TABLE III

TYPICAL DATA ON THE CONCENTRATION OF THE FACTOR BY COUNTERCURRENT DISTRIBUTION

Fraction	Microbiological activity, units
Start	35,100
1–3	880
4–6	2,960
7–9	5,100
10–12	6,300
13–15	6,750
16–18	5,460
19–21	3,270
22–24	1,930
25–27	1,090
28–30	430
Recovery	34,500 (98%)

The apparatus was arranged to run with 10 ml. of each phase in each tube. The distribution was carried through 265 transfers with effluent collected in a flask at the end. The tube contents were then combined by groups of five and assayed for microbiological activity. The combined tube content from tubes 131–135 and tubes 136–140 showed the highest potency. These contained, respectively, 14,700 units in a weight of 247 mg. or a potency of 59,500 units/g. and 16,700 units in a weight of 224 mg. or a potency of 74,500 units/g. These two fractions were combined with others of similar potency to give a total of approximately 106,600 units in a weight of 1.769 g. or a potency of 60,000 units/g. This preparation was distributed in the same solvent system and equipment by the previous technique until a total of 299 transfers had been made. The material of highest potency was found in plates 141 to 165. These were combined to give 85,600 units in 905 mg. or a potency of 94,000 units/g. This material was loaded into two tubes of the 200-tube countercurrent machine. The ends of the system were connected after the first 200 transfers. Then the contents of tubes 1 through 50 and 151 through 200 were removed (retaining tube contents of 51–150 in place) and fresh solvent phases were inserted. Operation was continued until a total of 600 transfers had been made; then tube contents were again replaced as above and operation was continued to a total of 1200 transfers. The material of highest potency occurred in tubes 150 to 179 where 34,500 units in 273.2 mg. or a potency of 126,000 units/g. was obtained.

Fractions from tubes 150 through 179, inclusive were combined and distributed again in the same solvent system. A total of 100 transfers was made in this distribution. Combinations of two tubes were made. Pertinent data are summarized in Table IV. When the weight distribution was

TABLE IV

PERTINENT DATA ON THE COUNTERCURRENT DISTRIBUTION OF HIGH POTENCY FACTOR

Tubes	Weight, mg.	Microbiological activity Units/fraction	Units/g.
37–38	2.1	220	104,000
42–43	9.9	1190	120,000
45–46	16.9	2210	131,000
47–48	21.4	3170	148,000
50–51	27.7	3770	136,000
52–53	26.3	3560	135,000
54–55	25.8	3190	123,000
57–58	18.6	2760	148,000
59–60	13.9	1550	111,000
62–63	7.5	760	101,000

plotted in the usual method the curve was very close to the theoretical distribution curve but not close enough to indicate complete purity. The contents of plates 45 to 58 inclusive, were combined and the solvents removed at reduced pressure. The nearly pure factor was a clear, almost colorless oil which weighed 150 mg. It was highly active in the microbiological assay, having a potency of about 140,000 units/g.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF CALIFORNIA AT LOS ANGELES]

Mold Metabolites. VIII. Contribution to the Elucidation of the Structure of Helvolic Acid[1]

BY DONALD J. CRAM AND NORMAN L. ALLINGER

RECEIVED APRIL 13, 1956

Helvolic acid, a $C_{32}H_{42}O_8$ metabolic product of *Aspergillus fumigatus*, has been thoroughly characterized through identification of its functional groups. That the molecule contains an α,β-unsaturated carboxyl group, two acetoxyl groups, one α,β-unsaturated ketonic function, an isolated ketonic function, one isolated double bond and four rings (probable) has been demonstrated. The relative positions of some of these functions have been deduced. Aromatization of the substance gave a minute yield of a hydrocarbon which possesses an ultraviolet absorption spectrum characteristic of the 11-naphtho[2,1-*a*] fluorene ring system. This observation coupled with the fact that Diels isolated a hydrocarbon containing this same ring system by aromatization of cholesterol suggests that helvolic acid possesses a steroid skeleton. The data can be interpreted in terms of a highly hypothetical steroidal structure for helvolic acid.

A solid material possessing antibiotic properties was isolated by Waksman, *et al.*,[2] from the culture filtrates of *Aspergillus fumigatus* and was given the name fumigacin. Chain, *et al.*,[3a] and Menzel, *et al.*,[3b] demonstrated that fumigacin was a mixture of gliotoxin (a known compound) and a previously unknown antibiotic which the former group designated as helvolic acid (I). This substance has been partially characterized chemically[3a,b,4] and biologically[3b,4c,5] and X-ray diffraction studies of both the acid and its methyl ester have been reported.[6]

(1) This work was generously supported by a grant and a sample of helvolic acid from the Upjohn Co., Kalamazoo, Michigan.

(2) S. A. Waksman, E. S. Horning and E. L. Spencer, *J. Bact.*, **45**, 233 (1943).

(3) (a) E. Chain, H. W. Florey, M. A. Jennings and T. I. Williams, *Brit. J. Exp. Pathology*, **24**, 108 (1943); (b) A. E. O. Menzel, O. Wintersteiner and J. C. Hoogerheide, *J. Biol. Chem.*, **152**, 419 (1944).

(4) (a) J. H. Birkinshaw, A. Bracken and H. Raistrick, *Biochem. J.*, **39**, 70 (1945); (b) W. H. Elliott, P. A. Katzman, S. A. Thayer and E. A. Doisy, *Fed. Proc.*, **6**, 250 (1947); (c) H. W. Flory, *et al.*, "Antibiotics," Vol. I, Oxford University Press, London, 1949, p. 332; (d) T. I. Williams, *Biochem. J.*, **51**, 539 (1952); (e) H. S. Burton, E. P. Abraham and H. M. E. Cardwell, *ibid.*, **62**, 171 (1956).

(5) E. A. Hall, F. Kavanagh and I. N. Asheshov, *Antibiotics and Chemotherapy*, **1**, 369 (1951).

(6) D. M. Crowfoot and B. W. Low, *Brit. J. Exp. Pathology*, **24**, 120 (1943).

Molecular Formula of Helvolic Acid (I).—Two molecular formulas have been proposed for helvolic acid (I), $C_{32}H_{42-44}O_8$[3a] and $C_{29}H_{38-40}O_7$.[3b] The latter is supported by molecular weights obtained by the Rast method and by titration. From the present work (see later section), it is clear that I splits into smaller fragments when heated, and consequently too low a molecular weight is obtained by the Rast method. The ease of reaction of I with base leads to neutralization equivalents which are too low and hence to a specious molecular weight. The C_{32} formula rests on the molecular weight determined by the X-ray method[6] and on the analysis of three compounds including a silver salt.[3b] In the present work the molecular weight was found by the Signer method[7a] to be 585 for the methyl ester of helvolic acid, a value in fair agreement with a molecular formula of $C_{33}H_{44}O_8$ (mol. wt. of 569) for the ester based on the $C_{32}H_{42}O_8$ formula (mol. wt. of 555) for helvolic acid. This last formula best interprets the values of 556 and 554 found, respectively, for helvolic acid and its methyl ester by the X-ray method.[6] These molecular formulas are also supported by the analysis of several derivatives including a *p*-bromophenacyl ester, a dinitrophenylhydrazone, a semicarbazone and several hydrogenation products. Of forty elemental analyses obtained in the present work on I and derivatives, twenty fit either a $C_{32}H_{42}O_8$ or a $C_{33}H_{44}O_8$ formula equally well, while fourteen favor the former and six the latter. Thus the bulk of the data strongly favors a $C_{32}H_{42}O_8$ formula for helvolic acid.

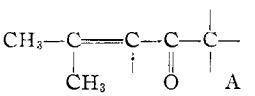
Functional Groups of Helvolic Acid (I).—Previous workers characterized I as follows[3,4]: The substance was reported to contain a titratable and esterifiable (diazomethane) carboxyl group,[3a] a lactone and two other functions, each of which consumed a mole of base on prolonged heating which resulted in the liberation of acetic acid and another unidentified volatile acid.[4c] The last claims were experimentally undocumented. Other authors stated that the hydrolysis resulted in the consumption of three moles of base with the liberation of two moles of acetic acid.[4b,d] Compound I gave a positive (Zimmerman) test for a ketone, and formed a monoöxime, a semicarbazone[3b] (no dinitrophenylhydrazone) and a dioxime.[4c,d] The compound gave positive Liebermann–Burchard (for a homoallylic acetate) and Charbrol–Charonnet (given by bile acids) tests, and the following negative tests: ferric chloride, Legal, fuchsin, Tollens, Molisch, Rosenheim, Hammerstein, Tortelli–Jaffe and digitonin.[7b] The compound was reported to absorb one mole of bromine but no hydrogen with platinum in alcohol.[3b] Later the hydrogenation of I to an octahydro derivative was reported.[4b]

The paper of Burton and co-workers[4e] appeared after the present work was completed. These workers have summarized the available data and accounted for all of the oxygen atoms present as follows: two keto groups, two acetoxyl groups and one carboxyl group. Insofar as our work overlaps theirs, the agreement is substantially complete.

(7) (a) E. P. Clark, *Ind. Eng. Chem., Anal. Ed.*, **13**, 820 (1941); (b) For a description and interpretation of these tests, see references 33 and 34.

In the present work preliminary experiments indicated that treatment of I with either acid or base or simply heating it seemed to give mainly resinous products. An attack on the molecule with very mild reactions and the utmost use of physical methods for structure elucidation appeared indicated.

We have found that the methyl ester of helvolic acid (II, or methyl helvolate) can be catalytically hydrogenated in a stepwise fashion to give crystalline di-, tetra-, hexa- and octahydro derivatives. A similar sequence was also applied to the parent acid I. From the differences between the ultraviolet spectrum of each successive reduction product, evidence regarding the nature of the chromophore reduced was obtained (Fig. 1).[8] Methyl dihydrohelvolate was obtained by the hydrogenation of II in methanol with palladium-on-charcoal by interrupting the reaction after one mole of hydrogen had been absorbed. The difference between the spectra of the dihydroester and II gives λ_{max} 236 mμ, ϵ 10,600 for the first chromophore hydrogenated. The reported spectrum of mesityl oxide[9] has λ_{max} 235 mμ, ϵ 14,000, a fact that suggests the first chromophore hydrogenated contains the unsaturated system of the structural unit A. The spectrum of methyl helvolate possesses a weak



carbonyl R-band at λ_{321}, ϵ 87, which is displaced to longer wave length and higher intensity as compared to simple ketones. Such a shift is typical of α,β-unsaturated ketones; for instance, the corresponding band in mesityl oxide is λ_{314}, ϵ 59.[9] Helvolic acid semicarbazone shows a maximum in the ultraviolet at 277 mμ, ϵ 14,900. The expected λ_{max} for the same derivative of compounds containing A is 260 mμ,[10] and the reason for the discrepancy is not known. Methyl helvolate 2,4-dinitrophenylhydrazone (red orange) possesses λ_{max} 376 mμ, ϵ 29,600, as compared to λ_{max} 377–379 mμ, ϵ 25,000–35,000, for suitable models.[11] The same derivative of methyl tetrahydrohelvolate (see below) was also prepared and gave λ_{max} 361 mμ, ϵ 26,000, which attests to the absence of structural unit A in the tetrahydroester. The infrared spectrum of the dihydro compound showed the characteristic bands of an isopropyl group[12] at 8.56 and 8.65 μ which were not present in II, a fact that suggests the two β-alkyl groups of A to be methyls. Ozonolysis of helvolic acid (I) gave acetone, identified as its 2,4-dinitrophenylhydrazone, whereas under the same conditions dihydrohelvolic acid gave no detectable water-soluble ketone. Clearly the double bond of structural unit A was reduced in going to the dihydroester.

Hydrogenation of the dihydroester to the tetrahydroester proceeded under the same conditions

(8) That these chromophores are isolated is indicated by the good correlation between the spectra of Fig. 1 and model spectra, and by consistency between the argument based on spectra and direct chemical evidence.

(9) J. Bielecki and V. Henri, *Ber.*, **47**, 1690 (1914).

(10) L. K. Evans and A. E. Gillam, *J. Chem. Soc.*, 565 (1943).

(11) E. R. H. Jones and E. A. Braude, *ibid.*, 498 (1945).

(12) N. Sheppard and D. M. Simpson, *Quart. Revs.*, **7**, 19 (1953).

as the conversion of I to the dihydroester. From the difference between the ultraviolet absorption spectra of the dihydro- and tetrahydroesters, the group hydrogenated in this case appears to be an isolated double bond which possessed no observable maximum in the ultraviolet but did have a strong end absorption with λ_{215}, ϵ 200; λ_{210}, ϵ 700; λ_{205}, ϵ 1600. Comparison of these values with those obtained for isolated double bonds in steroids[13] suggests that the double bond is disubstituted since its spectrum is nearly identical with that of a Δ^{11}-steroid. That the double bond of the dihydroester is probably in a cycle was shown by the fact that the substance gave a yellow color with tetranitromethane while the tetrahydroester did not. Quantitative ultraviolet measurements of the complex of tetranitromethane with methyl dihydrohelvolate were made and the following constants found: λ° 472 mμ, $S(\lambda^\circ)$, -1.9×10^{-2}. The values reported for 2-cholestene[14] are λ° 479 mμ, $S(\lambda^\circ)$ -1.7×10^{-2}. Thus the isolated double bond in helvolic acid appears both cyclic and disubstituted. Ozonolysis of methyl tetrahydrohelvolate was attempted but starting material was recovered, a fact indicating the lack of additional reactive double bonds in this compound.

Methyl tetrahydrohelvolate could not be hydrogenated further with palladium in methanol, but with platinum and methanol one additional mole of hydrogen was cleanly absorbed to give a hexahydro derivative. The ultraviolet spectrum of this substance was essentially identical with that of the tetrahydroester. Since hydrogenation of even an isolated double bond is easily detected in this way, the group which absorbed hydrogen in this reaction must have possessed practically no ultraviolet absorption from 205 to 260 mμ. This result coupled with the resistance of the tetrahydroester to ozonolysis suggests that this mole of hydrogen converted a ketone to an alcohol. Accordingly, methyl hexahydrohelvolate did not react with 2,4-dinitrophenylhydrazine, even under conditions more vigorous than those used to make the derivative of the tetrahydroester. The hexahydroester formed a crystalline acetate under conditions from which compound I could be recovered unchanged.[3b] The infrared spectrum of methyl hexahydrohelvolate showed a very weak, sharp band at 2.80 μ such as is usually found in steroidal alcohols,[15] while the more highly unsaturated esters did not show this band. It is considered likely that the keto group of structural unit A was reduced during the conversion of the tetrahydro- to the hexahydroester.

Hydrogenation of the hexahydroester with platinum and acetic acid gave the octahydroester, and the chromophore lost in this case had λ_{max} 222 mμ, ϵ 7,900. The commonly encountered chromophore which has this characteristic absorption and is also rather inert to ozone is an α,β-unsaturated acid having not more than one

(13) P. Bladon, H. B. Henbest and G. W. Wood, *Chemistry and Industry*, 866 (1951), and *J. Chem. Soc.*, 2737 (1952).

(14) E. Heilbronner, *Helv. Chim. Acta*, **36**, 1121 (1953).

(15) K. Dobriner, E. R. Katzenellenbogen and R. N. Jones, "The Infrared Absorption Spectra of Steroids—an Atlas," Interscience Publishers, Inc., New York, N. Y., 1953.

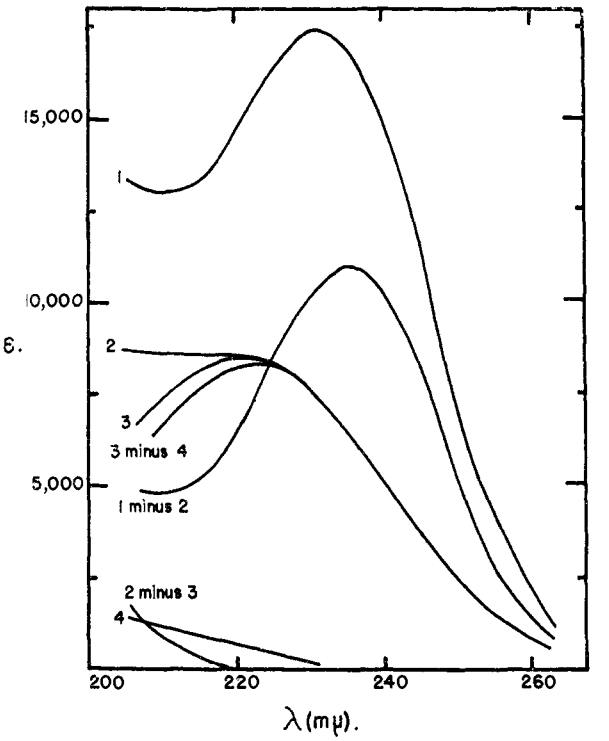


Fig. 1.—Ultraviolet absorption spectra in 95% ethanol (Cary spectrophotometer, model 11 PMS): curve 1, methyl helvolate; curve 2, methyl dihydrohelvolate; curve 3, methyl tetrahydrohelvolate and methyl hexahydrohelvolate; curve 4, methyl octahydrohelvolate.

hydrogen on the double bond. The possible alternatives are B and C, which in simple systems have virtually identical ultraviolet spectra (λ_{max} 219, ϵ 11,000),[16] and D, for which no simple model compound appears to be available.[16b] The relatively low extinction coefficient of the chromophore suggests that steric inhibition of resonance may be present, which if so might also lower the wave

RCH=CR'COOH RR'C=CHCOOH RR'C=CR"COOH
B C D

length of λ_{max}.[17] A sample of tetrahydrohelvolic acid was also prepared, and when heated at 260° for one hour, carbon dioxide was evolved slowly (identified as barium carbonate), a reaction characteristic of many α,β-unsaturated acids.[18] The infrared spectrum of the pyrolysis residue showed no carboxyl absorption in the 3–4μ region. Compounds of structure B are not usually decarboxylated under these conditions,[19] and the amorphous pyrolysis product showed no infrared absorption attributable to a terminal methylene group, which would have been expected from material possessing structural unit C. The most probable

(16) (a) J. Cason, N. L. Allinger and G. Sumrell, *J. Org. Chem.*, **18**, 850 (1953); (b) The dehydration product of ethyl 3-hydroxy-2,3,5,9-tetramethyldecanoate, which is presumably a mixture of the α,β,γ-trialkyl-α,β-unsaturated acid and its β,γ-isomer, showed λ_{max} 210 mμ, log ϵ 3.45 [P. C. Jocelyn and N. Polgar, *J. Chem. Soc.*, 132 (1953).]

(17) E. A. Braude, E. R. H. Jones, H. P. Koch, R. W. Richardson, F. Sondheimer and J. B. Toogood, *J. Chem. Soc.*, 1890 (1949).

(18) R. T. Arnold, D. C. Elmer and R. M. Dodson, THIS JOURNAL, **72**, 4359 (1950).

(19) J. Cason and C. F. Allen, *J. Biol. Chem.*, **205**, 449 (1953).

structure is D, and it is also likely that at least one hydrogen is attached to Cγ[18] as in >CHCR=CR″-COOH (D′).

The octahydroester contains a chromophore best defined as an isolated ketone group, λ_{max} 318, ϵ 52. This wave length is longer than is usual for simple ketones, possibly the result of either steric crowding[20a] of the function, or of the presence of another unsaturated function (*e.g.*, an acetoxyl) being pressed into the environment of the carbonyl.[20b] Such a steric constraint would also be consistent with the resistance of the carbonyl group to reaction with carbonyl reagents and to catalytic hydrogenation.

The hydrogenation of helvolic acid was also carried out in a stepwise fashion, and a similar series of hydrogenated derivatives were obtained. The properties of these acids are consistent with the conclusions derived from the properties of the corresponding esters.

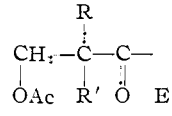
The reaction of helvolic acid (I) with base was examined in detail. The compound consumed a little more than one mole of base when titrated at room temperature, a second mole after a few minutes at 40° and a third mole when heated in diethylene glycol with strong base at 140° for a few minutes. The consumption of the first mole was an immediate neutralization, but neutralization equivalents were always low due to the ease with which the second equivalent was consumed in a saponification. The values for the saponification equivalent (mild conditions) came to 277 ± 5 (theory 278), and acidification of the mixture gave a solid compound (85% yield) to which the name helvolinic acid is assigned. This compound presumably corresponds to the "ring opened lactone" of Menzel, *et al.*[3b] Acetic acid, characterized as its *p*-phenylphenacyl derivative, was also isolated.

Although helvolic acid appeared to be an acetate of helvolinic acid, the analytical data for the latter conflicted with this conclusion. This anomaly was set aside as follows: Methyl helvolinate was prepared both from helvolinic acid (diazomethane) and from methyl helvolate (base), and the analysis corresponded to that of a desacyl derivative of methyl helvolate. The infrared spectrum of methyl helvolinate possessed an exceedingly strong, new band at 2.95 μ which appeared to obey Beer's law and which is attributed to an intramolecularly hydrogen bonded hydroxyl group.[21] The 2,4-dinitrophenylhydrazone and tetrahydro derivatives of methyl helvolinate were prepared and had the expected properties. Finally methyl helvolinate was converted to methyl helvolate with acetic anhydride in pyridine. The anomalous analytical data for helvolinic acid can perhaps be interpreted as a result of the compound containing one-half of a molecule of water of crystallization. Tetrahydro- and hexahydrohelvolinic acids likewise appear to be fractional hydrates, although water could not be removed by prolonged drying in vacuum.

Vigorous saponification of helvolic acid gave saponification equivalents of 183 and 186 (theory 185), and acetic acid was the only detectable volatile acid (characterized as its *p*-bromophenacyl ester). When helvolinic acid was heated above its melting point (230°), acetic acid was liberated in about 97% yield. These results indicate that helvolic acid contains two acetoxyl groups, one readily hydrolyzable, the other more difficultly.

The product obtained by vigorous saponification of helvolic acid is worthy of comment. Williams,[4d] on the basis of the carbon–hydrogen analysis, assigned a lactonized bisdesacetyl formula to this compound. In our hands the compound was obtained as an amorphous solid which failed to crystallize. The analysis, however, was in agreement with a simple bisacetyl formulation, and no evidence for lactone formation was noted. In cephalosporin P_1 such a lactone does appear to form.[4e] One difference between these compounds may be the position of this acetoxyl.

The nature of the environment of the hydroxyl group in helvolinic acid is of interest. This acid gives negative tests with both periodate and digitonin. Since the hydroxyl is very strongly intramolecularly hydrogen bonded, it is probably β to a carbonyl group. The extreme ease of saponification of the acetate giving rise to this hydroxyl suggests that the acetoxyl is attached to a primary carbon atom. These suggestions are summarized in structural unit E for helvolic acid.



These data demonstrate what other authors have suggested,[4e] namely, that helvolic acid contains two keto, two acetoxyl and one carboxyl groups, as well as two conjugated and one isolated double bonds. The eight oxygen atoms and all of the functional groups of the molecule have been identified, and a number of conclusions respecting their environments have been reached.

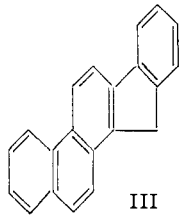
Ring System of Helvolic Acid (I).—The molecular formula of helvolic acid ($C_{32}H_{42}O_8$) indicates the presence of twelve sites of unsaturation in the molecule. The two acetoxyl and two keto groups, the carboxyl and the three double bonds account for eight of these sites, leaving four to be accounted for by rings. A selenium dehydrogenation at 370° was carried out in an effort to establish the character of the ring system. Octahydrohelvolic acid (24 g.) was first treated with lithium aluminum hydride and then with selenium under nitrogen to give, after extended purification, 15 mg. of a yellowish hydrocarbon, m.p. 170–195°. Further recrystallization of this gave 7 mg. of material, m.p. 195–210°. Although this sample was not pure, it was the best obtained. The ultraviolet absorption spectra of the two samples were nearly indistinguishable (Fig. 2) and characteristic of the 11-naphtho[2,1-*a*]fluorene ring system (III). Several alkyl derivatives containing this ring system have been reported,[22] and their detailed spectra differ

(20) (a) Ramart-Lucas, *Bull. soc. chim.*, **51**, 289 (1932); (b) D. J. Cram and H. Steinberg, THIS JOURNAL, **76**, 2753 (1954).

(21) R. N. Jones, P. Humphries, F. Herling and K. Dobriner, *ibid.*, **74**, 2820 (1952).

(22) (a) J. W. Cook, C. L. Hewett, W. V. Mayneord and E. Rode, *J. Chem. Soc.*, 1727 (1934); (b) O. Diels, W. Gadke and P. Kording, *Ann.*, **459**, 1 (1927); (c) E. Bergmann, THIS JOURNAL, **60**, 2305 (1938).

only slightly. Our dehydrogenation product seems clearly to belong to this group, only small spectral differences being apparent. The dehydrogenated material was a yellowish solid, and chromatography failed to yield a colorless sample. Cook, *et al.*,[22a] have reported that III was difficult to obtain pure

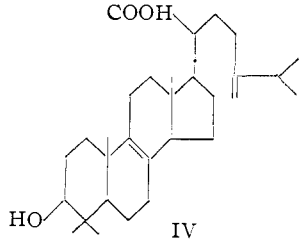


III

and that its solutions yellowed readily, presumably due to oxidation at the saturated carbon atom. The yield of the crude hydrocarbon obtained in the present work was only 0.1%, and the possibility that this small amount of material came from an impurity cannot be ruled out. However, the assumption is made in the subsequent discussion that the hydrocarbon is an authentic degradation product of helvolic acid.

Since the hydrocarbon contains five rings and helvolic acid only four, a ring closure during the aromatization is indicated. Diels[22b] isolated a hydrocarbon (poor yield) containing the same ring system as III from the aromatization of cholesterol, although the exact structure of the compound has never been demonstrated. The hydrocarbon from helvolic acid is possibly related to helvolic acid in the same (uncertain) way Diels' hydrocarbon is related to cholesterol. The crystal structure of methyl helvolate (II) is of the common cholesterol type[23] with space group $P2_1$. The molecular dimensions of II are similar to those of coprostane except that II is slightly wider and thicker. Thus although no piece of evidence taken alone places helvolic acid in the steroid class, this classification is supported by: (1) the presence of four carbocyclic rings in the molecule, (2) its degradation to a Diels-like hydrocarbon, and (3) the geometry of its unit cell.

Part Structures for Helvolic Acid.—The unsaturated ketone structure (A) must clearly be at the end of the steroid side chain. Helvolic acid contains one more carbon atom than does cholesterol, and such additional atoms are generally found as alkyl groups at C-24, as in ergosterol. The ultraviolet absorption spectra of I, II and of the dinitrophenylhydrazone of the latter suggest, however, that a hydrogen atom rather than an alkyl group occupies this position. On the other hand the infrared spectrum of I shows no band near



IV

(23) D. Crowfoot, "Vitamins and Hormones," Vol. II, Academic Press, Inc., New York, N. Y., 1944, p. 409.

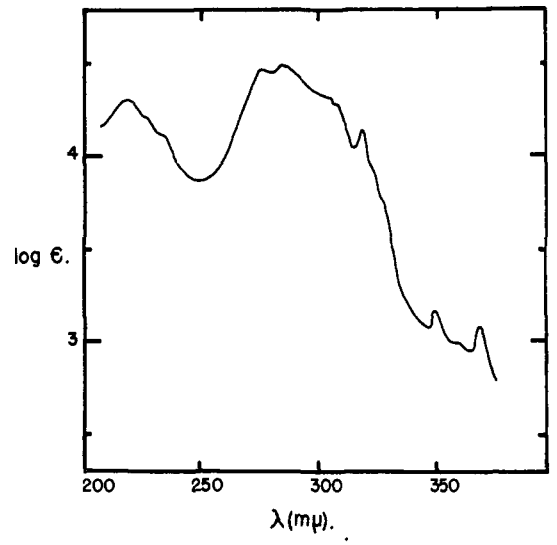
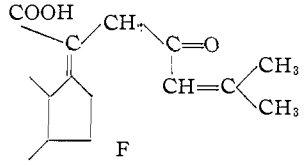


Fig. 2.—Ultraviolet absorption spectrum in 95% ethanol (Cary spectrophotometer, model 11 PMS) of dehydrogenation product of helvolic acid.

12μ, characteristic of a trisubstituted olefin. In an ordinary steroid with the end of the side chain accounted for, C-21 is the remaining position that can carry a carboxyl which is α,β-unsaturated. A carboxyl at this position is known in eburicoic acid (IV) and related compounds.[24] Possibly the carboxyl is so located in helvolic acid (I). This part structure (F) leaves the location of the extra carbon of I in doubt.



F

When helvolic acid was melted (*ca.* 210°), acetic acid was evolved (0.87 equivalent), and the residue could not be crystallized but could be sublimed to give a glass which is designated as pyrohelvolic acid. Methyl pyrohelvolate (diazomethane plus pyrohelvolic acid) and the free acid were both labile to air and analyzed low in carbon and hydrogen. The ester readily reduced with palladium and hydrogen (three moles) to give methyl hexahydropyrohelvolate which although amorphous gave a good analysis. The ultraviolet spectral properties of these three compounds proved informative. Pyrohelvolic acid possessed λ_{max} 232, ϵ 18,400, similar to I which indicated that in the former both the unsaturated ketone and unsaturated acid absorbing units remained intact. However, new bands appeared in pyrohelvolic acid: λ_{max} 299, ϵ 6,200; λ_{max} 287, ϵ 8,200; λ_{max} 275, ϵ 6,900. The number of bands and their relative positions and intensities are indicative of pyrohelvolic acid carrying a homoannular diene structural unit, similar to that in ergosterol but bearing one additional substituent.[25] Methyl hexahydropyrohelvolate possessed the anticipated spectrum, λ_{max} 220, ϵ 7,450.

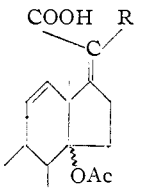
(24) J. M. Guider, T. G. Halsall, R. Hodges and E. R. H. Jones, *J. Chem. Soc.*, 3234 (1954).

(25) L. Dorfman, *Chem. Revs.*, **53**, 47 (1953).

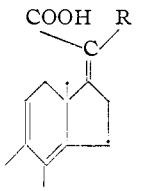
The three moles of hydrogen absorbed destroyed the conjugated diene and the double bond of the α,β-unsaturated ketone, leaving the α,β-unsaturated ester chromophore.

Dihydrohelvolic acid upon pyrolysis likewise formed a diene detected by its characteristic ultraviolet spectrum. Since tetrahydrohelvolic acid did not form any diene under these conditions, the isolated double bond in helvolic acid became one of the double bonds in the diene. Methyl helvolate (II) did not eliminate acetic acid, even at 300°, so the free carboxyl is evidently required for the reaction. Heating of this ester (II) at 300° with sebacic acid again resulted in no pyrolysis. Thus the carboxyl group of helvolic acid appears to serve an intramolecular catalytic role in promoting the loss of acetic acid from the molecule.

Tetrahydrohelvolic acid also lost acetic acid when heated above its melting point although it did not form a conjugated diene in so doing. Although the isolated double bond in helvolic acid is one of the bonds (possibly migrated) comprising the diene in pyrohelvolic acid, the elimination is not dependent on the presence of that particular bond but instead is dependent on the free carboxyl group. A possible explanation for these facts is that the double bond of the conjugated acid migrates to a β,γ-position when the acid is heated, placing it β to a tertiary acetoxyl group which then eliminates. Further double bond migrations then occur to give the diene and the α,β-unsaturated acid function. In agreement with these conclusions, helvolic and dihydrohelvolic acids give positive Lieberman–Burchard tests, while the esters and tetrahydrohelvolic acid give negative tests. A tentative disposition of these structural features in the steroid nucleus is set forth in G for helvolic acid and H for pyrohelvolic acid.



G

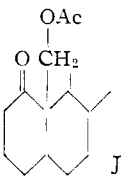


H

The most intense λ_{max} of the diene in pyrohelvolic acid occurs at 287 mμ (compared to 280 mμ for ergosterol), a fact suggesting this diene occupies the positions shown in H. The ultraviolet spectrum of such a system has not been reported, but the calculated value of λ_{max} is 293 mμ.[26] The isolated double bond in helvolic acid itself has, in general, the properties expected for a Δ^{11}-double bond,[27] including the infrared spectral (single) band of medium intensity at 13.10μ. This band is absent in the spectrum of methyl tetrahydrohelvolate. The contribution of this double bond to the molecular rotation (−17°) is not in good agreement with the expected value (+33°),[28] but the atypical structure of helvolic acid makes such small deviations insignificant.

Alternative structures for G and H would carry the isolated double bond at Δ^4 and the diene at $\Delta^{5,7}$. Such a formulation is grossly inconsistent with the contribution (−121°) the diene makes to the molecular rotation of methyl pyrohelvolate. The contribution of such a diene is usually about −538°. An additional fact that favors the diene of pyrohelvolic acid being placed other than at $\Delta^{5,7}$ is its failure to react with maleic anhydride as does ergosterol.[29]

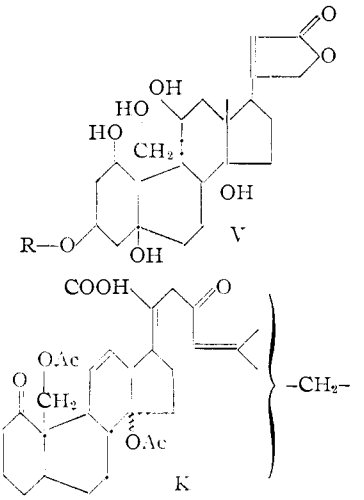
Helvolinic acid also lost acetic acid and formed a conjugated diene upon pyrolysis. The infrared spectrum of pyrohelvolinic acid thus obtained indicated that the carboxyl and the strongly hydrogen-bonded hydroxyl groups were unaffected by the reaction. Clearly the keto group in E is different from that in A, and E must be placed in the steroid nucleus in such a way as not to conflict with either A or F. Since the acetoxyl of E (or the corresponding hydroxyl) showed no tendency to eliminate upon pyrolysis, it seems probable that neither R or R′ of E is hydrogen. These facts taken in conjunction with the hindered character of the ketone of E suggest that the ketone function occupies carbon 1, and the acetoxyl carbon 19, as in structural fragment J. Natural steroids



J

oxygenated at C-1 and C-19 are uncommon but known. An example is the cardiac aglycone ouabagenin[30] (V).

The infrared spectra of helvolic acid, its derivatives and reduction products were all examined,



K

(26) Fieser (ref. 28, p. 187) calclated 293 mμ for this chromophore, assuming that the effect of a double bond exocyclic to a five-membered ring is equivalent to that of a double bond exocyclic to a six-membered ring. This assumption has been borne out; H. S. French and L. Wiley, THIS JOURNAL, **71**, 3702 (1949).

(27) H. B. Henbest, G. D. Meakins and G. W. Wood, *J. Chem. Soc.*, 800 (1954).

(28) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1949, p. 210.

(29) A. Windaus and A. Luttringhaus, *Ber.*, **64**, 850 (1931).

(30) (a) R. P. A. Sneeden and R. B. Turner, THIS JOURNAL, **77**, 130 (1955); (b) T. Reichstein and O. Schindler, *Helv. Chim. Acta*, **38**, 147 (1955), and earlier papers of this series.

and they gave considerable support to the argument for the various functions of A–H. This evidence is set forth in the Experimental.

Structure K summarizes the argument presented for the structure of helvolic acid. This structure is of course provisional, and serves more as a vehicle for focusing the data than as a structure unequivocally demanded by the facts.

Experimental Part[31]

Infrared Spectra.—The spectrum of helvolic acid (I) showed general absorption in the 3–4 μ region characteristic of a carboxyl group. This absorption disappeared when compound I was esterified to give II. No O–H stretching band was observed in either I or II. A strong band at 8.05 μ (I and II) is assigned to the acetoxyl groups, whereas the strong bands at 7.92, 8.20 and 9.64 μ are probably due to various C–O motions. The bands at 7.3 μ are not sufficiently resolved to be useful.

A comparison of the spectra in the 5–6 μ region was made for II and derived compounds. Since many of the bands could not be resolved, their integrated intensities (only approximate) were used for interpretive purposes (see Table I). The bands shown by methyl helvolate can be assigned: *a*, acetoxyl; *b*, unresolved multiplet due to acetoxyl, unsaturated methyl ester and isolated ketone; *c*, unsaturated ketone. In the dihydroester, the unsaturated ketone has been saturated, and *c* has moved to lower wave length and is detectable only as an enhanced absorption of *b*. Since conversion of the di- to the tetrahydroester involved saturation of an isolated double bond, the two compounds should and do possess identical spectra in this region. In the hexahydroester, a decreased intensity in *b* is noted, a fact consistent with the reduction of a ketone to an alcohol. It is not possible to determine with certainty from the spectra which of the two keto groups in the molecule was reduced here. However, in view of the reactivity of one toward carbonyl reagents originally (part of structure A) and the lack of reactivity of the other (that of structure E), the former is probably the one reduced. This part of the spectrum shows no detectable change when the hexahydroester is further reduced. Since in the last reduction a double bond becomes saturated, the only large change expected is a decrease in the wave length of the carbonyl band of the ester conjugated with it. This change is not detectable because of the intensity of band *b*.

TABLE I

Methyl ester	Relative band area *a*	*b*	*c*	λ_{max}, μ *a*	*b*	*c*
Helvolate	1.0	2.0	1.1	5.72	5.80	5.96
Dihydrohelvolate	1.0	2.5	0.0	5.73	5.83	..
Tetrahydrohelvolate	1.0	2.5	.0	5.73	5.83	..
Hexahydrohelvolate	1.0	1.8	.0	5.74	5.82	..
Octahydrohelvolate	1.0	1.8	.0	5.74	5.80	..
Helvolinate	0.0	2.1	1.0	..	5.81	5.93
Pyrohelvolate	1.5	0.7	1.0	5.72	5.81	5.98
Hexahydropyrohelvolate	1.0	1.0	0.0	5.72	5.85	..

Methyl helvolinate differs in this portion of the spectrum from methyl helvolate in that the band at 5.72 μ has vanished, a fact in accord with the loss of an acetoxyl group.

The spectrum of methyl pyrohelvolate is essentially as expected. Evidently one of the bands, which in methyl helvolate contributed to band *b*, is in this compound not resolved from *a*. The sum of intensities of *a* and *b* is appreciably less relative to *c* in the pyroester than in II, and clearly a carbonyl (acetoxyl) has been lost in the latter. The group lost here is not the same acetoxyl lost in the saponification of helvolic to helvolinic acid. Methyl hexahydropyrohelvolate is comparable with methyl tetrahydrohelvolate, except one acetoxyl band is gone, and one of the bands contributing to *b* in the latter now contributes to *a*.

Helvolic Acid (I).—The sample of the natural product available to us was found to be about 90% pure, m.p. 199–203°, $[\alpha]^{25}D$ −126.2° (chloroform 0.8). The bulk of the material was purified by recrystallizing it three times from aqueous acetic acid. The material thus obtained was shown to be homogeneous by chromatographing a 1-g. sample on 30 g. of neutral alumina (activity III)[32] with chloroform followed by chloroform–acetic acid. Only one compound was evident, and recrystallization of the individual fractions furnished material identical with that which was obtained directly by crystallization. The pure compound was obtained as fine needles, m.p. 211.3–212.1° dec., $[\alpha]^{25}D$ −124° (chloroform 1). The reported physical constants are $[\alpha]^{25}D$ −132°,[3b] m.p. 220, 226,[4a] 212,[3a] and 215–220°.[3b] The ultraviolet spectrum[31] showed maxima as follows: λ_{322}, ϵ 98; λ_{231}, ϵ 17,300.

Titration of 30-mg. samples gave equivalent weights of 500–510. A sample was allowed to stand at room temperature for 12 hours with 3 equivalents of base, and the saponification equivalent was then determined as 277. Saponification with 1 *N* potassium hydroxide in diethylene glycol at 140° for 5 minutes gave saponification equivalents of 183 and 186. The theoretical values for the uptake of one, two and three moles of base are, respectively, 555, 277 and 185.

Helvolic acid gave a strong red color in the Liebermann–Burchard test,[33] an orange color in the Zimmermann test,[34] a yellow color with tetranitromethane,[35] and it failed to consume periodate.[36]

Anal. Calcd. for $C_{32}H_{42}O_8$: C, 69.28; H, 7.63. Found: C, 69.14, 69.60, 68.95; H, 7.57, 7.34, 7.46; C-methyl, 9.80% or 3.62 CH_3–C/mole.

The semicarbazone was prepared by heating 58 mg. of I in a filtered solution of semicarbazine in alcohol for 24 hours. Three crystallizations of the product from aqueous ethanol gave tiny needles, m.p. 215–216° dec. The ultraviolet spectrum of the semicarbazone showed λ_{max} 277, ϵ 15,300; λ_{max} 230, ϵ 13,600.

Anal. Calcd. for $C_{33}H_{45}O_8N_3$: C, 64.79; H, 7.41; N, 6.87. Found: C, 64.71; H, 7.64; N, 6.70.

Reaction of Helvolic Acid (I) with 2,4-Dinitrophenylhydrazine Reagent.—When a sample of I was treated with the 2,4-dinitrophenylhydrazine reagent and the solution was allowed to stand, a red gel separated. After 1 hour this material was filtered, and after the solution had stood for several hours, yellow-orange needles were obtained, m.p. 185.5–186.0°. The ultraviolet spectrum of this material showed λ_{max} 372.5, ϵ 18,300; λ_{max} 252, ϵ 9,800; λ_{max} 218, ϵ 16,300.

Anal. Calcd. for $C_{38}H_{46}O_{11}N_4$: C, 62.11; H, 6.31. Found: C, 61.26; H, 6.69.

Helvolic Acid Dibromide.—To 42 mg. of I in 10 ml. of chloroform was added dropwise one equivalent of bromine in chloroform. The solvent was removed and the residue was taken up in 3 ml. of benzene. The addition of 10 ml. of pentane caused the precipitation of a white powder, m.p. 153–155° dec. Crystallization of this material from benzene–hexane gave white crystals, m.p. 153.5–154.5° dec.

Anal. Calcd. for $C_{32}H_{42}O_8Br_2$: C, 53.70; H, 5.90. Found: C, 52.53; H, 6.40.

Ozonolysis of Helvolic Acid (I).—A stream of ozone and oxygen was passed into a solution of 35 mg. of I in 10 ml. of methylene chloride at 0° until the absorption of ozone was incomplete. The resulting solution was added to 10 ml. of boiling water containing 50 mg. of zinc dust and a trace of silver nitrate. After cooling the mixture, the zinc was collected and the phases of the filtrate were separated. The aqueous phase was treated with the 2,4-dinitrophenylhydrazine reagent to give 10 mg. of a yellow powder, m.p. 110–116°. After three recrystallizations of the material from aqueous ethanol, 2 mg. of yellow needles was obtained, m.p. 124.5–125.1°, undepressed by admixture with an au-

(31) All m.p.'s are corrected. Ultraviolet spectra were determined on a Cary recording spectrophotometer, model 11 PMS, in 95% ethanol, and λ's are recorded in mμ. The infrared spectra were recorded on a Perkin–Elmer recording spectrophotometer, model 21, 10% solutions in chloroform unless otherwise specified. Optical rotations were taken in chloroform (concn. 0.5–1.0).

(32) H. Brockmann and H. Schodder, *Ber.*, **74B**, 73 (1941).

(33) Reference 28, p. 100.

(34) Reference 28, p. 101.

(35) I. Ostromisslensky, *J. prakt. Chem.*, **84**, 489 (1911).

(36) E. L. Jackson, "Organic Reactions," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 341.

thentic sample of acetone 2,4-dinitrophenylhydrazone (m.p. 124.6–125.2°).

p-Bromophenacylhelvolate.—Sodium helvolate was converted to its *p*-bromophenacyl ester in the usual manner.[37] The ester was chromatographed on alumina (activity I)[32] with chloroform, and the center fraction was twice recrystallized to furnish needles, m.p. 201–202.5°.

Anal. Calcd. for $C_{40}H_{47}O_9Br$: C, 63.91; H, 6.30; Br, 10.63. Found: C, 63.55, 63.91; H, 6.28, 6.47; Br, 10.36, 10.16.

Methyl Helvolate (II).—The acid I was esterified with an excess of diazomethane in methylene chloride at 0° for 15 minutes. The crude ester obtained by evaporation of the solvent was crystallized from aqueous methanol and was obtained as fine needles, yield 91%, m.p. 262.0–262.6°, $[\alpha]^{26}D$ −134° (chloroform, 0.8); reported[3b] m.p. 260–261°, $[\alpha]^{25}D$ −150°. The ultraviolet spectrum showed λ_{max} 321, ϵ 87; λ_{max} 232, ϵ 17,400. In contrast to the free acid, the ester gave only an insignificantly weak brown color in the Liebermann–Burchard test. An attempt was made to pyrolyze II, but after heating at 300° for 15 minutes (under nitrogen), no change was observed, and the starting material was recovered unchanged.

Anal. Calcd. for $C_{33}H_{44}O_8$: C, 69.69; H, 7.80; mol. wt., 569. Found: C, 69.78, 70.15, 70.20; H, 7.91, 8.05, 7.84; mol. wt. (Signer), 585. Calcd. for $C_{32}H_{41}O_7$–OCH_3: –OCH_3, 5.46. Found: –OCH_3, 5.99, 5.21.

The 2,4-dinitrophenylhydrazone of II was formed in the usual way and was recrystallized from alcohol to yield an orange powder, m.p. 245–250° dec. The ultraviolet spectrum showed λ_{max} 376, ϵ 30,400; $\lambda_{shoulder}$ 256, ϵ 15,500 (chloroform).

Anal. Calcd. for $C_{39}H_{48}O_{11}N_4$: C, 62.55; H, 6.46; N, 7.48. Found: C, 62.42, 63.04; H, 6.41, 6.59; N, 7.23.

Methyl Dihydrohelvolate (VI).—Methyl helvolate (II), 254 mg., was hydrogenated in 40 ml. of absolute methanol using 12 mg. of 10% palladium-on-carbon catalyst at room temperature and atmospheric pressure. After 45 minutes one mole of hydrogen had been taken up and the reaction was stopped. The catalyst was removed and the solution was concentrated to a volume of 20 ml. After addition of a little water to the hot solution, the product crystallized as fine needles, wt. 226 mg. (89%), m.p. 214.6–215.5°, $[\alpha]^{25}D$ −82.2° (chloroform, 0.9). The compound gave a yellow color with tetranitromethane. The ultraviolet spectrum showed a broad, flat maximum at 212–220 mμ, ϵ 8,600 and λ_{max} 312, ϵ 58. Pyrolysis of VI gave a diene which was detected by its ultraviolet spectrum.

Anal. Calcd. for $C_{33}H_{46}O_8$: C, 69.45; H, 8.12. Found: C, 69.39, 69.45; H, 8.24, 8.34.

Ozonolysis of VI was carried out in a manner similar to that described for I. No 2,4-dinitrophenylhydrazone could be formed from the aqueous phase after treatment of the ozonolysis mixture with zinc dust.

Methyl Tetrahydrohelvolate (VII).—Hydrogenation of II, 1.78 g., was carried out as described for the preparation of VI but was allowed to proceed until the hydrogen uptake ceased. Under these conditions two moles of hydrogen were absorbed. The catalyst was removed from the solution by filtration and, after addition of a little water to the filtrate, the product VII crystallized and was obtained as fine needles (methanol), m.p. 208.2–208.9° (no melting point depression with VI), wt. 1.60 g. (90%), $[\alpha]^{25}D$ −79.2° (chloroform 0.8). The compound showed no color with tetranitromethane. Pyrolysis of 10.7 mg. of VII at 220° for 10 minutes under vacuum gave 0.154 meq. (81%) of the theoretical amount of acetic acid by titration. Another sample of VII (95 mg.) was held at 260° for one hour during which time a slow stream of nitrogen was passed through the reaction vessel and then through a barium hydroxide solution. The barium carbonate was removed by filtration and, after drying, the substance weighed 15.3 mg. (47%). The infrared spectrum of the residue was determined, and it showed no trace of a carboxyl group in the 3–4 μ region. No olefin band was observed in the 10–12 μ region. The ultraviolet spectrum of VII showed λ_{max} 219.5, ϵ 8,500; λ_{max} 311; ϵ 64.

(37) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1948, p. 157.

Anal. Calcd. for $C_{33}H_{48}O_8$: C, 69.20; H, 8.45. Found: C, 69.35, 69.45; H, 8.96, 8.64.

When the compound was treated with ozone in the usual manner, it was recovered unchanged.

The 2,4-dinitrophenylhydrazone of IV was prepared in the usual way, and after recrystallization from methanol was obtained as a yellow-orange powder, m.p. 262.2–263.0° dec. The ultraviolet spectrum showed λ_{max} 361.5, ϵ 24,300 (chloroform).

Anal. Calcd. for $C_{39}H_{52}O_{11}N_4$: C, 62.22; H, 6.96. Found: C, 62.50; H, 7.18.

Methyl Hexahydrohelvolate (VIII).—A sample of VII, 57 mg., was hydrogenated as previously described, only 10 mg. of PtO_2 was used as catalyst. The hydrogen uptake ceased after 4 hours, one mole being consumed. The product was isolated as usual, needles from methanol, m.p. 169.8–170.5° with previous sintering, $[\alpha]^{25}D$ −87.2° (chloroform, 0.4). The ultraviolet spectrum of VIII was identical with that of VII.

Anal. Calcd. for $C_{33}H_{50}O_8$: C, 68.96; H, 8.77. Found: C, 69.20, 69.15; H, 9.14, 8.72.

Attempts to prepare the dinitrophenylhydrazone of VIII under the conditions used above gave no reaction, the starting material being recovered unchanged. After prolonged standing with dinitrophenylhydrazine reagent (several days), the starting material could not be recovered, but there was no evidence for derivative formation.

An acetate of VIII was obtained readily. Compound VIII (26 mg.) was dissolved in 2 ml. of dry pyridine and, after cooling the solution to 0°, 2 ml. of acetic anhydride was added. The solution was left at room temperature for 18 hours and was then diluted with water. The crystals were collected, wt. 24 mg., m.p. 216–220°. Recrystallization of this material from aqueous methanol furnished 20 mg. of white needles, m.p. 219.3–220.6°.

Anal. Calcd. for $C_{35}H_{52}O_9$: C, 68.16; H, 8.50. Found: C, 68.55; H, 8.62.

Methyl Octahydrohelvolate (IX).—Ester VIII (175 mg.) was hydrogenated with 20 mg. of PtO_2 in 10 ml. of pure acetic acid, one mole of hydrogen being absorbed. The solution was filtered and frozen, and the solvent was removed by lyophilization. The residual white crystalline solid (IX) had m.p. 78–91°. The compound was crystallized from methanol at −80°, and yielded a white solid, m.p. 87–91°. The ultraviolet spectrum showed λ_{max} 317, ϵ 52, and end absorption, λ_{230}, ϵ 270; λ_{220}, ϵ 594; λ_{215}, ϵ 910; λ_{210}, ϵ 1,190; λ_{205}, ϵ 1,440.

Anal. Calcd. for $C_{33}H_{52}O_8$: C, 68.72; H, 9.08. Found: C, 69.23; H, 9.25.

Dihydrohelvolic Acid.—Hydrogenation of helvolic acid was carried out as described for hydrogenation of the ester. The dihydro acid was obtained as white needles from aqueous acetic acid, m.p. 198.5–200° dec., yield 77%. The compound gave a deep red color in the Liebermann–Burchard test.

Anal. Calcd. for $C_{32}H_{44}O_8$: C, 69.04; H, 7.97. Found: C, 68.90; H, 8.37.

Tetrahydrohelvolic Acid.—The tetrahydro acid was obtained from I by hydrogenation as described for the preparation of the corresponding ester VII. Crystallization from aqueous methanol gave white needles in 85% yield, m.p. 195–196.5°, negative Liebermann–Burchard test.

Anal. Calcd. for $C_{32}H_{46}O_8 \cdot H_2O$: C, 66.64; H, 8.39. Found: C, 66.59; H, 8.07.

Hexahydrohelvolic Acid.—The tetrahydro acid was converted to the hexahydro acid under the conditions described for the preparation of the corresponding ester. The product was obtained as white needles from aqueous methanol, m.p. 192.5–193.5°, yield 90%.

Anal. Calcd. for $C_{32}H_{48}O_8$: C, 68.54; H, 8.63. Found: C, 68.47; H, 8.65.

Octahydrohelvolic Acid.—The hexahydro acid (44 mg.) was reduced to the octahydro derivative as described for the corresponding ester. The crude product was crystallized twice from acetic acid–water to give 8 mg. of chunky crystals of octahydrohelvolic acid, m.p. 214–220° (literature reported m.p. 223.5–224.5°[4b]). The ultraviolet absorption spectrum gave λ_{max} 317, ϵ 48, and end absorption: λ_{240}, ϵ 130; λ_{230}, ϵ 360; λ_{220}, ϵ 670; λ_{210}, ϵ 1,090.

Helvolinic Acid (X).—Helvolic acid, 184 mg., was dissolved in 10 ml. of 0.1 *N* sodium hydroxide, and the solution was held at 40° for 5 minutes. The solution was then cooled and acidified with dilute hydrochloric acid. The solid was collected and was crystallized from aqueous acetic acid as needles, m.p. 196–197.5° dec., wt. 145 mg. (85%), $[\alpha]^{24}D$ −86.9° (chloroform, 0.96). The ultraviolet spectrum had λ_{max} 232, ϵ 16,300; λ_{max} 324, ϵ 76. The infrared spectrum showed an exceedingly strong, sharp band at 2.94 μ, and a typical broad carboxyl band in the 3–4 μ region. The compound showed no reaction with periodate or bismuth trioxide, and it gave no precipitate with digitonin.

Repeated attempts to prepare a *p*-bromophenacyl ester led only to gummy products.

Anal. Calcd. for $C_{30}H_{40}O_7 \cdot {}^1/_2H_2O$: C, 69.07; H, 7.92. Found: C, 69.23, 69.37, 69.07; H, 7.75, 8.22, 7.75.

Attempts (see below) were made to oxidize the primary hydroxyl group of helvolinic acid to a carbonyl group, but these attempts failed. A previous instance of the failure of a 19-hydroxy steroid to react under these conditions has been recorded.[38]

Helvolinic acid (32 mg.) was dissolved in 5 ml. of pyridine containing 29 mg. of chromic oxide. After 18 hours, the mixture was diluted with water and the solid was collected. Crystallization of this material provided 26 mg. of starting material, m.p. 190–191°. The infrared spectrum of this material was identical with that of helvolinic acid.

To ascertain the nature of any volatile product resulting from the above hydrolysis, the acidified filtrate from the saponification was distilled. The first 5 ml. of distillate was neutralized and was treated with *p*-phenylphenacyl bromide, 50 mg., in 20 ml. of ethanol. The product was obtained by dilution of the solution with water and crystallization, wt. 50 mg., m.p. 96–100°. Two recrystallizations of this material from ethanol raised the m.p. to 105.6–107.8°. An authentic sample of *p*-phenylphenacyl acetate had m.p. 110.0–110.4°, and the mixed melting point was 107.8–109.3°.

Hexahydrohelvolinic Acid.—Hexahydrohelvolic acid (42 mg.) was allowed to stand with 2.5 equivalents of dilute aqueous sodium hydroxide for 18 hours at room temperature. Titration gave a saponification equivalent of 266. The product of the reaction was isolated and crystallized from acetic acid. There was obtained 35 mg. of a fine white powder, m.p. 192.5–193.5°. The infrared spectrum showed a strong, sharp band at 2.96 μ.

Anal. Calcd. for $C_{30}H_{46}O_7 \cdot {}^1/_2H_2O$: C, 68.28; H, 8.98. Found: C, 68.81; H, 8.75.

Methyl Helvolinate.—Helvolinic acid (26 mg.) was esterified with diazomethane in methylene chloride at 0° for 10 minutes. After evaporation of the solvent *in vacuo*, the residue was crystallized from aqueous methanol. The fine needles were collected, wt. 21 mg., m.p. 202.7–203.5°, $[\alpha]^{25}D$ −100.0° (chloroform, 0.66). The infrared spectrum showed a sharp strong band at 2.94 μ.

Anal. Calcd. for $C_{30}H_{39}O_6$–OCH_3: C, 70.69; H, 8.04; $-OCH_3$, 5.89. Found: C, 70.78, 71.10; H, 8.02, 7.80; $-OCH_3$, 5.62.

Methyl helvolinate was also prepared by the hydrolysis of methyl helvolate, and the compound obtained in that way was identical in all respects with the sample described above.

The dinitrophenylhydrazone was prepared in the usual way and was recrystallized from aqueous ethanol. The compound was obtained as orange needles, m.p. 155–157°. The ultraviolet spectrum showed λ_{max} 374, ϵ 30,200; $\lambda_{shoulder}$ 256, ϵ 16,700; λ_{max} 220, ϵ 26,700.

Anal. Calcd. for $C_{37}H_{46}O_{10}N_4$: C, 62.87; H, 6.56; N, 7.93. Found: C, 62.67, 62.78; H, 6.56, 6.76; N, 7.77.

Methyl Helvolinate Acetate.—Methyl helvolinate (24 mg.) was dissolved in 0.5 ml. of dry pyridine and, after cooling the solution to 0°, 0.5 ml. of acetic anhydride was added. The resulting solution was allowed to stand at room temperature for 24 hours, and was then diluted with water. The crystals were filtered and recrystallized from aqueous methanol to furnish fine needles, 15 mg., m.p. 263.5–265°. The infrared spectrum of the compound was identical with that of II, and the two samples showed no mixed melting point depression.

(38) G. W. Barber and M. Ehrenstein, *J. Org. Chem.*, **20**, 1253 (1955).

Anal. Calcd. for $C_{33}H_{44}O_8$: C, 69.69; H, 7.80. Found: C, 69.95; H, 7.79.

Methyl Tetrahydrohelvolinate.—Hydrogenation of methyl helvolinate with palladium-on-carbon in methanol in the usual way furnished the tetrahydro derivative as a crystalline powder (methanol), m.p. 122–125°, yield 80%.

Anal. Calcd. for $C_{30}H_{43}O_6$–OCH_3: C, 70.16; H, 8.74; $-OCH_3$, 5.85. Found: C, 70.17; H, 8.87; $-OCH_3$, 5.38.

Reaction of Helvolic Acid with Excess Base.—Twenty mg. of helvolic acid was heated under reflux for one hour in a solution containing 20 mg. of potassium hydroxide in 2 ml. of water. The cooled solution was acidified and the solid was collected, m.p. 113–116° dec. It did not prove possible to obtain a crystalline sample of this material, so the washed and dried precipitate was analyzed directly. The ultraviolet spectrum showed λ_{max} 223, ϵ 17,900.

Anal. Calcd. for $C_{28}H_{38}O_6$: C, 71.46; H, 8.14. Found: C, 71.81, 71.60; H, 7.85, 7.91.

A similar hydrolysis was carried out with a 170-mg. sample of helvolic acid. After removal of the solid from the acidified solution, the filtrate (total volume 15 ml.) was partially distilled and three 3-ml. fractions were collected. The *p*-bromophenacyl ester was formed from each fraction; the melting points were in the range 71–75°. The combined crude *p*-bromophenacyl esters were chromatographed on aluminum with ether. There was obtained 23 mg. of *p*-bromophenacyl acetate, m.p. and mixed m.p. 82–84° and 3 mg. of lower melting material.

Methyl Pyrohelvolate (XI).—Helvolic acid, 558 mg., was converted to the pyro acid by heating at 230° for 10 minutes at a pressure of 5 mm. The acetic acid evolved was collected in a Dry Ice trap, and titration showed that the amount collected amounted to 87% of the theoretical.

The identification of the distillate as acetic acid was made on material obtained from a similar run. The sample had m.p. 15–18°, and its smell and infrared spectrum were identical with those of an authentic sample.

The pyro acid was obtained as a yellow glass, m.p. 122–127°. The ultraviolet spectrum showed the following maxima: λ_{max} 299.5, ϵ 6,200; λ_{max} 287, ϵ 8,200; λ_{max} 275, ϵ 6,900; λ_{max} 231, ϵ 18,400. A small sample of the compound was readily sublimed at 250° (2 mm.). The ultraviolet spectrum of the sublimate was unchanged, m.p. 107–110°. Attempts to crystallize this material were unsuccessful.

Anal. Calcd. for $C_{30}H_{38}O_6$: C, 72.84; H, 7.74. Found: C, 71.88; H, 7.67.

Pyrohelvolic acid (5.0 mg.) and 4 mg. of maleic anhydride were heated at 180° for 5 minutes. The mixture was cooled and its ultraviolet spectrum was examined. No observable change in the 260–330 mμ region was noted, and thus little or no Diels–Alder reaction could have occurred.

The bulk of the material was converted to the methyl ester with diazomethane. Evaporation of the solvent gave XI as a colorless glass, m.p. 78–81°, yield 96%. The ultraviolet spectrum of the ester was identical with that of the acid. The material was freed of solvent by heating at 100° at a pressure of 1 mm. for several hours. The analysis of the ester was not satisfactory and, since the sample stood for some weeks before being analyzed, it is suspected that the low carbon value found is due to air oxidation of the diene system; $[\alpha]^{25}D$ −103.6 (chloroform, 1.1).

Anal. Calcd. for $C_{31}H_{40}O_6$: C, 73.20; H, 7.93. Found: C, 72.18; H, 8.02.

Methyl Hexahydropyrohelvolate.—A freshly prepared sample of ester XI, 297 mg., was hydrogenated with 20 mg. of palladium-on-carbon in 20 ml. of methanol. The total hydrogen uptake was 36.1 ml. (86% of three moles), and was complete in 12 hours. After filtration of the solution, the product was taken up in ether, and the ether phase was washed free of methanol and was dried. Removal of the solvent gave the product as a white foam, m.p. 83–87°. The ultraviolet spectrum of this material showed the diene system had been lost, λ_{max} 220, ϵ 7,450; $[\alpha]^{25}D$ +79.4° (chloroform, 0.4).

Anal. Calcd. for $C_{31}H_{46}O_6$: C, 72.34; H, 9.01. Found: C, 72.49; H, 9.00.

Pyrolysis of Dihydrohelvolic, Tetrahydrohelvolic and Helvolinic Acids.—The pyrolyses of these compounds were carried out as with helvolic acid, about 2 mg. of compound

being used. The residue was in each case a glass. The ultraviolet spectra of these glasses were determined and those of dihydrohelvolic and helvolic acids were qualitatively the same as the spectrum of pyrohelvolic acid in the 260–300 mμ region. Pyrotetrahydrohelvolic acid showed no absorption in this region. The pyrolyses were then repeated with 10–30 mg. samples. The volatile distillate from helvolinic acid was isolated, and its infrared spectrum proved to be identical with that of acetic acid. The distillate from another sample of helvolinic acid was titrated and found to correspond to 96% of one mole of acetic acid per mole of helvolinic acid taken. A similar titration showed that tetrahydrohelvolic acid evolved 81% of one mole of acetic acid under these conditions.

Selenium Dehydrogenation of I.—A 24.0-g. sample of I was first reduced to the octahydro derivative with 1 g. of platinum oxide in 60 ml. of acetic acid. The reduction was complete in 3 hours and, after removal of the catalyst by filtration, the solvent was distilled in vacuum. The residual white froth was powdered and the powder was added portionwise to a suspension of 9 g. of lithium aluminum hydride in 800 ml. of ether. The mixture was heated under reflux for 3 hours, and the complex was then decomposed by the addition of 10 ml. of ethyl acetate followed by water. After the proper amount of water was added, the ether phase was decanted from the solid sludge and the solvent was evaporated. The residue was dried at 100° under vacuum, and then was mixed with 21 g. of selenium powder. The mixture was kept at 150–250° for one hour and then at 360–370° for 28 hours under a nitrogen atmosphere. The cooled residue was extracted with ether and, after filtration of the solution, the volume was reduced to 5 ml. The concentrated solution was then placed on a column containing 10 g. of neutral alumina of activity I,[32] and the material was eluted with pentane, then pentane–ether, 20-ml. fractions being collected. The first three fractions contained oils which were not investigated. Fractions 4 and 5 contained a total of about 150 mg. of a brown semi-solid. Later fractions contained only tarry materials and were not investigated. Fractions 4 and 5 were combined and rechromatographed on a similar column of alumina with 10% ether in pentane. In this case fractions 2–4 contained about 50 mg. of a gummy yellow solid. Ten ml. of ethanol was added to the solid and, after warming, the solution was decanted from the insoluble tar. The solution was diluted with a little water and allowed to cool. The yellow solid was collected by filtration, wt. 15 mg., m.p. 170–195°. This material was recrystallized and gave 7 mg. of a yellow solid, m.p. 195–210°. The ultraviolet spectra were determined for both of the samples and were identical (Fig. 2).

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C^{14} Isotope Effect on the Ion-exchange Chromatography of Amino Acids

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The ion-exchange chromatography on Dowex 50 of amino acids labeled with C^{14} was studied. Labeling of the carbon adjacent to an ionized atom, such as the 1- or 2-position in an α-amino acid, resulted in slower movement of the labeled molecules on the column with the result that the specific activity of successive fractions within the amino acid band increased. The presence of the isotope in positions separated from a charged center, such as serine-3-C^{14} and valine-4-C^{14}, had no effect on the ion-exchange behavior. The possibility is discussed that an inductive effect related to the heavier isotope is responsible for the observed differences.

In a preliminary communication,[1] we reported a systematic effect of C^{14}-labeling on the ion-exchange chromatography of randomly labeled[2] amino acids. The labeled amino acid molecules traveled somewhat more slowly on a column of Dowex 50 eluted with a citrate buffer (*p*H gradient) with the result that the specific activity of successive fractions within a peak was not constant but increased progressively. The fact that the magnitude of the isotope effect was related (inversely) more closely to the number of carbon atoms than to the molecular weight suggested that the effect of a C^{14} atom depended on its occupying a particular position in the molecule rather than solely on its higher mass. As here reported, this hypothesis has been confirmed by chromatographing specifically labeled amino acids.

Experimental

Ion-exchange Chromatography.—The ion-exchange methods were the same as those devised by Moore and Stein[3] except as indicated. For the separation of the acidic and neutral amino acids, *p*H gradient elution[4] of a 100 × 0.9 cm. column of Dowex 50-x12, minus 400 mesh[5] at 50° was used. The *p*H gradient was obtained by an equal level arrangement[4] employing a 300-ml. straight-sided bottle with an inside diameter of 56 ± 0.5 mm. for the mixing chamber and a 500-ml. Florence flask with a 12 cm. neck, 26 ± 0.5 mm. inside diameter, for the reservoir. The reservoir was filled with 0.25 *N* NaOH (containing 1% BRIJ 35 solution)[3] and the mixing chamber with 250 ml. of citrate buffer, *p*H 3.10 (prepared from 245 g. of sodium citrate (dihydrate), 600 g. of citric acid (monohydrate), 50 ml. of thiodiglycol, water to make 10 l. and 1% BRIJ 35 solution). Pressure was applied to both flasks to maintain a flow rate of 6 ml./hr. (about 2.5 lb. at 50 cm. below the top of the column). This provided a very gradual, slightly concave *p*H gradient, starting at *p*H 3.10 and reading *p*H 3.3 at about 200 ml. After 150 ml. of effluent had been collected, the mixing chamber was closed to provide a constant volume mixer,[4] which produced a sharply increasing gradient reaching *p*H 6 at about 330 ml. of effluent.

Collection of fractions was stopped at this point, after the emergence of phenylalanine. Elution was continued for at least 50 ml. after the effluent became alkaline. The column was washed with about 50 ml. of the *p*H 3.10 buffer before starting the next analysis.

The following specifically labeled amino acids were chromatographed in the amounts indicated (the figures showing

(1) K. A. Piez and H. Eagle, *Science*, **122**, 968 (1955).

(2) The expression "randomly labeled" is suggested as more accurate than the more common "uniformly labeled," since the latter term is true only in a statistical sense and does not necessarily apply to the individual molecule. In fact, in the usual sample of C^{14}-labeled material the level of activity is such that most of the labeled molecules contain only one C^{14} atom, randomly placed.

(3) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(4) K. A. Piez, *Anal. Chem.*, **28**, 1451 (1956).

(5) Rescreened (wet) through 200 mesh. It has been our experience that approximately 90% of the resin as received can be washed through a 200 mesh sieve with a jet of water. One lot contained only 50% that passed 200 mesh. It was not suitable for chromatography, giving very poor resolution. The cause appeared to be too narrow a size range, that is, nsufficient very fine resin to give a solidly packed column.