[CONTRIBUTION FROM THE DEPARTMENTS OF BIOLOGICAL CHEMISTRY AND MEDICINE, HARVARD MEDICAL SCHOOL, AND THE MEDICAL CLINIC, MASSACHUSETTS GENERAL HOSPITAL]

Studies of the Pernicious Anemia Principle in Liver. III. The Isolation and Properties of a Substance with Primary Therapeutic Activity[1]

BY Y. SUBBAROW, BERNARD M. JACOBSON AND VILMA PROCHOWNICK

This communication is a detailed account and an extension of results previously reported in a preliminary form.[2]

It has already been found[3] that a therapeutically active extract of liver can be prepared by adsorption of Cohn's fraction G on norit, elution of the adsorbate by hot ethyl alcohol, and subsequent concentration of the elute by vacuum distillation (fraction B). The copious precipitate appearing on further concentration of the elute (fraction C) has been characterized as a complex purine.[4] The filtrate (fraction D) from 100 g. of fresh liver concentrated to 3 cc. is light brown in color, and contains 10 to 12 mg. of total nitrogen. This filtrate is the starting point of the preparation described below.

Experimental

After acidification of 150 cc. of the filtrate (derived from 5 kg. of liver) to *p*H 2 with hydrochloric acid, 16 g. of English fuller's earth was added, and the mixture was stirred mechanically for thirty minutes at room temperature. The precipitate was filtered off and washed once with 50 cc. of water. To the filtrate and washings were added 10 volumes of 95% ethyl alcohol and 10 volumes of ether, and the mixture was left in the cold room for twenty-four to thirty-six hours. The resulting precipitate was then filtered off. The precipitate (fraction H[5]) was obtained in a yield of 1 g. (20 mg. from 100 g. of liver) and contained 12 to 13% nitrogen.

One gram of this precipitate was dissolved in 50 cc. of water and the solution was brought to *p*H 3 with 10 *N* sulfuric acid. A crystalline precipitate immediately separated. This precipitate consisted mainly of calcium sulfate, and on the basis of spectroscopic examination contained traces of silver, copper, iron, aluminum and lead, all of the latter in a concentration of less than 0.01%, and probably derived from the fuller's earth. The filtrate of the calcium sulfate precipitate was then added to 40 cc. of water containing 1 g. of Reinecke salt[6] at 40°. After remaining in the cold room for twenty-four hours a crystalline precipitate formed which was filtered off and washed once with 50 cc. of ice-cold water. The precipitate was suspended in 300 cc. of 0.03 *N* sulfuric acid at 30–35°, and the Reinecke acid was removed by repeated extraction with 500 cc. of a mixture of equal volumes of amyl alcohol and ether. After several such extractions the color was entirely removed, and the solution was concentrated *in vacuo* to a volume of 25 cc. To this concentrate were added 10 volumes of acetone and 10 volumes of ether, and the mixture was kept in the cold room for forty-eight hours. A microcrystalline precipitate separated in a yield of 100 mg. (2 mg. from 100 g. of fresh liver). This precipitate is termed fraction I.

Fraction I was also prepared without the use of fuller's earth. To 1 liter of fraction D were added 7 liters of 95% ethyl alcohol and the mixture was kept at room temperature for five hours. The resulting precipitate, which contain none of fraction I, was filtered off. To the filtrate 3 liters of ethyl alcohol and 10 liters of ether were added, and the mixture kept in the cold room for forty-eight hours. The resulting precipitate, termed fraction E, was filtered off and dissolved in 500 cc. of water. To this was added 200 cc. of a 5% solution of rhodanilic acid in methyl alcohol. After forty-eight hours in the cold room a crystalline precipitate separated which was filtered off, and freed of rhodanilic acid by means of pyridine, with subsequent removal of the pyridine by ether, the method of regeneration described by Bergmann.[7] The regenerated rhodanilate solution was then precipitated by Reinecke salt as described above, and the subsequent procedure was followed. The yield of fraction I was approximately the same as that obtained by the previous method.

Properties of Fraction I.—The microcrystalline material, derived from either the crystalline rhodanilate or directly from the crystalline reineckate, is white and dissolves readily in water, forming a colorless solution. The sulfate is readily soluble in dilute alcohol, but is very little soluble in absolute alcohol. The sulfate can be precipitated from an aqueous solution acidified to *p*H 2.5 with sulfuric acid by the addition of acetone and ether. Repeated precipitation did not change the nitrogen content. The sulfate decomposes without melting above 290°. In 0.8% aqueous solution the sulfate has an optical activity $[\alpha]^{28}D$ −85.4 ±2°. *Anal.* Found: C, 41.56; H, 6.74; N, 13.13; S, 4.6. 3.4% of the 4.6% S is present as inorganic sulfate. The ash content of this sample was 1.6%. Spectroscopic examination disclosed that almost all of the ash consisted of chromium, evidently derived from the decomposition of the Reinecke salt. The amino nitrogen (Van Slyke[8]) was determined after interaction with nitrous acid at the end of

(1) This investigation has been supported by grants from the Ella Sachs Plotz Foundation, the Proctor Fund of the Harvard Medical School, the Milton Fund of Harvard University and the Lederle Laboratories, Inc.; and by Therapeutic Research Grant No. 267 of the Council on Pharmacy and Chemistry of the American Medical Association.

(2) Subbarow and Jacobson, *J. Biol. Chem.*, **114**, cii (1936).

(3) Subbarow, Jacobson and Fiske, *New Eng. J. Med.*, **214**, 194 (1936).

(4) Subbarow, Jacobson and Fiske, *ibid.*, **212**, 663 (1935).

(5) Due to the possibility of conflict in terminology with the fraction G of Cohn, we have omitted the use of the letter G in designating our fractions.

(6) Dakin and West, *J. Biol. Chem.*, **109**, 489 (1935).

(7) Bergmann, *ibid.*, **110**, 471 (1935).

(8) The authors are indebted to Mr. Leon Rosenfeld for assistance in these determinations.

five minutes and at the end of sixty minutes, with the following results:

PER CENT. AMINO N FOUND	5 min.	60 min.
Before hydrolysis	5	14.7
After hydrolysis for 6 hours in 2 *N* HCl	75	87

Under the usual conditions fraction I does not show a ninhydrin reaction, but after neutralization to *p*H 7 a slight reaction is observed. Fraction I reduces the Folin–Ciacalteau phenol reagent,[9] gives a positive orcin test, and a weak diazo reaction. The Sagakuchi, Millon, glyoxylic acid and biuret tests are negative.

Fraction I is readily precipitated by phosphotungstic acid and is partially precipitated by Hopkins' reagent ($HgSO_4$ in H_2SO_4). The reineckate can be recrystallized readily from hot water, but due to the decomposition products formed during the successive regenerations this salt is not suitable for further purification. A crystalline rufianate is readily obtained with the following composition: C, 46.5; H, 4.84; N, 4.23; S, 6.6. For further purification the rufianate is also not practicable due to the necessity of using large amounts of amyl alcohol in regeneration. Furthermore, if the rufianic acid is removed with barium the resulting barium rufianate precipitate adsorbs the fraction I.

An aqueous solution of fraction I, when exposed to ultraviolet light, exhibits an intense blue fluorescence. The absorption spectrum in the ultraviolet of an aqueous solution of fraction I (C 0.5 mg. per cc., L 1 cm.) as depicted in Fig. 1, shows an inflection between 2480 and 2560 Å.[10]

Therapeutic Activity.—That fraction H is therapeutically active is evidenced by satisfactory clinical and hematological improvement in four patients suffering from pernicious anemia, following the administration of the material by intramuscular injection. The therapeutic activity of fraction I has been tested similarly in two patients, with resulting moderate clinical and hematological improvement. The data are presented in Table I.

It is to be noted that all of the experimental periods of therapeutic trial are of short duration, and are not adequate for an estimate of the capacity of fraction H and of fraction I to induce a complete clinical and hematological remission. It has already been reported[11] that the complete therapeutic activity of commercial liver extract probably depends upon its content of several chemically distinct substances. Fractions H and I, in the light of the present evidence, probably exert a primary or initiating therapeutic action, which is augmented by the addition of several other substances derived from crude liver extract. The evidence for this view, and the details of the therapeutic activities of fractions H and I, will be discussed in a forthcoming publication.

TABLE I

THE THERAPEUTIC ACTIVITY OF FRACTION H AND FRACTION I

Fraction no.	H	H	H	H	I	I
Patient no.	16	15	21	22	23	13
Total amount fraction administered, mg.	60	20	20	20	7.6	4
R. b. c. at beginning, millions per cu. mm.	1.05	1.28	1.15	0.88	2.41	1.40
R. b. c. at end, millions per cu. mm.	2.42	1.93	1.80	1.60	3.10	2.35
Reticulocytes at peak, %	31.0	24.4	30.2	24.6	6.6	12.0
Day of reticulocyte peak	6th	7th	5th	5th	4th	5th
Length of period, days	18	8	8	8	10	10

Since this paper has gone to press fraction I regenerated from the rufianate has been tested for its therapeutic activity on two patients. The resulting hematopoietic responses were similar to the responses to fraction I described in Table I.

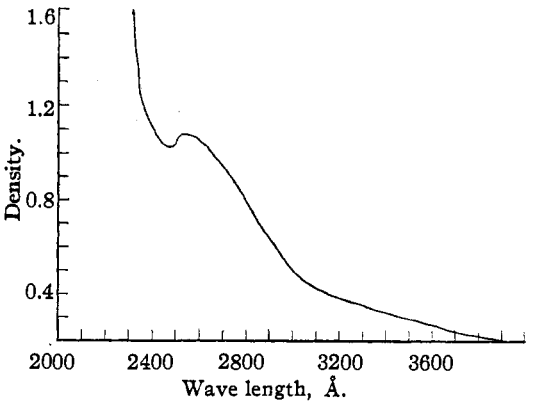

Fig. 1.

(9) Folin and Ciacalteau, *J. Biol. Chem.*, **73**, 627 (1927).

(10) For the determination of the absorption spectrum the authors are indebted to Prof. George R. Harrison of the Massachusetts Institute of Technology.

(11) Fiske, Subbarow and Jacobson, *J. Clin. Investigation*, **14**, 709 (1935).

There has recently been reported by Laland and Klem[12] the separation from liver of an amorphous, reddish-yellow material, in a yield of 0.35 mg. from 100 g. of fresh liver, with the following composition: C, 53.64; H, 6.85; N, 13.33; S, 0.74 (ash content 2.05%). The absorption spectrum in the ultraviolet showed two points of inflection, one between 2500 and 2650 Å. and another between 3450 and 3500 Å. The similarity between the nitrogen content and one point of inflection of the absorption spectrum of the material of Laland and Klem and fraction I is worthy of note. The material of Laland and Klem has been found to be therapeutically active, in doses of 0.7 mg., by Strandell, Poulsson and Schartum-Hansen.[13] Proof is still lacking that the chemically active material reported in this paper is a single pure chemical substance.

(12) Laland and Klem, *Acta Med. Scand.*, **88**, 620 (1936).

Summary

There is described the isolation from commercial liver extract of a microcrystalline white substance, as a sulfate, in a yield of 2 mg. from 100 g. of fresh liver, exhibiting intense blue fluorescence when exposed to ultraviolet light, and exerting therapeutic activity in pernicious anemia.

(13) Strandell, Poulsson and Schartum-Hansen, *ibid.*, **88**, 624 (1936).

BOSTON, MASS. RECEIVED AUGUST 19, 1936

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF COLUMBIA UNIVERSITY]

Investigations in the Retene Field. VII. Certain Fluorenones and Phenanthridones from Retenediphenic Acid

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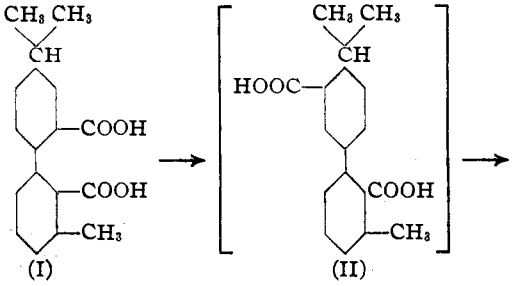
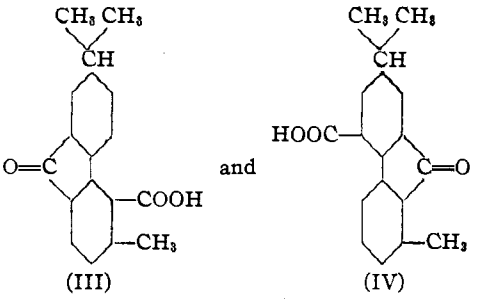
In a previous publication[2] it was shown that the oxidation of retenequinone in glacial acetic acid solution with 30% hydrogen peroxide yielded retenediphenic acid. This paper is concerned with the preparation and proof of structure of some additional derivatives of retenediphenic acid and with the establishment of the configuration of compounds previously reported.[2]

When retenediphenic acid (I) was treated with 95% sulfuric acid, a rotation of one of the rings of the biphenyl nucleus took place and the hypothetical intermediate (II) thus formed lost a molecule of water in two ways to yield 6-methyl-2-isopropylfluorenone-5-carboxylic acid (III) and 1-methyl-7-isopropylfluorenone-5-carboxylic acid (IV).



(1) Fritzsche Fellow in Organic Chemistry, Columbia University, 1935–1936.

(2) Adelson, Hasselstrom and Bogert, THIS JOURNAL, **58**, 871 (1936).



At 110° the principal product was the keto acid (III), while the isomeric acid (IV) was isolated when the reaction was carried out at room temperature. At 60° there resulted a mixture of acids which could not be separated by fractional crystallization; at 175° complete sulfonation took place. These keto acids, (III) and (IV), were characterized by conversion into the oximes, the methyl esters and the oximes of the latter.

At 110° the action of 95% sulfuric acid on the 3-methyl-4′-isopropyl-2′-carbomethoxybiphenyl-2-carboxylic acid (V) resulted in a mixture of the keto acids, (III) and (IV), and the methyl ester of (IV). This is similar to the experience of Underwood and Kochmann,[3] who studied the action of sulfuric acid on methyl acid diphenate. At room temperature, however, 95% sulfuric acid converted the acid ester (V) into the methyl ester

(3) Underwood and Kochmann, *ibid.*, **46**, 2074 (1924).