assay of the residual DPN. Production of the yellow color in alkali after the incubation was subsequently found to depend on (1) the active DPNase, (2) added DPN, (3) INH and (4) the disappearance of DPN during the incubation. For example, the presence of nicotinamide during the incubation prevented DPN breakdown, and, to the same extent, prevented appearance of the yellow compound. Figure 1 illustrates the direct relationship which appears to exist between the disappearance of DPN and the appearance of the yellow compound during an incubation with human prostate homogenate. The yellow compound has an absorption maximum in alkali at 385 mμ, the height of this peak being *p*H dependent; thus while it is maximal in 0.1 *N* NaOH it disappears (reversibly) in acid. That the yellow color is attributable to an N-substituted INH moiety in the DPN analog is strongly suggested by the fact that N'-methyl INH shows practically identical changes in absorption spectrum on treatment with alkali and acid.

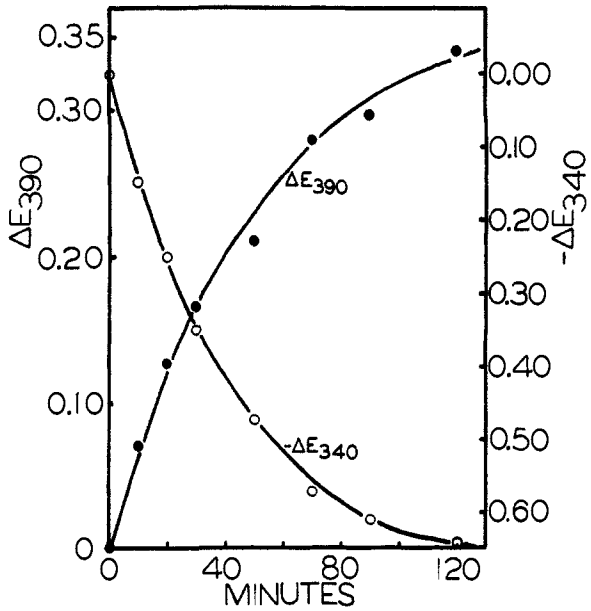


Fig. 1.—Time relationship between disappearance of DPN and formation of analog. Concentrations in 3.6 ml. reaction mixture: 0.02 *M* phosphate buffer *p*H 7.2; 6×10^{-4} *M* DPN; 2×10^{-2} *M* INH; 1.5 ml. 10% human prostate homogenate; temperature, 38°. Aliquots analyzed for (a) DPN, by addition of crystalline yeast alcohol dehydrogenase and ethanol to the aliquot in glycine/NaOH buffer *p*H 9.5 and determining the increase in optical density at 340 mμ, expressed as E_{340}, and (b) the analog, by determining the optical density at 390 mμ in the glycine/NaOH buffer *p*H 9.5, expressed as E_{390}. Changes in these values during the incubation are expressed as $-\Delta E_{340}$ and ΔE_{390}, respectively.

It has been found that the yellow compound is formed by all "INH-insensitive" DPNases tested with the exception of the *Neurospora* enzyme (which is unaffected by 1.3×10^{-1} *M* INH). This latter observation is of particular significance because the *Neurospora* enzyme does not promote the exchange reaction between free nicotinamide and the bound nicotinamide of DPN.[2]

A more detailed study of the reaction involving the production of the yellow compound has been carried out using pig brain as the enzyme source. These experiments have resulted in the isolation, from a reaction mixture originally containing 1000 mg. of DPN (Sigma "90"), of 774 mg. of a yellow product which appears to be the proposed analog of DPN, *i.e.*, the compound in which the nicotinamide moiety of DPN has been replaced by INH. An analysis of the compound has yielded the data shown in Table I—data which conform to the expected values which would obtain for an INH analog of DPN. Paper chromatographic techniques have shown that while the analog spot (R_f = 0.40 in EtOH: 0.1 *N* acetic acid, 1:1) gives a positive picryl chloride test for the hydrazide grouping,[4] prior hydrolysis in acid or alkali abolishes the analog spot and simultaneously releases free INH (R_f = 0.74).

TABLE I

	Moles per mole of analog					
	Ribose	P	INH	5-adenylic acid	Nicotinamide	DPN
Calcd. for $ARPPR(INH)^+$	2.0	2.0	1.0	1.0	0	0
Observed	2.2	2.1	0.88	0.84	0.11	0.14

Preliminary investigations of the properties of the analog in enzyme systems have shown it to be a potent inhibitor of the INH-sensitive beef spleen DPNase—at least twice as potent as free INH. Further studies are now being carried out dealing in particular with the possible role of the analog in the antituberculous action of INH and more generally with the significance of such analogs of essential coenzymes in the mechanism of drug action.

McCOLLUM-PRATT INSTITUTE
THE JOHNS HOPKINS UNIVERSITY
BALTIMORE 18, MD.
LEONARD J. ZATMAN
NATHAN O. KAPLAN
SIDNEY P. COLOWICK
MARGARET M. CIOTTI

RECEIVED MAY 21, 1953

(3) L. J. Zatman, S. P. Colowick, N. O. Kaplan and M. M. Ciotti, *Bull. Johns Hopkins Hosp.*, **91**, 211 (1952).

(4) W. F. J. Cuthbertson and D. M. Ireland, *Biochem. J.*, **52**, xxxiv (1952).