The "synthetic" bottromycin thus obtained was compared by means of paper chromatography with authentic bottromycin. The following eluent systems were used: water saturated 1-butanol–*p*-toluenesulfonic acid–piperidine (300:6:2.4); *t*-butyl alcohol–formic acid–water (70:15:15), and 1-butanol–acetic acid–water (1:2:4). All three autobiograms showed only a single spot of biologically active compound on the agar test plates. The R_f values of corresponding spots, as obtained with the authentic and "synthetic" bottromycin, were also identical.

Preparation of the Salicylic Acid Salt of "Synthetic" Bottromycin (Methyl Ester of Kunz Bottromycin).—One g. of "synthetic" bottromycin was dissolved in 50 ml. of ether. After filtration 200 mg. of salicylic acid, dissolved in 10 ml. of ether, was added. A spontaneous precipitate of the amorphous salt was formed which, after storage in the refrigerator and isolation, yielded 1.02 g. of a white product. It could be obtained in crystalline form by crystallization from 20 ml. of ethyl acetate; yield 734 mg., decomposition point 158–160°. Biological activity of this salicylate was identical with that of the salicylate of authentic bottromycin.

Anal. Calcd. for $C_{38}H_{59}N_7O_7S \cdot C_7H_6O_3$: C, 60.40; H, 7.16; N, 10.96; S, 3.58. Found: C, 60.26; H, 7.17; N, 11.28; S, 3.66.

II. Preparation of the Ethyl Ester and its Salicylic Acid Salt.—Two and a half g. of Kunz bottromycin was dissolved in 750 ml. of 0.1 *N* ethanolic hydrochloric acid solution (water-free) and kept for 72 hours at room temperature. The ethyl ester was isolated by the same technique as described above for the methyl ester. One and twenty-five hundredths g. of a white amorphous compound was obtained with a biological activity of the same order as that of bottromycin. Its infrared spectrum also showed again absorption at 5.72 μ (1745 cm.$^{-1}$). One g. of this ethyl ester dissolved in 50 ml. of ether was treated with 200 mg. of salicylic acid dissolved in 10 ml. of ether. One and four hundredths g. of salicylic acid salt was obtained and after crystallization from 20 ml. of ethyl acetate 742 mg. of crystalline salt was obtained with a decomposition point of 152–156°. Biological activity of the salicylic acid salt of the ethyl ester of Kunz bottromycin also appeared to be of the same order as obtained for bottromycin itself.

Anal. Calcd. for $C_{39}H_{61}N_7O_7S \cdot C_7H_6O_3$: C, 60.73; H, 7.37; N, 10.78; S, 3.52. Found: C, 60.35; H, 7.32; N, 11.08; S, 3.84.

III. Preparation of the *n*-Butyl Ester and its Salicylic Acid Salt.—By the same procedure as described for the methyl- and ethyl ester 3.02 g. of amorphous white butyl ester was prepared from 3.3 g. of Kunz bottromycin. However, its biological activity was only 30% of that of bottromycin.

In the same way a salicylate of this ester was prepared. One g. of butyl ester was dissolved in 60 ml. of ether, 200 mg. of salicylic acid dissolved in 10 ml. ether was added. Nine hundred and seventy-five mg. of amorphous salicylate was obtained which after crystallization from 20 ml. of ethyl acetate yielded 810 mg. of an acicular crystalline compound. Its decomposition range was 150–160°. Microbiological activity of this compound was only 45% of the activity of bottromycin.

Paper chromatographic studies on the *n*-butyl ester of Kunz bottromycin proved that this compound was not identical with bottromycin since two distinct spots were obtained on autobiograms when both were mixed. The following elution system was used: 1-butanol–acetic acid–water (1:2:4) in combination with Whatman paper no. 20.

Anal. Calcd. for $C_{41}H_{63}N_7O_7S \cdot C_7H_6O_3$: C, 61.60; H, 7.38; N, 10.48; S, 3.43. Found: C, 61.43; H, 7.33; N, 10.92; S, 3.52.

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DELFT, HOLLAND

[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY, BIOCHEMISTRY AND PEDIATRICS, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA, AND THE SLOAN KETTERING INSTITUTE FOR CANCER RESEARCH]

A New Antioxidant from Yeast. Isolation and Chemical Studies[1]

BY MARTIN FORBES, FRIEDRICH ZILLIKEN, GLYN ROBERTS AND PAUL GYÖRGY

RECEIVED JULY 11, 1957

A new antioxidant of the empirical formula $C_{16}H_{12}O_5$ has been isolated in crystalline form from different types of yeast. The method of isolation of the substance, its biological activity and its physical and chemical properties as well as those of a crystalline dimethoxy derivative ($C_{17}H_{14}O_5$) and a crystalline degradation product are described.

Introduction

Dietary supplements of ethanolic extracts of British baker's yeast, of Fleischmann's baker's yeast, as well as the ether extracts of the ethanol soluble fractions of these, were found to prevent the occurrence of hemorrhagic liver necrosis in rats, kept on a diet which regularly induces this fatal liver injury.[2] Like vitamin E, these extracts prevented also the characteristic susceptibility[3] to hemolysis by dialuric acid of the red blood cells of vitamin E deficient rats. Paper chromatographic analysis of these ether extracts of yeast, however, showed no spots corresponding to α-, β-, γ- or δ-tocopherol but indicated the presence of a substance giving a positive reaction in the Emmerie–Engle test and exhibiting a strong blue fluorescence when exposed to long wave ultraviolet light. An eluate of the section (R_f = 0.84) of the paper chromatogram incorporating the blue fluorescent material, in contrast to all other sections of the chromatogram, when admixed *in vitro* to red blood cells of vitamin E depleted rats, was found to prevent their hemolysis by dialuric acid.[2] This communication deals with the isolation and crystallization of the blue fluorescent compound from the ether soluble fraction of yeast and its chemical and physical properties.

Results and Discussion

Isolation.—A typical experiment showing the isolation of the hemolysis preventing principle is presented in Tables I and II. The methanol step does not substantially enrich the activity but is

(1) Supported in part by the Subcommittee on Liver of the Advisory Committee on Metabolism, through Army Contract No. DA-49-007-MD-78, Office of the Surgeon General of the United States Army, and by the American Cancer Society Research Grant (C-2367) from the National Cancer Institute of National Institutes of Health, United States Public Health Service.

(2) M. Forbes and P. György, *J. Nutr.*, in press (1957).

(3) C. S. Rose and P. György, *Blood*, **5**, 1062 (1950).

useful in the removal of sterols and lipids which later tend to interfere with the chromatography.

TABLE I

EXTRACTION OF "HEMOLYSIS-PREVENTING ACTIVITY" FROM YEAST

	Extraction step	Dry wt. extracted, g.	Unit/mg.	Total units
(1)	10 kg. yeast extr. with 50 l. 95% ethanol	140	5	700,000
(2)	Methanol soluble fraction after removal of sterols	115	6	690,000
(3)	Ether extract of the ethanol and methanol soluble material suspended in water	13	33	429,000

TABLE II

CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE ETHANOL SOLUBLE YEAST FRACTION[a]

Fraction	Solvent	Volume, ml.	Dry wt., mg.	Units/mg.	Total units	R_f values
1	Pet. ether	250	113	..		
2	Pet. ether	250	75	..		
3	Pet. ether	500	78	100	7,800	0.84
4	Pet. ether	500	26	2000	52,000	.84
5	Pet. ether + 0.1% EtOH	500	89	400	35,600	.84
6	Pet. ether + 0.5% EtOH	250	660	..		
7	Ethanol	500	570	..		
					95,400	

[a] Material subjected to chromatography on the Florisil column (3 × 16½ cm.) was a petroleum ether solution containing 4.3 g. of solute; the activity totalling 140,000 units amounted to 33 units per mg.

Chromatography of the ether extract on Florisil reveals, in addition to the blue fluorescent material of R_f = 0.84, a series of distinct bands. When after development with petroleum ether the column is exposed to long wave ultraviolet light six separate zones can be detected. The fastest moving material represents a yellow band followed by a sand colored zone. Distinctly separated from the latter follows the dark blue fluorescent band of R_f 0.84 which is flanked by two narrow zones of yellow fluorescence. Finally after the blue fluorescent band has been eluted and the column is further developed with petroleum ether and 0.5% ethanol, a broad brown band at the top of the column becomes mobile.

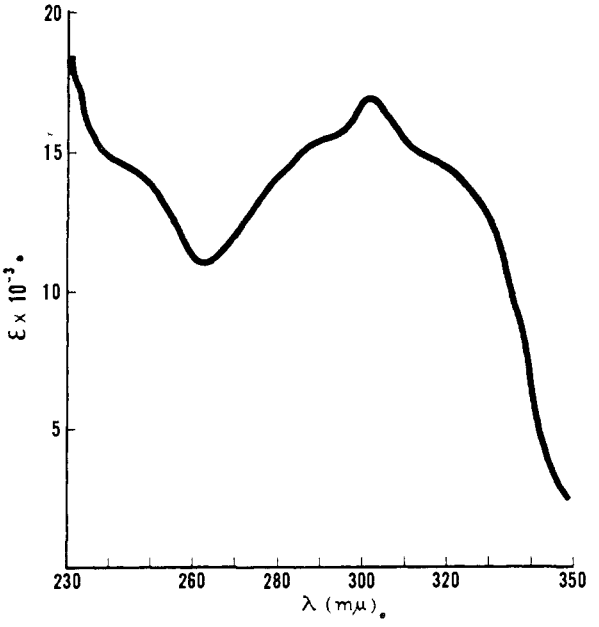

Fig. 1.—Ultraviolet absorption spectrum of $C_{16}H_{12}O_5$, m.p. 118°, in absolute ethanol; 3.5×10^{-5} molar.

The blue fluorescent substance crystallized readily from petroleum ether upon concentration of the active fractions, in a yield of 0.5–2 mg. per pound of dry yeast. Identical crystals were isolated from Fleischmann's baker's and brewers' yeast and from British baker's yeast. In Torula yeast the presence of the same compound could be demonstrated.

Biological Activity.—As little as 0.2 μg. of the crystals gave complete protection in the hemolysis test, compared to 0.5 μg. of α-tocopherol. Since α-tocopherol is quite insoluble in the aqueous reaction mixture, Tween 80 must be added. The yeast factor in contrast is fully active with or without the addition of Tween 80.

On the basis of the hemolysis test and the Emmerie–Engel reaction—but without isolation—the occurrence of a similar substance in yeast has been indicated recently by Cowlishaw and Prange.[4]

The biological effectiveness of the new antioxidant as the active constituent in the ether extract of yeast has still to be demonstrated on rats kept on a necrogenic yeast diet.

Chemical and Physical Properties.—For characterization of the compound, the colorless slender needles—showing an even angle of extinction in the polarizing microscope—were recrystallized from dry petroleum ether (b.p. 30–60°) until the m.p. remained unchanged at 118°, (Berl, uncor.). A solution of the crystals exhibits a blue fluorescence which becomes much more intense upon the addition of alkali and is extinguished upon subsequent acidification. The substance gives a reddish-brown $FeCl_3$ reaction at room temperature and reduces Fe^{+++}. Paper chromatographic analysis in 75% ethanol, according to Eggitt and Ward,[5] revealed a single spot R_f = 0.84 giving a positive Emmerie–Engle test. The ultraviolet spectrum is shown in Fig. 1. In absolute ethanol the substance shows a maximum absorption between 300–303 mμ (ϵ_{302} 1661) and a minimum at 262 mμ (ϵ_{262} 874). Analysis revealed the empirical formula to be $C_{16}H_{12}O_5$. The molecular weight, according to Rast, was found to be 279–286. The substance is optically inactive. A Zeisel determination revealed the presence of one methoxyl group, whereas oxidation with chromic acid (Kuhn–Roth) yielded no volatile acid, indicating the absence of any C-methyl group.

The analysis of the infrared spectrum, Fig., 2 is set out in Table III. The spectrum was obtained

(4) B. Cowlishaw and I. Prange, *Biochim. Biophys. Acta*, **23**, 663 (1957).

(5) P. W. R. Eggitt and L. D. Ward, *J. Sci. Food Agric.*, **4**, 176 (1953).

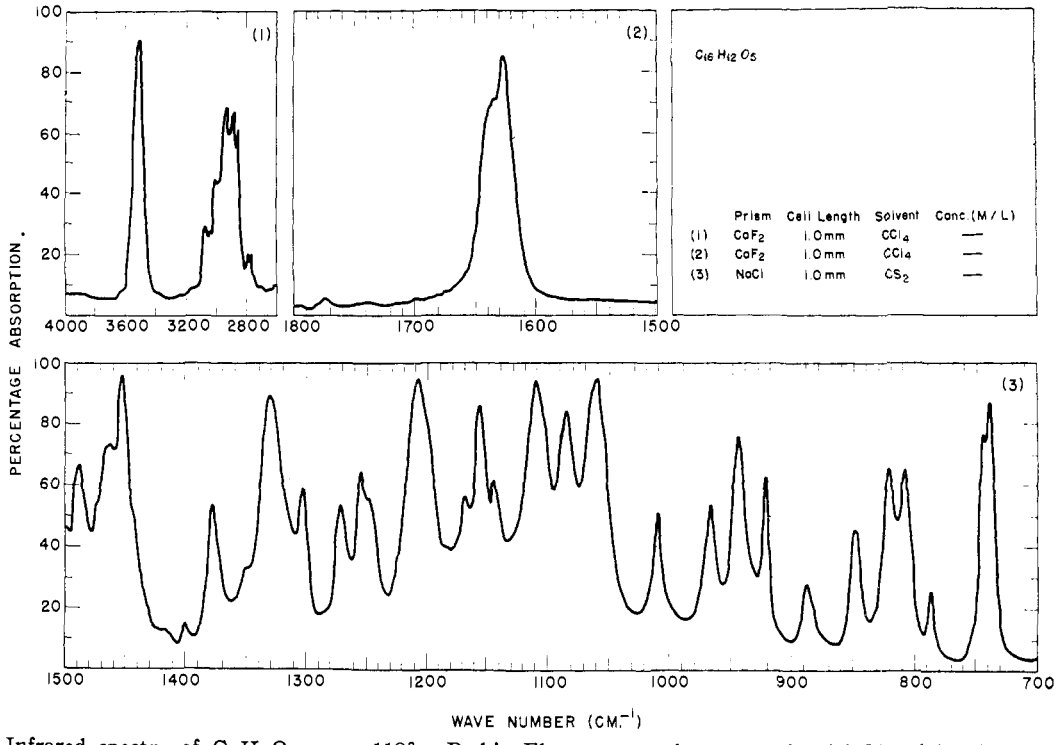

Fig. 2.—Infrared spectra of $C_{16}H_{12}O_5$; m.p. 118°; Perkin–Elmer spectrophotometer Model 21: (1) prism, CaF_2; cell length, 1.0 mm.; solvent, CCl_4; (2) prism, CaF_2; cell length, 1.0 mm.; solvent, CCl_4; (3) prism, NaCl; cell length, 1.0 mm.; solvent, CS_2.

as solutions in carbon disulfide, carbon tetrachloride and as a dispersion in potassium bromide.

Microhydrogenation in glacial acetic acid, with PtO_2 as the catalyst, resulted in a consumption of 9.1 moles of hydrogen within 2–3 hr., whereas in absolute ethanol, using 5% Pd adsorbed on $BaSO_4$ as the catalyst, only 4 moles of hydrogen was consumed over a period of 120 hr.

Considering the oxygen functions, 2 of the 5 oxygen atoms were accounted for, one as a methoxy group and one as a phenolic hydroxyl group, whereas the 3 remaining oxygen atoms were neither hydroxyl groups nor carbonyl groups from infrared evidence, and the conclusion was drawn that these must be in the form of other ethers. This concept received further support from the isolation of a crystalline dimethoxy derivative $C_{17}H_{14}O_5$, m.p. 92° (Berl, uncor.). The latter was obtained by treating the original compound with diazomethane. The dimethoxy derivative gave a negative Emmerie–Engle test (R_f = 0.73) and, like the original compound, exhibited a blue fluorescence. Its activity in the hemolysis test was only one fifth of that of the initial material. The ultraviolet spectrum of the dimethoxy derivative revealed the same absorption λ_{max} = 301–302 mμ (Fig. 1) as the original substance. The infrared spectrum of the dimethoxy derivative showed a complete absence of the hydroxyl function as evidenced by the disappearance of the band at 3510 $cm.^{-1}$.

Oxidation of the original compound with stoichiometric amounts of $FeCl_3$ under nitrogen yielded a red amorphous product $C_{16}H_{10}O_5$, m.p. 185–187° (dec.).

Fusion with potassium hydroxide at 200° under nitrogen, followed by high vacuum sublimation at 10^{-4} mm. of the acidic ether extract yielded, in addition to unchanged starting material, a crystalline aromatic carboxylic acid, m.p. 190–191°. The infrared analysis of the crystalline degradation product revealed the absence of any hydroxyl groups as evidenced by the absence of absorption in the neighborhood of 3500 $cm.^{-1}$ (KBr). The broad absorption extending from 3400 to 2200 $cm.^{-1}$ (KBr), with sub-maxima at 2670 and 2445 $cm.^{-1}$, was characteristic of the O–H stretching vibrations of a carboxylic acid. The carbonyl region (1800–1600 $cm.^{-1}$) was complex with the band center at 1692 $cm.^{-1}$. Together with a subsidiary band at 1703 $cm.^{-1}$, the spectrum resembled that exhibited by benzoic acid. The interpretation was made that the carboxyl group was directly attached to the unsaturated nucleus.[6] This would indicate that the new antioxidant contains at least two aromatic rings, one of which is unsubstituted and the other bears the phenolic hydroxyl, the methoxyl group and the remaining oxygen in ether linkage.

Experimental

Solvents and Adsorbent.—Absolute methanol and ethanol were purified by distillation. Peroxide-free ether was used. Petroleum ether (b.p. 30–60°) was purified by shaking with sulfuric acid and washing with water, followed by distillation. The necessity to exercise precautions in the use of solvents was indicated when β-phenylnaphthylamine, a contaminant of benzene, was isolated in the early experiments. Florisil 60/100 mesh (Floridin Co., Warren, Pa.) was washed with methanol and dried overnight at 110°.

(6) Although not stated at the time, it is evident in retrospect that the spectrum is characteristic of an *ortho*-substituted benzene nucleus (prominent band at 738 $cm.^{-1}$).

TABLE III

Frequency ($cm.^{-1}$)	Solvent	Deduction
3510	CCl_4	O–H stretching vibration of an associated hydroxyl group. The simplicity of the contour indicated that here was only one hydroxyl group present. There was no change in the position of the OH band on dilution, which was interpreted as evidence that the hydroxyl group was bonded intramolecularly with a functional group in the ortho position
3070	CCl_4	C–H stretching of an aromatic ring
3010		
3000–2800	CCl_4	The intensity of the bands in this region relative to the 3070 and 3010 $cm.^{-1}$ bands indicated that there were more satd. carbon atoms than can be accounted for by one methoxy group
1632	CCl_4	C=C vibrations of an aromatic system
1625		
1499		
1487		
2000–1632	CCl_4	The absence of absorption in this region eliminated carbonyl groups such as those present in acids, esters, ketones, aldehydes and lactones
744, 738	CS_2, KBr	Out-of-plane bending of C–H bonds in an aromatic ring. This doublet together with the absence of bands below 700 $cm.^{-1}$ strongly indicates an *o*-substituted phenyl derivative
849, 820, 808	CS_2	Out-of-plane bending of C–H bonds in an aromatic ring. These suggested a 1,2,4-substituted phenyl derivative, though tetra- and penta-substituted compd. were not positively eliminated

Detection and Determination of the Substance.—The active fraction was detected by its blue fluorescence in long wave ultraviolet light and the red color it developed upon treatment with the Emmerie–Engel reagent (0.5%, α,α-dipyridyl and 0.2% ferric chloride in alcoholic solution). Paper chromatography was performed according to Eggitt and Ward.[5] Quantitative determination of the substance was carried out by means of the hemolysis test[7] with slight modifications.[2]

Biological Activity.—Activity was expressed as the number of "hemolysis prevention" units/mg., 1 unit being the minimal weight of material preventing the hemolysis by dialuric acid of erythrocytes from vitamin E-deficient rats. α-Tocopherol and β-phenylnaphthylamine were used as standards. The former requires the addition of Tween 80 to the test system.

Yeasts.—Four different yeasts were investigated: British bakers' yeast (Distillers Co., Ltd., Glasgow), Fleischmann's bakers' yeast, type 5009, Fleischmann's brewers' type yeast 2019 (Standard Brands, Inc., N. Y.) and Torula yeast (Lake States Yeast Corp., Rhinelander, Wis.).

The infrared spectra were obtained with a Perkin–Elmer spectrophotometer Model 21 equipped with sodium chloride and calcium fluoride optics.

Method of Isolation.—Ten kg. of yeast was refluxed for 8 hr. with 50 l. of redistilled 95% ethanol (Publickers). The alcoholic extract was filtered and the filtrate was kept overnight at +5°. It then was freed of an inactive crystalline precipitate and the clear yellow filtrate was concentrated to a sirup *in vacuo* under nitrogen at a temperature not exceeding 50°. The residue (140 g.) was dissolved in 1.5 l. of redistilled dry methanol and was kept overnight at −40°. The precipitates consisting of sterols and other lipids were centrifuged off at −20° and washed once with cold methanol. The supernate and the combined washings were evaporated to dryness under nitrogen *in vacuo* and the residue resuspended in water to make a 10% (w./v.) suspension. The *p*H was then adjusted to 8.0 with 4 *N* NaOH and the suspension extracted with ether continuously for 96 hr. The ether extracts were dried over sodium sulfate, filtered and evaporated *in vacuo* under nitrogen to a sirup. The residue (13 g.) was then dissolved in 130 ml. of dry petroleum ether (b.p. 30–60°) and chromatographed on Florisil. A portion of the petroleum ether solution corresponding to 4.3 g. of solute was adsorbed on a 3 × 16.5 cm. column, containing 60 g. of Florisil. In the beginning, the flow rate was adjusted to 30 ml./hr. After development of the bands, visible in long wave ultraviolet light, the flow rate was changed to 60–90 ml./hr. Fractions containing the characteristic blue fluorescent bands, possessing "hemolysis preventing" activity, were combined and evaporated to a volume of about 15 ml. Colorless slender needles began to crystallize from the pale yellow solution when kept at +5°. The combined fractions from 3 such columns, representing ten kg. of yeast, yielded after recrystallization from the same solvent 10.6 mg. of crystals, m.p. 118° (Berl, uncor.).

Crystalline Antioxidant.—For all microanalytical purposes the colorless slender needles were recrystallized from petroleum ether (b.p. 30–60°) and dried at room temperature over NaOH and paraffin at 0.1 mm. until constant weight was achieved, m.p. 118° (Berl, uncor.); yield 0.5–2 mg. per pound of dry yeast; $[\alpha]^{25}D$ 0 (*c* 3, methanol). Calcd. for $C_{16}H_{12}O_5$ (284.3): C, 67.59; H, 4.25; O, 28.10; OCH_3, 10.93. Found: C, 67.58 (67.72); H, 4.54 (4.41); O, 27.10 (27.65); OCH_3, 10.18 (10.47); $C(CH_3)$ negative; mol. wt., 279, 286. The crystals are very soluble in methanol, ethanol, ethyl ether, much less soluble in petroleum ether and only sparingly soluble in water. Recrystallization also can be achieved from ethanol–H_2O or acetic acid–H_2O.

Catalytic Hydrogenation.—The crystals, 1.4 mg., were dissolved in 2.0 ml. of glacial acetic acid and hydrogenated in the presence of 7.6 mg. PtO_2. Within the first hour, a rapid uptake of hydrogen occurred which came to a standstill after 2–3 hr. Assuming a molecular weight of 284, at normal temperature and pressure 9.1 moles of hydrogen was consumed. In another microhydrogenation involving 1.570 mg. in 2.0 ml. of absolute ethanol + $Pd/BaSO_4$ (5%), 4 moles of hydrogen was consumed over a period of 120 hr.

Dimethoxy Derivative.—One hundred mg. of the original product (m.p. 118°) was dissolved in 10 ml. of absolute ethyl ether and chilled to +5°. An ether solution of diazomethane, obtained from 10 g. of nitrosomethylurethan, was added in small portions. After addition of a few drops of dry methanol the color was discharged. The solution was kept at +5° for several hours with occasional shaking and then evaporated *in vacuo*. The colorless residue was dried and subsequently extracted three times with dry petroleum ether (b.p. 30–60°). The combined extracts were filtered and evaporated to a volume of 15 ml. Upon chilling at 0° for several hours, colorless needles were obtained. After two recrystallizations from the same solvent, the melting point remained unchanged. The crystals were dried over NaOH and paraffin at room temperature and 0.1 mm. until constant weight was achieved; yield 76 mg.; m.p. 92° (Berl, uncor.); mixed m.p. 78–79° (with starting material); $[\alpha]^{25}D$ 0 (*c* 3, methanol).

Anal. Calcd. for $C_{17}H_{14}O_5$ (298.3): C, 68.44; H, 4.74; OCH_3, 20.83. Found: C, 68.12; H, 4.94; OCH_3, 19.93 (20.3).

The substance gave a negative Emmerie–Engel test; upon catalytic microhydrogenation with PtO_2 in glacial acetic acid, 8.95 moles of hydrogen was consumed at normal temperature and pressure. The ultraviolet spectrum is identical with that shown in Fig. 1. The infrared spectra were obtained in KBr, CS_2 and CCl_4, R_f = 0.73 on paper chromatograms according to Eggitt and Ward. The "hemolysis-preventing" activity[2] was 1 μg.

Oxidation with $FeCl_3$.—One hundred and sixty mg. of original product was dissolved in 10 ml. of 80% ethanol and

(7) C. S. Rose and P. György, *Amer. J. Phys.*, **168**, 414 (1952).

treated with 302 mg. of $FeCl_3 \cdot 6H_2O$ at room temperature in an atmosphere of nitrogen. After a few minutes a brilliant red color was observed which appeared to be stable. After 1 hr., water was added and the solution extracted with ether. The combined ether extracts were washed with water, dried over sodium sulfate and evaporated to dryness. Even after chromatography on Alumina, the red colored oxidation product remained amorphous, m.p. 185–187° (under dec.).

Anal. Calcd. for $C_{16}H_{10}O_5$ (282.2): C, 68.09; H, 3.54. Found: C, 67.94; H, 3.69.

The ultraviolet spectrum revealed absorption at λ_{max} 209, 245 and 282 mμ.

Degradation with Alkali.—Fifty mg. of the original product was fused with 10 pellets of KOH for 1 hr. at 200° in an atmosphere of nitrogen. The brown melt was taken up in water and extracted with ether. From the alkaline ether extract 25 mg. of starting material was recovered in crystalline form, m.p. 117–118°. The alkaline aqueous solution was acidified with dilute HCl and continuously extracted with ether. The dried and evaporated ether extracts were subjected to high vacuum sublimation at 1×10^{-4} mm. in a Willstätter tube. At an air-bath temperature of 120–125°, a colorless crystallized sublimation product was found in the first receiver. After recrystallization from ether–petroleum ether, 4 mg. of prismatic needles was obtained, m.p. 190–191° (Berl, uncor.).

The infrared analysis offered conclusive evidence that the alkaline degradation product was a non-substituted aromatic carboxylic acid. This is substantiated by the broad absorption extending from 3400 to 2200 cm.$^{-1}$ with submaxima at 2670 and 2445 cm.$^{-1}$. The absence of any absorption from 3400–4000 cm.$^{-1}$ provides evidence that the acid does not contain a phenolic or alcoholic hydroxyl group.

PHILADELPHIA, PA.

[CONTRIBUTION NO. 1447 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

On the Aggregation of Bovine Serum Albumin in Acid Solutions[1,2]

BY P. BRO,[3] S. J. SINGER AND J. M. STURTEVANT

RECEIVED JUNE 7, 1957

Bovine serum albumin forms aggregates in 0.10 *M* NaCl–HCl solutions below *p*H 3.4. The aggregation reaction appears to be reversible with respect to *p*H, but the rate of the reaction in both directions is subject to erratic fluctuations. The reaction is inhibited by excess mercuric ion. The aggregates can be converted to monomer on an ion exchange column in the presence of thioglycolate. The experimental evidence available is consistent with the hypothesis that the reaction is a thiol–disulfide exchange.

Introduction

It has been observed by several investigators that bovine serum albumin (BSA) may aggregate in acid solutions. Reichmann and Charlwood,[4] observed aggregates at *p*H 1.9 in the absence of added salts. In 0.10 *M* KCl solutions aggregates did not appear to form, whereas they did form in 0.50 *M* KCl solutions. Saroff, Loeb and Scheraga[5] found that aggregates appeared in 0.10 *M* NaCl and sodium acetate solutions of BSA. The aggregation phenomenon was investigated in some detail by Kronman, Stern and Timasheff[6] who attempted to establish the conditions under which the aggregation reaction occurs. Their findings were inconclusive in that no clearly defined physical and chemical factors could be isolated which were responsible for the formation of aggregates. Aggregates also were observed by the present authors,[7] who reported on the sedimentation behavior, and made the observation that the aggregate appeared to undergo a reversible swelling similar to that observed with monomeric BSA.

During a series of calorimetric studies on serum albumin, the question arose as to the effects which the aggregation reaction might have on the thermochemical behavior of the protein at low *p*H. In order to answer that question it was necessary to learn more about the chemical nature of the aggregates and to devise a method for changing the aggregates back to monomer.

Experimental

Crystalline BSA was purchased from Armour and Co. (Lot G-4302) and from Pentex, Inc. (Lots A-1201 and B-12016 P). Crystalline bovine mercaptalbumin (BMA) was prepared by way of its mercury dimer by the method described by Dintzis.[8] Analytical grade reagents were used throughout. The investigations were carried out with approximately 1% protein in NaCl–HCl solutions 0.10 *M* in Cl^-, unless otherwise stated, at approximately 25°. The protein concentration of the various solutions was measured with a Phoenix B-S differential refractometer calibrated with albumin solutions of known concentration as determined by the micro-Kjeldahl method. The nitrogen content of the albumin was taken to be 16.07%. The experimental results are reported in terms of the *p*H of the solutions as measured with a Beckman model G *p*H-meter at 25°, the *p*H-meter being standardized with a 0.10 *M* ionic strength acetate buffer having a *p*H of 4.65.[9]

The extent of the aggregation reaction was measured with a Spinco Model E ultracentrifuge operated at 59,780 r.p.m. Ultracentrifuge cells with plastic centerpieces were used. In analyzing the sedimentation patterns, it was assumed that the refractive increments of the various aggregates are the same as that of the monomer, and the areas under the partially resolved peaks of the sedimentation patterns were divided symmetrically. The ratios of the areas were taken as the ratios of the concentrations of the various components. The usual correction for the dilution effect was introduced. The errors in the observed areas due to the Ogston–Johnston[10] effect were within the experimental error. On the basis of the analysis of 24 different sedimentation patterns, the standard deviation in the determination of the aggregate to monomer ratio was found to be 0.04 and to be independent of the value of the ratio.

The aggregrate under investigation here, al-

(1) From a dissertation submitted by P. Bro in partial fulfillment of the requirements for the Ph.D. degree, June, 1956.

(2) This research was aided by grants from the National Science Foundation (G 179) and the United States Public Health Service (RG 3996 C).

(3) General Electric Co. Fellow, 1954–1956.

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