the dihydrobromide of *p*-cymylene-3,5-dibromo-2,6-diamine and the acetyl and benzoyl derivatives of the free base.

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THE BIOCHEMISTRY OF SULFUR. I. THE IDENTITY OF ERGOTHIONEINE FROM ERGOT OF RYE WITH SYMPECTOTHION AND THIASINE FROM PIGS' BLOOD

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RECEIVED DECEMBER 21, 1926 PUBLISHED FEBRUARY 5, 1927

This paper is the first contribution from this Laboratory to a new series of publications dealing with the chemistry of sulfur compounds having biological significance. The work which led to the chemical study discussed in this paper was the independent discoveries by Hunter and Eagles[2,3] and Benedict, Newton and Behre[4,5] of new organic sulfur compounds occurring in the corpuscles of pigs' blood. Hunter and Eagles[6] have assigned to their compound, which was originally called "substance X," the name "sympectothion," having the formula $C_{18}H_{32}N_6S_2O_5$. Benedict and his co-workers[5] assigned to their product the name "thiasine," having the formula $C_{12}H_{20}N_4O_3S$. At the time that this special research was undertaken in this Laboratory, which was on October 1, 1926, we had no positive evidence of the chemical structure of either of these two products. In fact, from what has been learned by experiment since we became interested in this subject we are now able to conclude that the original formulas assigned were incorrect in both cases. Before we began our research we were fortunate in receiving from Dr. George Hunter of Toronto University and Dr. S. R. Benedict of Cornell University small samples of their respective compounds to aid us in the elucidation of their chemical structure. A careful comparison of the solubility of the two products, their behavior towards alkali and the fact that neither compound revealed the presence of amino nitrogen and that both gave the same color reactions with phosphotungstic acid indicated that the same organic structure functioned in both compounds. We are now able to report positively that Hunter and Eagles, and Benedict and his co-workers were dealing with the same compound, but one having a different empirical formula than those reported in their last publications.

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[2] Bulmer, Eagles and Hunter, *J. Biol. Chem.*, **63**, 17 (1925).

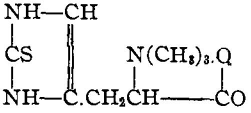
[3] Hunter and Eagles, *ibid.*, **65**, 623 (1925).

[4] Benedict, *ibid.*, **64**, 215 (1925).

[5] Benedict, Newton, and Behre, *ibid.*, **67**, 267 (1926).

[6] Hunter and Eagles, *ibid.* (in press).

The formula to be assigned to their respective compounds is represented by the expression $C_9H_{15}N_3SO_2$, and it is now proved by the writers to be identical in structure with ergothioneine first isolated by Tanret from ergot of rye.[7] Barger and Ewins[8] showed that this sulfur compound is to be represented structurally as the betaine derivative of thiol-histidine (I).



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The chemical evidence on which we have based this conclusion is given in detail in the experimental part of this paper. In the light of these results and in order to avoid confusion in nomenclature, we suggest that this naturally occurring sulfur compound isolated from pigs' blood be given the original name proposed by its discoverer, Tanret.

Experimental Part

Correction of the Formula Assigned to Sympectothion.—In the purification of this substance Hunter and Eagles[3] added to a concentrated aqueous solution of their compound from blood a definite volume of absolute alcohol, whereupon the compound separated quickly but gradually in a microcrystalline condition. The compound thus obtained does not contain water of crystallization. It does, however, absorb moisture which is difficult to remove in an evacuated desiccator over calcium chloride. When crystallized from water the compound separates with two molecules of water in accordance with Tanret's description. It was due to the fact that proper attention was not paid to the conditions necessary for absolute drying that the original analytical results did not agree with the theoretical for ergothioneine. After thoroughly drying the compound, we have repeated the determinations of nitrogen and sulfur and have obtained results more in accord with those calculated. It is interesting to note that the value for sulfur runs low, probably due to the firmness with which the element is linked in the ergothioneine molecule.

Anal. Calcd. for $C_9H_{15}N_3SO_2$: N, 18.34; S, 13.97. Found: N, 18.05; S, 13.48.

In the case of thiasine, Benedict and co-workers reported in their last paper[5] a value for sulfur of 10.8%. The final method of purification applied by these workers to thiasine was crystallization of the hydrochloride as the free base from pyridine.

Preparation of Ergothioneine from Ergot of Rye

In order to establish the identity of sympectothion with ergothioneine, it was necessary to obtain some of the latter substance for purposes of

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[7] Tanret, *J. pharm. chim.*, [VI] **30,** 145 (1909).

[8] Barger and Ewins, *J. Chem. Soc.*, **99,** 2336 (1911).

comparison and identification. We therefore turned to ergot of rye as the only source of material and applied the method of separation first described by Tanret.[7] We were successful in isolating the compound, but not in a sufficiently pure condition to compare with sympectothion. By modification of Tanret's technique we finally succeeded in obtaining a pure product. The details of this improved technique will be discussed in a future paper of this series.[9]

Comparison of Tanret's Ergothioneine and Hunter and Eagles' Sympectothion

Ergothioneine prepared in the above manner agreed in every respect, chemically and physically, with sympectothion. Both compounds are neutral to litmus and are very soluble in water and slightly soluble in hot alcohol. They both give a blue color with phosphotungstic acid in the presence of sodium carbonate, a cherry-red color with Pauly's diazo reagent and a yellow color with Koessler and Hanke's reagent.[10] The sulfur is not removed by digestion with alkali, but in both cases trimethylamine is evolved almost quantitatively. Amino nitrogen is not revealed by action of nitrous acid. Potassium permanganate and bromine water are decolorized by both compounds. Both compounds show the same behavior towards iodine and also are characterized by the firmness of the linkage of sulfur as exhibited on fusion with sodium. Tanret[7] reports a specific rotation of $[\alpha]_D = +110°$, while our value for sympectothion is $+115°$. Both substances could be made to melt anywhere between 259° and 282° in a capillary tube, depending upon the rate of heating. Tanret gave the melting point as 290°, but determined this on a Maquenne block. The melting point obtained by us, 276°, was obtained by heating the samples for 195 seconds. A mixture of ergothioneine and sympectothion melted at the same temperature.

Optical Evidence in Support of the Identity of Ergothioneine with Sympectothion

Specimens of both compounds were submitted to Dr. Edgar T. Wherry of the Bureau of Chemistry, United States Department of Agriculture, Washington, D. C., for a comparison of their crystallographic and optical properties. His report confirms the chemical evidence supporting the identity of the two compounds. We are greatly indebted to Dr. Wherry for his report to us, which was written on December 8, 1926, and is given below.

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[9] For the supply of ergot of rye necessary for the isolation of ergothioneine, we wish to express our thanks to the Upjohn Co. of Kalamazoo, Michigan, and Sharp and Dohme of Baltimore, Maryland. From the stock supplied by both companies we were able to isolate ergothioneine.

[10] Koessler and Hanke, *J. Biol. Chem.*, **39**, 497 (1919).

"I am glad to be able to state that the optical evidence definitely confirms the chemical data, that the two supposedly distinct substances, A and B, are identical. It should be noted that C and D show exactly the same properties and do not appear significantly better for optical study.[11] As from what you told me many people are likely to wish to identify these substances, I have made the following description rather full. This description is submitted to you for quotation in whatever paper you may write upon the substance.

"The substance proves to be insoluble in the organic liquids commonly used in the immersion method. It consists of fairly well developed plates and rods, small, but easily studied, using a 4mm. objective. The crystal system appears to be monoclinic, the class the anaxial or domatic. The crystal habit is rather characteristic, many of the grains showing distinct hemimorphic development, that is, the terminal faces at opposite ends slope at different angles, or the grain tapers from one end to the other. The grains are often aggregated into stellate groups. An additional feature is the frequent presence of conical cavities extending from the end more or less deeply into the grain, the material sometimes being frayed out around the orifice, and the cavities again being so well developed that in certain conditions of illumination they have the appearance of an hour-glass.

"On examination as to their optical properties, the grains were found to have a negative elongation, that is, to exhibit their lowest refractive index lengthwise. The lowest value which it has been possible to find is $n_D = 1.582$, and this may be taken as α for the substance. Most grains exhibit, however, a slightly higher value, some, indeed, showing different values at opposite ends, and for identification purposes they are most conveniently immersed in a liquid of refractive index 1.585. For the convenience of workers who may wish to identify the substance, but have no series of immersion liquids at hand, it may be mentioned that both bromoform and *o*-bromophenol have approximately this index, at least in their commercial state, although a mixture of 2 parts of α-chloronaphthalene and 1 part of mineral oil of the 'liquid petrolatum' type, brought to the desired index by adding minute amounts of one or the other component, with repeated trials on a refractometer, is commonly used. The refractive index shown by the grains crosswise varies widely from one to another, averaging 1.65, but extending at least to 1.61 (β) on the one hand and 1.70 (γ) on the other. Immersion liquids with these three values can be made up by mixing the α-chloronaphthalene with mineral oil for the lower, and with methylene iodide (n, 1.74) for the two higher values. The only other noteworthy optical property is the extinction observed between crossed

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[11] A, B, C and D were used merely to label the samples submitted for study and no information was given to Dr. Wherry regarding the sources of the samples.

nicols, which ranges from parallel on some grains to inclined at an angle of around 5° on many others, and to as high as 20° on a few.

"The procedure recommended for identifying this substance is as follows.

"Immerse a small amount of the material in a drop of oily liquid, $n_D = 1.585$, on a microscope slide, cover, place on the microscope stage, examine between crossed nicols to find a field containing a fair number of grains, take out the analyzing nicol, and cut down the illumination by means of the substage diaphragm. Rotate the stage, and if the substance here described is represented, the grains will successively disappear as they approach parallelism with the cross-hair corresponding to the vibration plane of the polarizing nicol. In raising the microscope tube slightly by means of the fine-adjustment screw, a movement of light into or out of the grain may be observed, but it will be at most very faint, indicating practical identity of the refractive index of the grains lengthwise with that of the liquid, 1.585.

"Repeating the procedure with the immersion liquid, $n_D = 1.65$, and observing the grains as they turn into approximately perpendicular position with respect to the same cross-hair, it will be found that some of them practically disappear, although now raising the tube will bring out many cases of deviation from a match with the liquid. On trying liquid 1.61, nearly all the grains will show, on raising the tube, a band of light entering them and illuminating their centers, but careful search from one to another will ultimately bring to light a few which practically match the liquid so that no movement of light occurs. On similarly trying liquid 1.70, nearly all the grains will show the light band passing out, but ultimately matching will again be found in a few of them, if the substance under discussion is represented.

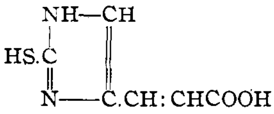
"This procedure I have followed with all four samples submitted, and all proved to show the exact features described, including the quantitative measurement of refractive-index values represented by the matching of immersion liquids. There can be no further question as to the identity of the two compounds supposed to be represented."

Behavior of Sympectothion toward Alkali

1.034 g. of this compound was distilled with 22 cc. of 50% potassium hydroxide solution and the distillate was collected in 10 cc. of 1 *N* hydrochloric acid. Titration of an aliquot portion of this solution revealed a 99.6% decomposition of the sulfur compound. The acid solution was evaporated to dryness, the hydrochloride redissolved in water and the base (trimethylamine) precipitated as the chloroplatinate; yield, 83.4%; m. p., 245°.

Anal. Calcd. for $[N(CH_3)_3.HCl]_2.PtCl_4$: Pt, 36.93. Found: 36.76.

Isolation of Mercaptoglyoxaline Acrylic Acid



The alkaline solution from the digestion above was colored deep orange-red. Following the procedure used by Barger and Ewins,[8] the thio acid was separated and obtained in an amorphous condition. This acid was obtained in a crystalline form by acidifying a 0.1% solution of its sodium salt with hydrochloric acid; m. p., 307°, with decomposition.

Anal. Calcd. for $C_6H_6N_2SO_2$: N, 16.47. Found: 16.30.

Conversion of the Mercapto Acid to Urocanic Acid

The procedure of Barger and Ewins[7] was followed. A 70% yield of urocanic acid nitrate was obtained, showing the characteristic crystalline habit described by Hunter.[12] The nitrate melts from 192–208°, depending on the rate of heating. Decomposition of the nitrate with sodium carbonate gave urocanic acid melting at 229–230°.

Urocanic acid prepared from casein and kindly furnished to us by Professor Andrew Hunter of Toronto University melted at the same temperature. When mixed together no lowering of the melting point was observed.

Anal. Calcd. for $C_6H_6N_2O_2$: N, 20.29. Found: 20.40. Calcd. for $C_6H_6N_2O_2.2H_2O$: H_2O, 20.69. Found: 21.10.

The Identity of Thiasine with Sympectothion and Ergothioneine

The specimen of thiasine submitted for our examination by Dr. Benedict did not possess the crystalline properties shown by sympectothion; but it agreed in its chemical properties with this substance. In order to be certain that our comparison would reveal identity, 0.4 g. of the thiasine sample was taken and subjected to a precipitation with phosphotungstic acid, and the free base isolated in exactly the same manner as that utilized for the preparation of sympectothion. It was then obtained in a crystalline condition, melting at the same temperature as sympectothion and ergothioneine, namely, 276°. Mixtures of the respective compounds did not show a lower melting point.

Summary

1. Sympectothion from the Laboratory of Professor George Hunter of the University of Toronto has been shown to be identical with ergothioneine.

2. Thiasine from the Laboratory of Professor Benedict of Cornell University is identical with sympectothion, and consequently all three of these substances are to be represented by the same constitution.

3. An improved method of isolating ergothioneine was used, but the description of the technique has been reserved for a future paper.

4. It is our plan to develop in the immediate future a semi-commercial process for obtaining ergothioneine from blood in quantity. We are now engaged in a study of methods of synthesizing this compound.

New Haven, Connecticut

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[12] Hunter, *J. Biol. Chem.*, **11**, 537 (1912).