molecules reacting/100 eV., which agrees with the values given by Allen (1947) for organic liquids.

These observations are all indicative of an indirect action of high-voltage cathode rays on these amino-acids. For ionic yields higher than 1.00, the same explanation as given previously for histidine (Bhatia & Proctor, 1951) would probably be valid, namely, that molecular fragments of the amino-acids resulting from cathode-ray treatment competed for the free radicals.

Evolution of hydrogen sulphide from cystine indicates that the molecule of cystine is probably decomposed at the disulphide linkage.

SUMMARY

1. Aqueous solutions of L-cystine, DL-phenylalanine, L-tyrosine, and DL-tryptophan in various concentrations were irradiated with high-voltage cathode rays at doses ranging from 10^6 to 10^8 r.e.p. Determinations of the amino-acid contents of the

solutions were made microbiologically after irradiation.

2. All four amino-acids were decomposed upon irradiation, and the decomposition was related exponentially to the dose.

3. Ionic yields were determined for the four amino-acids over a wide range of concentrations.

4. Data are presented to show that the action of high-voltage cathode rays on these amino-acids took place through free radicals being intermediates.

5. Evolution of hydrogen sulphide from the cystine solution indicated that cystine was probably decomposed at the disulphide linkage upon irradiation with high-voltage cathode rays.

The authors gratefully acknowledge the co-operation of Prof. J. G. Trump and Mr K. A. Wright of the Department of Electrical Engineering, Massachusetts Institute of Technology, who placed the electrostatic generator at their disposal.

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Some Chemical Properties of Helvolic Acid

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(Received 15 October 1951)

Burton & Abraham (1952) reported that cephalosporin P₁, an antibiotic produced by a species of *Cephalosporium*, resembled helvolic acid (Chain, Florey, Jennings & Williams, 1943). Further work by Burton, Cardwell & Abraham (1951) has confirmed this resemblance and has allowed a simple interpretation of the results of a chemical investigation into helvolic acid, completed by the author in 1945 and described in this paper.

The isolation of helvolic acid from culture filtrates of *Aspergillus fumigatus* mut. *helvola* Yuill has been

described by Chain *et al.* (1943). Helvolic acid was shown to be a colourless crystalline compound, melting point 212°. It is readily soluble in most organic solvents, except light petroleum, but is insoluble in water; its sodium salt is readily soluble in water. Helvolic acid was assigned the formula C₃₈H₄₄O₈. It titrated as a monobasic acid and a monomethyl ester was prepared by the action of diazomethane.

These preliminary observations on the chemical properties of helvolic acid were confirmed and extended in the investigation now described, which was directed primarily to the discovery of the role of the oxygen atoms in the molecule.

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The helvolic acid required in the present investigation was produced by the method previously described (Chain *et al.* 1943). From a total of 5200 l. of crude culture filtrate, worked up in twenty-seven batches, 5.5 g. of pure helvolic acid were obtained. Attempts to increase this yield by varying the culture medium and the conditions of growth were unsuccessful.

An investigation of the rate of development of anti-bacterial activity, assayed by the ordinary plate-and-cylinder method used for penicillin, showed that when the mould was grown as a surface culture at 25°, in a synthetic medium of glucose and mineral salts, a peak activity was reached after about 8 days' growth. Thereafter, the activity fell considerably to a minimum at 16–18 days. This was followed by a second increase to a fairly constant value at 21 days, at which time the crop was harvested for the isolation of helvolic acid: it was found that the antibacterial activity of cultures of 6–8 days' growth was due, not to helvolic acid, but to gliotoxin. An account of this work has been published elsewhere (Glister & Williams, 1944).

Waksman, Horning & Spencer (1943) described an antibiotic, which they named fumigacin, produced by *A. fumigatus* Fres. Menzel, Wintersteiner & Hoogerheide (1944) showed that fumigacin was a mixture of gliotoxin and helvolic acid; their account of helvolic acid, as far as it goes, agrees in almost all essentials with the findings of the present author.

A point of discrepancy, however, is the value of the specific rotation. Chain *et al.* (1943) gave the value $[\alpha]_D^{25} - 49.4^\circ$ in chloroform; Menzel *et al.* gave $[\alpha]_D^{25} - 132 \pm 2^\circ$. Two separate, highly purified, samples have now been found to give $[\alpha]_D^{25} - 113^\circ$ (c, 3.1) and -117° (c, 2.6) respectively. The difference still existing may be due to contamination with gliotoxin of the material used by these workers; gliotoxin is strongly laevorotatory ($[\alpha]_D^{25} - 254^\circ$). The possibility of contamination was raised by Menzel *et al.* and not entirely disproved. Their product reduced Fehling's solution slowly at 100°. Our purified samples have no action on Fehling's solution even on heating for 1 hr. at this temperature. Gliotoxin, however, readily reduces Fehling's solution.

RESULTS

Nature of oxygen atoms. All the analytical and degradative evidence supports the formula $C_{33}H_{42-44}O_8$. For helvolic acid, Menzel *et al.* proposed a formula $C_{39}H_{38-40}O_7$; several objections to this will be discussed later.

The absence of enolic or phenolic properties in helvolic acid show that the acidic properties are due to a carboxyl group. This is confirmed by the preparation of a monomethyl ester (m.p. 262°) by the action of diazomethane on the acid. Analysis of the

ester gave results in conformity with a formula $C_{33}H_{44-46}O_8$. The molecular weight was found by an X-ray crystallographic method (Crowfoot & Low, 1943) to be 556 ± 14 ($C_{33}H_{44}O_8$ requires 570).

A monosemicarbazone and a dioxime of helvolic acid, and a monosemicarbazone of its methyl ester, have been prepared. The absence of reducing properties and the positive Zimmermann reaction indicate that at least one of the carbonyl groups is ketonic. Menzel *et al.*, using a different preparative method, obtained a monoxime.

An acetoxyl determination by the standard (acid hydrolysis) method of Elek & Harte (1936) gave values of 8.3 and 7.8 % acetoxyl for the free acid and 7.9 % for the methyl ester. Menzel *et al.* record similar results for this method. The calculated values, assuming the formula $C_{33}H_{44}O_8$ for the acid, are 7.74 and 7.54 % respectively for one acetoxyl group. The volatile acid was identified as acetic acid. Drs Weiler and Strauss (Dyson Perrins Laboratory, Oxford) carried out an acetoxyl determination on helvolic acid by hydrolysing it for 15 min. at 100° with 0.1N-ethanolic sodium hydroxide. After acidification, the volatile acids were removed by steam distillation and collected in excess of standard alkali. They obtained values of 16.3 and 15.6 % acetoxyl, i.e. roughly twice those given by the method of Elek & Harte. These results suggest that, while helvolic acid contains two acetoxyl groups, one is much more readily split off than the other. Since helvolic acid is virtually insoluble in acid solution, the conditions of the Elek & Harte method of estimation do not favour the removal of an unreactive acetoxyl group.

If helvolic acid is hydrolysed in 0.1N-alkali for 36–48 hr. at 37°, approximately one equivalent of alkali is taken up, in addition to that required to neutralize the carboxyl group, showing the liberation of a second acidic grouping. This probably corresponds to hydrolysis of the more reactive of the two acetoxyl groups. A crystalline sodium salt of the new acid is precipitated during hydrolysis. The corresponding acid has been obtained in a crystalline form. Its equivalent weight by direct titration was 590; on again treating with excess alkali at 37°, its equivalent was very nearly half this, namely 298. Further hydrolysis with alkali gave, after acidification, a substance which no longer dissolved readily in alkali and which analysed for $C_{28}H_{36}O_5$. This suggests that hydrolysis of the second acetoxyl group gives an hydroxyl so situated that it readily lactonizes with the free carboxyl group (Burton *et al.* 1951).

On the evidence adduced above, all the eight oxygen atoms known to be present in the helvolic acid molecule may be satisfactorily accounted for. Two occur in a carboxyl group, two in carbonyl groups, and the remaining four are distributed

between two acetoxyl groups, one of which is much more resistant than the other to acid hydrolysis. The hydroxyl group liberated on hydrolysis of this second acetoxyl group is so situated that it readily lactonizes with the carboxyl group.

Reaction with bromine. Helvolic acid behaves as an unsaturated substance, decolorizing bromine water and permanganate in the cold and reacting readily with ozone. When the acid is treated in the cold with excess bromine in glacial acetic acid, four atoms of bromine react per molecule of acid, but the low bromine content of the product indicates that the main reaction here is oxidative.

Oxidation with hydrogen peroxide. Helvolic acid is readily and smoothly oxidized by hydrogen peroxide (perhydrol) or by periodic acid, to the same neutral compound, m.p. 229–230°, the properties of which are consistent with a formula $C_{32}H_{46}O_{11}$.

One possible explanation is that this compound results from the formation of a diglycol at the site of the two double bonds, followed by loss of water to form a lactone ring.

On prolonged hydrolysis at 37° with 0.1 N-alkali, the compound slowly dissolves; its equivalent weight under these conditions is 160. The molecular weight corresponding to $C_{32}H_{46}O_{11}$ is 606 and thus four equivalents of alkali are apparently involved in the alkaline hydrolysis of the perhydrol oxidation product; this has been characterized as a dioxime (additional evidence for the presence of two carbonyl groups in helvolic acid itself) and as the benzoyl and 3:5-dinitrobenzoyl esters. With ketene, a monoacetyl derivative was obtained. In view of the evidence that helvolic acid itself contains two acetoxyl groups, it seems that the two remaining hydroxyl groups believed to be present in the perhydrol product are, therefore, rather inert.

Further oxidation with lead tetraacetate. Recognition of the presence of two double bonds led to attempts to split up the molecule by attacking it at these points. With cold alkaline permanganate an immediate reaction takes place, involving ten atoms of oxygen per molecule; there is a further slow reaction in which more than twenty oxygen atoms per molecule are involved. Oxidation with chromium trioxide appears to follow a similar course. It was not possible, however, to characterize any degradation products. With lead tetraacetate, a neutral crystalline derivative was obtained; melting point above 200° (decomp.). Analysis indicated a formula $C_{32}H_{44}O_{11}$; the molecular weight by X-ray crystallography (Crowfoot & Rogers, 1944) is 595 ± 15 ($C_{32}H_{44}O_{11}$ requires 604).

Helvolic acid and its methyl ester react readily with ozone. The acid itself appears to yield two

acidic fractions; the methyl ester yields a neutral product. The products could not, however, be characterized.

Helvolic acid is rapidly oxidized by selenium dioxide in ethanol at 100°, but again it was not possible to isolate any degradation product.

The possibility of dehydrogenation with selenium was explored, but preliminary experiments, and the experience of other workers with different substances, indicate that this method is not likely to be fruitful unless the oxygen content of the molecule can first be reduced.

Reduction. Helvolic acid is very resistant to reduction; after attempted reduction with phosphorus and iodine (6 hr. at 100°) unchanged acid was recovered in good yield.

DISCUSSION

So far as common ground is covered, the results described here are, in almost all respects, in agreement with those of Menzel *et al.* (1944). On questions of fact they differ significantly only in the finding that helvolic acid has no reducing properties. The evidence is entirely consistent with a formula $C_{32}H_{42-44}O_8$ for helvolic acid. Menzel *et al.* are inclined to favour a formula $C_{29}H_{38-40}O_7$, but there are several objections to this. In particular, it corresponds to a molecular and equivalent weight (498 or 500) well outside the limits (562 ± 12) set by the X-ray crystallographers (Crowfoot & Low, 1943). The American workers themselves record equivalent weights of 514, 560 and 591; their molecular weights were determined by the Rast method, which is very much less reliable than X-ray crystallography. A further objection to the C_{29} formula is that we have found good evidence for the existence of eight oxygen atoms in the molecule. The analytical results obtained by Menzel *et al.* agree almost equally well with either formula, but for the silver salt they record values practically identical with the theoretical values for $C_{32}H_{43}O_8Ag$.

The eight oxygen atoms required by the formula $C_{32}H_{44}O_8$ can be satisfactorily accounted for as follows: two in a carboxyl group; four in acetoxyl groups; two in carbonyl groups (at least one ketonic). Helvolic acid behaves as an unsaturated compound, and the course of the reaction with perhydrol, as well as other evidence, suggests that two double bonds are present. The fact that the perhydrol oxidation product is a neutral compound, and that there is evidence that it contains a lactone ring, makes it probable that one double bond is in a position γ or δ to the carboxyl group.

No direct evidence has been obtained about the kind of carbon skeleton present in the molecule. Dr R. E. Richards, of the Physical Chemical Laboratory, Oxford, investigated the infrared

spectrum of helvolic acid and certain of its derivatives. The complexity of the spectrum, which can be attributed to the number of unsaturated carbon-oxygen linkages, prevents many definite conclusions being reached, but the spectra can be satisfactorily interpreted on the lines of the conclusions reached from chemical evidence. Of particular interest is the absence of absorption corresponding to aromatic nuclei. It appears, therefore, that helvolic acid has a partially unsaturated, alicyclic structure.

EXPERIMENTAL

Unless otherwise stated, elementary analyses and determinations of molecular weight by the Rast method were made by Drs Weiler and Strauss, Dyson Perrins Laboratory, Oxford.

Optical activity. Determinations were made with two highly purified samples of helvolic acid with the following results: specimen 1 (c, 3.1 in CHCl_3), $[\alpha]_D^{25} = -113^\circ$; specimen 2 (c, 2.6 in CHCl_3), $[\alpha]_D^{25} = -117^\circ$. For methyl helvolate (c, 2.6 in CHCl_3) a value $[\alpha]_D^{25} = -133^\circ$ was found.

Carbon-methyl determination. Found 11.1% C-Me. $\text{C}_{33}\text{H}_{44}\text{O}_8$ requires 9.7% for two C-Me groups and 14.5% for three.

Carboxyl derivatives

Helvolic acid semicarbazone. This was prepared by a method described by Hopper (1929). Helvolic acid (21.6 mg.) was dissolved in 0.5 ml. of pure pyridine and 11.5 mg. of semicarbazide hydrochloride were added as a concentrated aqueous solution. The mixture was kept at room temperature for 16 hr. On diluting the mixture with water, the almost pure semicarbazone slowly crystallized out. It was recrystallized from ethanol (75%, w/v). Yield 12.1 mg. *Helvolic acid semicarbazone* had m.p. 240–250° (decomp.). (Found: C, 64.6; H, 7.3; N, 6.9. $\text{C}_{33}\text{H}_{47}\text{O}_8\text{N}_3$ requires C, 64.6; H, 7.7; N, 6.9%.)

Helvolic acid dioxime. This was prepared by a method similar to that used for the semicarbazone except that the reaction was carried out in a sealed tube at 100°, heating being continued for 3 hr. The product crystallized from ethanol in long needles. *Helvolic acid dioxime* had m.p. 189°. (Found: C, 65.1; H, 7.6; N, 4.0, 4.7. $\text{C}_{32}\text{H}_{44}\text{O}_8\text{N}_2$ requires C, 65.6; H, 7.8; N, 4.8%.)

Methyl helvolate semicarbazone. This was prepared in exactly the same way as the semicarbazone of helvolic acid. It crystallizes from ethanol in long needles. *Methyl helvolate semicarbazone* had m.p. 212°. (Found: C, 65.2; H, 7.7; N, 5.6. $\text{C}_{34}\text{H}_{48}\text{N}_3\text{O}_8$ requires C, 65.1; H, 7.8; N, 6.7%. Lack of material prevented repetition of the determination of N.)

Silver helvolate

This was prepared by the method described by Menzel *et al.* (1944). Helvolic acid (56.7 mg.) was dissolved in 5 ml. ethanol, and the solution was neutralized by addition of the calculated quantity of 0.1N-NaOH. 10 ml. of 1% aqueous AgNO_3 were added. The precipitated Ag salt was washed with water, dried, and recrystallized by precipitation from CHCl_3 solution by addition of ether. The freshly prepared

salt is colourless; it darkens on keeping. (Found: C, 58.2; H, 6.5; Ag, 15.3. $\text{C}_{32}\text{H}_{43}\text{O}_8\text{Ag}$ requires C, 57.9; H, 6.5; Ag, 16.3%.)

Acetyl determinations

Method of Elek & Harte (1936)

The experimental procedure prescribed by these authors was rigidly followed.

Helvolic acid (two samples, hydrolysed for 65 and 70 min. respectively) contained 8.26 and 7.76% acetyl; $\text{C}_{33}\text{H}_{44}\text{O}_8$ requires 7.74% acetyl for 1 Ac/molecule. Methyl helvolate (hydrolysed for 80 min.) contained 7.94% acetyl; $\text{C}_{33}\text{H}_{46}\text{O}_8$ requires 7.54% acetyl for 1 Ac/molecule.

Identification of volatile acid formed in Elek & Harte determination. A method designed by Feigl (1937) was used. The volatile acid from 10.5 mg. of helvolic acid was collected in distilled water at 0° and the solution neutralized with excess CaCO_3 . After evaporating to dryness, the residue was heated in a small tube and the vapour evolved allowed to fall on paper moistened with a saturated solution of o-nitrobenzaldehyde in 2N-NaOH. The blue colour of indigo developed. The method failed to detect 0.3 mg. of acetic acid, but gave a decisive colour with 0.9 mg.

Determination by alkaline hydrolysis

This determination was made independently by Drs Weiler and Strauss, Dyson Perrins Laboratory, Oxford. Helvolic acid was hydrolysed for 15 min. at 100° with 0.1N-ethanolic NaOH. The reaction mixture was acidified with H_2SO_4 , steam distilled, and the volatile acids were absorbed in excess standard alkali. Values of 16.3 and 15.6% acetoxy were found for two separate samples.

Alkaline hydrolysis at 37°

The acid (two samples hydrolysed for 56 and 144 hr. respectively) absorbed 2.28 and 3.52 equivalents of alkali.

Helvolic acid was dissolved in 3 ml. of 0.1N-NaOH and the residual alkali was titrated after a known interval.

When the first titration above had been completed, excess acid was added, when a white curdy precipitate appeared. This was taken up in CHCl_3 , and the CHCl_3 layer repeatedly washed with distilled water until the washings were neutral. The CHCl_3 solution was then evaporated to dryness and the equivalent of the amorphous acidic residue was determined by direct titration with the micro-burette (Linderström-Lang & Holter, 1931). (Found: C, 70.5; H, 8.2; equiv. 566, 615 (mean 590). $\text{C}_{30}\text{H}_{40}\text{O}_7$ requires C, 70.3; H, 7.9%; equiv. 513.)

The hydrolysis product (29.3 mg.) was dissolved in 3 ml. of 0.1N-NaOH and kept at 37° for 48 hr. The equivalent weight was then determined by estimating the remaining alkali with 0.1N-HCl; equiv. found 298. Hydrolysis of helvolic acid with 0.1N-NaOH at 100° gave on acidification a material which was not readily soluble in alkali and was presumed to be a lactone. (Found C, 74.5; H, 8.8. $\text{C}_{28}\text{H}_{34}\text{O}_8$ requires C, 74.3; H, 8.0%.)

Reaction with bromine

When treated with excess 0.01N- Br_2 in glacial acetic acid at room temperature, helvolic acid reacted with 3.71 and 3.81 atoms of Br/molecule respectively in two separate experiments. A non-crystalline product containing about 5% Br was isolated.

*Reaction of helvolic acid with perhydrol
and with periodic acid*

Oxidation proceeds smoothly, giving the same product in either case. The following procedures are typical:

(1) Helvolic acid (65.9 mg.) was dissolved in 7 ml. of glacial acetic acid and 1 ml. perhydrol added. The mixture was allowed to stand for 16 hr. at room temperature and then evaporated to dryness in an evacuated desiccator. The residue was crystallized from 70% acetic acid. Yield 28.8 mg.

(2) The reagent was a 2.5% solution of sodium periodate in 75% acetic acid. Helvolic acid (23.6 mg.) was dissolved in 2.5 ml. of reagent and solution allowed to stand for 40 hr. at 37°. The mixture was diluted with 5 ml. of water and the precipitate dissolved in CHCl_3 . The CHCl_3 layer was separated, washed and evaporated at room temperature. The residue was recrystallized from 70% acetic acid. Yield 10 mg.

The identity of the product obtained by the two methods was proved by analysis, identity of melting points, and mixed, melting point. It was finally proved (Crowfoot & Rogers, 1944) by the complete identity of the X-ray crystallographic pictures. The product is neutral, crystallizing from acetic acid as small, colourless needles, m.p. 229–230°. It is insoluble in water. (Found: C, 64.4, 63.0, 64.4; H, 7.6, 7.6, 8.0; mol.wt. (Rast), 560. $\text{C}_{32}\text{H}_{46}\text{O}_{11}$ requires C, 63.4; H, 7.6; mol.wt. 606.) The perhydrol product dissolves slowly in 0.1N-NaOH. 15.5 mg., treated for 7 days at 37° with 0.1N-NaOH, went completely into solution. The quantity of alkali used up corresponded to an equivalent of 160.

A dioxime was prepared by the method used for helvolic acid. (Found: N, 4.3. $\text{C}_{32}\text{H}_{46}\text{O}_{11}\text{N}_2$ requires N, 4.4%.)

By reaction with excess ketene in ethereal solution at room temperature, a crystalline monoacetyl derivative was obtained; m.p. above 225° (decomp.). (Found: for two specimens C, 64.8, 64.2; H, 7.6, 7.6; Ac, 22.3. $\text{C}_{34}\text{H}_{48}\text{O}_{12}$ requires C, 64.0; H, 7.4; Ac, 19.8%.) By reaction with benzoyl chloride for 16 hr. in pyridine solution at room temperature, a crystalline monobenzoyl derivative was obtained, m.p. 248°. (Found: C, 66.8; H, 6.4. $\text{C}_{39}\text{H}_{50}\text{O}_{12}$ requires C, 65.9; H, 7.0%.)

A 3:5-dinitrobenzoyl derivative was prepared in the same way, m.p. above 215° (decomp.). (Found: N, 3.6. $\text{C}_{39}\text{H}_{48}\text{O}_{16}\text{N}_2$ requires N, 3.5%.)

Further oxidation of perhydrol oxidation product with lead

tetraacetate. The reagent used was a saturated solution of lead tetraacetate in glacial acetic acid. Helvolic acid (92.9 mg.) was converted to perhydrol oxidation product, and the latter was dissolved in 5 ml. of the tetraacetate reagent. After standing at 37° for 16 hr. the mixture was diluted with 5 vol. distilled water and shaken for 3 hr. to decompose excess tetraacetate. The solution was extracted with 5 ml. CHCl_3 and the CHCl_3 extract evaporated to dryness. The residue (72.8 mg.) was crystallized twice from hot ethanol. A neutral product was obtained, crystallizing in short needles, melting point indeterminate (decomp.). (Found: for two specimens C, 62.1, 63.5; H, 6.8, 7.2. $\text{C}_{38}\text{H}_{44}\text{O}_{11}$ requires C, 63.6; H, 7.3%.) Molecular weight was found by X-ray crystallography (Crowfoot & Rogers, 1944) to be 595 ± 15 ($\text{C}_{32}\text{H}_{44}\text{O}_{11}$ requires mol.wt., 604).

SUMMARY

1. All analytical and degradative evidence suggests that helvolic acid has the formula $\text{C}_{32}\text{H}_{42-44}\text{O}_8$. It titrates as a monobasic carboxylic acid.

2. The following derivatives of helvolic acid have been prepared and characterized: the semicarbazone, dioxime, methyl ester, silver salt, and a product obtained by oxidation with either perhydrol or periodic acid. The latter appears to be a lactone.

3. From the methyl ester, a semicarbazone has been prepared. From the perhydrol oxidation product, a dioxime, an acetoxyl, a benzoyl, and a 3:5-dinitrobenzoyl derivative have been prepared.

4. The behaviour of helvolic acid and certain of its derivatives on alkaline hydrolysis has been studied. Results indicate the presence of two acetoxyl groups in the original molecule.

5. Helvolic acid behaves as an unsaturated substance containing two double bonds.

It is a pleasure to record my thanks to Dr E. Chain, F.R.S., now of the Istituto Superiore di Sanità, Rome, for his encouragement and advice while the original experimental work was being done.

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