because of greater reduction of unsaturation by the methyl group in the former than by the hydrogen in the latter.

Similarly, large values for formates and small values for branched isomers have been observed in eight pairs of esters by Wender[16] in a study of parachors previously determined or calculated from previously recorded surface tensions.

Experimental.—Surface tensions were measured by Sugden's[3] modification of the method of maximum bubble pressure, using *m*-xylene[13] in the manometer and carefully purified benzene as a standard[17] for calibration of the apparatus. To ensure equal hydrostatic pressures, a maximum stable bubble was left on the large capillary while bubble pressures were being measured from the small capillary.[13] The manometer readings were taken with a cathetometer with an accuracy of 0.01 cm. Temperatures were controlled to ±0.08°. After calibration of the bubbler using accepted values of benzene (28.88 dynes/cm. at 20°),[18] readings were taken to check the calibration of CCl_4 and C_6H_5Cl. The observed values of 26.77 and 33.25 (20°) agree with accepted values (26.81 and 33.2).

(16) Simon Wender, Emory University thesis, 1935, unpublished.

(17) S. Sugden, *J. Chem. Soc.*, **119**, 1483 (1921).

Summary

1. The surface tensions of 15 normal isomeric esters ($C_{16}H_{32}O_2$) have been measured at 25, 35, 50, and 65° by the method of maximum bubble pressure.

2. The calculated parachors were found to decrease gradually from maxima of 695.3 and 699.1 at the ends of the series to a minimum of 689.3 near the center of the series.

3. Molecular volumes calculated from densities taken at constant surface tension (27 dynes/cm.) varied similarly to parachor.

4. The parachors were found to increase, in general, with the temperature.

(18) Richards and Carver, THIS JOURNAL, **43**, 827 (1921).

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[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF OREGON STATE COLLEGE]

Pantothenic Acid. II. Its Concentration and Purification from Liver

BY ROGER J. WILLIAMS, JOHN H. TRUESDAIL, HARRY H. WEINSTOCK, JR., EWALD ROHRMANN, CARL M. LYMAN AND CHAS. H. MCBURNEY

In the first article of this series[1] was announced the discovery of a growth determinant of universal biological occurrence, which was named "pantothenic acid." At the time of this initial report no progress had been made toward the concentration or isolation of the substance and the evidence for the existence of a single effective substance in the various sources was based upon electrolytic, diffusion, hydrogenation, esterification and other experiments in which the behavior of the physiologically active principle was the crucial question.

The concentration of a substance offering the particular difficulties encountered in connection with pantothenic acid had never been accomplished previously and much exploration was required before effective methods could be devised. The difficulties mentioned are based upon the following facts. (1) It is predominantly an acid rather than a basic substance and hence the techniques and precipitants extensively useful with nitrogenous bases cannot be applied except indirectly. (2) The richest convenient source (liver) contains on the order of 40 parts per million, and hence 250 kg. of liver after an extended process yielded only about 3 g. of crude (approximately 40%) material from which the remaining impurities were removed with great difficulty. (3) The substance is highly hydrophilic and we have yet to find any salt or simple derivative which is not highly soluble in water. (4) Due, presumably, to the presence of several types of functional groups, even after purification the substance has failed to crystallize.[2] In this respect it appears to behave somewhat like β-hydroxyglutamic acid, which, according to Dakin,[3] makes up 10% of casein; yet is not available on the market at any price. (5) Pantothenic acid is itself

(1) Williams, Lyman, Goodyear, Truesdail and Holaday, THIS JOURNAL, **55**, 2912 (1933).

(2) We are indebted to Professor Linus Pauling for X-ray examination of a sample which was suspected of possessing crystalline character. No diffraction pattern was observed and further study confirmed the non-crystalline character of the material.

(3) H. Dakin, *Biochem. J.*, **12**, 290–317 (1918).

unstable and can be handled safely only as a neutral salt. It is readily broken down by acids or bases and is particularly susceptible to destruction by acidic alcohol. (6) Unlike riboflavin and ascorbic acid, pantothenic acid could be tested for by biological tests only, since it has no color, reducing properties or other known outstanding property which can be used as a guide during its isolation. (7) Unlike the amino acids and biotin,[4] pantothenic acid has not been purified successfully by distillation of its ester. Whereas in later studies we have found it possible to esterify it and hydrolyze the ester without great loss, the process is attended by complete destruction unless carefully controlled, and the use of the ester (which seems scarcely more volatile than the acid itself) did not offer promise as a means of purification.

The crucial problem was first to devise some method whereby the acid could be separated from a variety of sugars and other neutral or basic water-soluble material. A solution to this problem was found in converting the acid into its brucine salt (as explained below) and extracting with chloroform, which fails to dissolve the sugars and similar compounds. It was found better to adsorb the acid on charcoal and elute first and later apply the brucine salt conversion. The essential steps in the process as applied to a 9-kg. batch of liver are given below. Sheep liver was used because of its uniformity and convenience. Beef or hog liver can be used. It was necessary, of course, that biological tests be made continuously during the process of concentration. For this purpose "G. M." yeast, the medium previously described[5] and the techniques previously developed[5,6] were used. As a standard material we used first a stable preparation made by the extraction of rice bran with 60% methanol, and assigned to this dry material a "potency" of 1. Other preparations were compared to this *at low dosage levels*. Later in our work a highly concentrated material dissolved in a concentrated sucrose solution was used as a secondary standard, but the ultimate standard remained the rice bran extract tested at a low dosage level. A "unit" of pantothenic acid is that amount which when tested as indicated is equivalent to 1 g. of the dry rice bran extract. The high potency of the material as compared to the low toxicity of brucine for the yeast, allowed us to test brucine salts directly for their effect on yeast growth.

(4) Kögl and Tönnis, *Z. physiol. Chem.*, **242**, 43 (1936).
(5) Williams and Saunders, *Biochem. J.*, **28**, 1887 (1934).
(6) Williams, McAlister and Roehm, *J. Biol. Chem.*, **83**, 315 (1929).

Preparation of Autolysis Extract.—Nine kilograms (20 lb.) of ground sheep liver was mixed thoroughly with 20 liters of water (40°) in a ten-gallon milk can. One liter of benzene was then added and stirred thoroughly into the mixture, which was allowed to autolyze for twenty-four hours at 37°. The vessel was closed to avoid evaporation of benzene.

After twenty-four hours the vessel was placed in a larger barrel or other suitable vessel containing water and the water in the outer jacket was heated as rapidly as possible by means of live steam until its temperature reached 95–100°. In the meantime the liver mixture was stirred frequently in order to heat evenly and prevent local coagulation on the walls of the container. When the liver mixture reached a temperature of 75–80° a coagulum formed. Between 80–86° some frothing took place due to evaporation of benzene. (Inhaling the vapor was avoided.) Finally all the benzene was removed in ten to fifteen minutes at 97° by passing live steam directly into the extract. (For some unknown reason the extract is not consistently obtained in a suitable condition if the amount of benzene originally used is materially decreased.)

While still hot the coagulum was filtered off through muslin with greatest ease, using large funnels. The filtrate was perfectly clear, orange colored and possessed a greenish fluorescence. The "potency" of the extract (19 liters) on a moist basis under most conditions was 0.3 and therefore it contained 5700 "units" of pantothenic acid. Under less favorable conditions (presumably due to the liver) the units obtained might be as low as one-half the above value.

Fuller's Earth Adsorption.—In order to remove organic bases which would interfere with later purification, the solution was treated while still warm with 360 g. of technical fuller's earth (B-K-H). The solution was stirred thoroughly with the earth for fifteen minutes and then filtered. The solution loses substantially no pantothenic acid by this treatment and its appearance remains about the same except for the loss of fluorescence.

Adsorption and Elution from Norite.—Previous to adsorption with Norite the *p*H of the solution was brought approximately to 3.6 after thorough mixing, by the addition of 10–30 ml. of 1–1 sulfuric acid. A flocculent precipitate formed and was filtered off using kieselguhr as a filtering medium.

At this stage the filtrate was cooled (some dilution with ice is permissible) to about 15°, after which about 450 g. (2 g. per 100 cc.) of Norite was added and the mixture kept agitated for two hours. The Norite was then filtered off and the colorless filtrate containing very little pantothenic acid was discarded.

The activated Norite should be eluted immediately after adsorption. It is not necessary, nor desirable, to dry the charcoal before elution. If the charcoal is heated, satisfactory elution becomes impossible. In each of the three elutions, 1500 ml. of 1.6 *N* ammonium hydroxide solution was used. For elution, the mixture was suspended (using a rolling machine) for two hours. The filtration was carried out at the pump on hardened filter paper (S & S 602 h). The combined elutions and rinse

were brought to neutrality (*p*H *ca.* 6.5) by adding a saturated water solution of oxalic acid.

The amount of activity recovered at this stage varied considerably with different batches. In typical cases 85–95% of the active substance was adsorbed by the charcoal and about 60% of that adsorbed was recovered in the eluate. This initial loss of about 50% of the activity was compensated for by the fact that the introduction of the charcoal adsorption step made the material easier to purify later. An average batch of eluate from 9 kilos of liver contained, therefore, about 3000 units.

Brucine Treatment.—To the eluate was added 30 g. of oxalic acid in water solution, then 180 g. of brucine alkaloid in methanol solution, and the *p*H of the resulting solution adjusted to 7. This solution was then evaporated to dryness after adding 225 g. of kieselguhr during the latter part of the evaporation. The evaporation was accomplished by using specially constructed water-jacketed evaporating pans. By use of electric fans the temperature was kept from 50–70°.

During the evaporation, acid production from the liver constituents evidently takes place[7] and it is necessary to add brucine from time to time to keep the solution neutral. Approximately 10 g. of brucine may be required for this purpose. In the end there was obtained from an average batch about 700 g. of dried pulverized material with a "potency" of 3.5 which was dried in a desiccator and ground in a ball mill.

Chloroform Extraction.—The dry kieselguhr residue above was extracted successively three times with 700-ml. portions of chloroform for an hour each. The chloroform solutions were extracted successively with 70, 70 and 35 ml. portions of water and the water solutions combined. Upon partial evaporation (or previously during extracting) some brucine oxalate crystallizes out and can be discarded. The final mixture of dry brucine salts obtained from a sample batch weighed about 20 g. and had a potency of 80. Over-all yields up to this point were in some cases much higher than this, but relatively large losses during the preceding three stages seemed inescapable.

Fractionation of the Brucine Salts.—This step in the process cannot be carried out economically with small batches and hence is described in terms of the material from 250 kg. of liver. The fractionation involved starting with approximately 600 g. of brucine salt mixture and from it obtaining about 9 g. of brucine salts containing over 50% of the activity and therefore about thirty times as concentrated as the original material. Most of the remaining active material was saved in the form of partially concentrated material. The process extended through many months and involved the carrying out of thousands of culture tests. Altogether about 700 fractions were separated besides numerous discards which were not numbered. To give a complete picture of how the fractionation was carried out would be impossible, but we feel that it will be useful to outline briefly the method of attack.

From the standpoint of novelty of operation, probably the most outstanding feature is the fact that we made extractions using quantities of solvents which superficially might seem absurd. For example, if the extraction demanded it we did not hesitate to extract 2 liters of (water-saturated) chloroform with 0.5 ml. of water. Even in such an extreme case the manipulation is not difficult.

When relatively pure brucine pantothenate is shaken with equal volumes of chloroform and water, only about one part in a thousand will remain in the chloroform, *i. e.*, its distribution coefficient is roughly 1000. However, the presence of other salts in crude material alters the characteristics of the solvents so that the distribution coefficient of brucine pantothenate under these conditions may be as low as 70.

The crude mixture of brucine salts obtained from sheep's liver contains about 5% of material with a higher distribution coefficient than brucine pantothenate and about 95% of material with a lower coefficient. The former are termed type *w* salts due to their greater tendency to go into the water layer. The latter are termed type *c* material since they have a greater tendency to go into chloroform solution.

The success of this process depends on taking several fractions in such a manner that the type *w* and type *c* materials can be sorted separately according to potencies, fractionated and discarded independently, at the same time losing but a small amount of activity.

All the crude brucine salt was put through the following first step in batches of about 50 g. The crude material was dissolved in chloroform and shaken with successive small portions of water. The volumes of water taken were small enough so that approximately 20–25% of the original activity would be found in the water layer. (To judge these amounts required extensive experience and many physiological tests.) Under these conditions type *w* material was obtained largely in the first fractions and type *c* material in the later.

The next steps in the process involved combination of type *c* salts (which are large in amount) of similar potency and their refractionation in a manner similar to that described above.

Continued fractionation by dissolving type *c* material in chloroform and extracting with water, would lead to an accumulation of type *w* material and a piling up of the active principle in these fractions. In order to avoid this the extraction procedure was reversed as the process demanded—that is, the material was dissolved in water and extracted with chloroform containing 1% of brucine alkaloid.

Throughout the whole fractionation process very small losses were sustained, but to work up all the material into "high potency" fractions involved an inordinate length of time and hence we were content with a yield of approximately 18,000 units in this form out of about 34,000 units of starting material. Though we were constantly on the lookout for such phenomena, we observed at no time during this fractionation any results which suggested that two or more substances were being concentrated simultaneously.

Conversion to Calcium Salts and their Fractionation.—Brucine salts at any stage of purification were converted readily into calcium salts by treating in solution with an excess of lime water and freeing from brucine by filtration and repeated extraction with chloroform. The purified brucine salts from the fractionation procedure were, therefore, treated in this way and the alkaline solution immediately freed from excess calcium (final *p*H about 7.5) by the

(7) Kapeller-Adler and Luisada, *Biochem. Z.*, 269–397 (1934).

use of oxalic acid. Solutions containing the calcium salts were then completely decolorized with high-grade blood charcoal and the almost colorless calcium salts were obtained by evaporation, in the form of a varnish. From 9 g. of brucine salts, potency 1600–2300, there was obtained a little over 3 g. of calcium salts, potency about 5000.

We shall not attempt to mention all the devices attempted before we developed the process outlined for the further concentration of pantothenic acid. The procedure adopted for the purification of calcium pantothenate consisted in (a) the precipitation of the active principle from concentrated aqueous solution with absolute alcohol, (b) treatment with mercuric chloride in aqueous and alcoholic solution to remove the presumably basic impurities still present in the concentrate, (c) a thrice repeated fractional precipitation of the calcium salt from an aqueous alcohol solution with isopropyl ether, and finally (d) a fractional precipitation from pyridine with acetone.

Precipitation from Aqueous Solution with Alcohol.—Calcium pantothenate, 431 mg. of "potency" 5700, was dissolved in 3 ml. of water in a 15-ml. centrifuge tube. There was added 10 ml. of absolute ethanol dropwise and with vigorous stirring. A precipitate which was formed was centrifuged from the solution and washed twice with 10 ml. of 95% ethanol. On addition of the washing liquor and 10 ml. additional absolute ethanol to the solution, a further slight precipitate was formed. These low activity fractions weighed 104 mg. and had a potency of 270.

Mercuric Chloride Treatment.—The solution was evaporated almost to dryness in a 15-ml. centrifuge tube, and 3 ml. of ethanol added. An excess (800 mg.) of mercuric chloride was added, the suspension allowed to stand overnight, and the precipitate centrifuged. The precipitate was washed three times with 5-ml. portions of ethanol. The washings were added to the solution, which was then evaporated almost to dryness. On addition of 10 ml. of water, a further precipitate formed, which was centrifuged. The mercuric chloride precipitate retained approximately 3% of the total activity. The solution was saturated with hydrogen sulfide, the mercuric sulfide washed twice with 1-ml. portions of water. The solution and washings were evaporated to half volume to remove the hydrogen sulfide. There was added 500 mg. of silver oxide. This was allowed to stand for two hours, the precipitate being broken up frequently with a stirring rod. On removal of the precipitate of silver chloride and excess silver oxide, the *p*H of the solution was found to be 5.2. It was adjusted to 7.2 with calcium hydroxide solution, and evaporated to dryness.

The above two steps were carried out, in six separate runs, on 3.16 g. of average potency 5000, containing 15,800 units. Ninety-three per cent. of the activity was accounted for, 85% being in the high activity fractions, weight 1.76 g. The potency of these fractions varied from 6500–8900. The remainder of the recoverable activity was found in the low activity fractions and the mercuric chloride precipitate.

Ethanol–Isopropyl Ether Fractionation.—The isopropyl ether used in this fractionation was treated with slightly acid ferrous sulfate solution, sodium carbonate solution, and dried over anhydrous sodium carbonate. Isopropyl ether was used in preference to ethyl ether because of its lower volatility and hygroscopicity. Calcium pantothenate, 216 mg., potency 7600, was dissolved completely in 0.30 ml. of water in a centrifuge tube. There was added 4.0 ml. of absolute ethanol in 0.2-ml. portions. A slight precipitate formed, which was not removed. There was added slowly dropwise, with vigorous stirring, 1.0 ml. of isopropyl ether, to yield fraction A. This was removed by centrifugation. Further addition of 1.2 cc. of isopropyl ether precipitated fraction B. Evaporation of the solution gave fraction C.

Fraction	Weight, mg.	Potency
A	83.5	6250
B	36.9	8250
C	88.9	9100

More of the calcium salt, 485 mg., was treated similarly in three runs, with essentially the same results.

The combined fractions C were again fractionated in the same manner with ethanol and isopropyl ether.

Fraction	Weight, mg.	Potency
D	37.8	9330
E	63.0	10300
F	56.5	10400
G	64.3	8160

A third ethanol–isopropyl ether precipitation of fractions E and F caused no further purification.

Acetone–Pyridine Fractionation.—The pyridine used in these fractionations was dried by allowing to stand over fused potassium hydroxide and distilling from barium oxide. The acetone was distilled from calcium carbonate. Acetone was added slowly dropwise to 95.2 mg. of fractions E and F above, in a solution of 0.15 ml. of water and 2.0 ml. of pyridine. Precipitates were removed at intervals.

Fraction	Weight, mg.	Potency
H	5.6	10,300
I	21.3	10,800
J	55.3	10,250
K	12.6	11,000

These highly active fractions of calcium pantothenate were prepared in the form of a clear, colorless varnish, very difficult to obtain in an entirely anhydrous condition. Pulverization gave a white powder, which was found to be quite hygroscopic. It took up 4–6% moisture under ordinary laboratory conditions.

Fraction J after being pulverized and dried at 56° *in vacuo* was found to have a potency of 11,100. As indicated in succeeding papers this fraction represents nearly pure material. We are able to get a quantitative measure of the amount in a culture solution when only 0.0005 γ per ml., or 5 parts per 10 billion, is introduced.

We desire to acknowledge our indebtedness to Anne King Stout and Ruth Carleton Scott for capable and painstaking help in connection with the concentration and the accompanying physiological tests.

We wish also to express our sincere thanks to those who have supported this work financially. This includes: the local institution; the National

Research Council; Standard Brands Inc. of New York, who have supported the investigation generously for many years; and the Rockefeller Foundation, who have made possible the large scale work by a substantial grant.

Summary

The concentration and purification of pantothenic acid from liver is described. Because of the character of the material this has proved an unusually difficult task. The final preparation (amorphous) has a potency of 11,100 as compared with a standard rice bran extract which was chosen as unity. Further fractionation of this material resulted in no increased purity. For this and other reasons it is thought to be substantially pure. Its presence can be determined quantitatively when only 5 parts is present in 10 billion parts of culture medium (0.0005 γ per ml.).

CORVALLIS, ORE. RECEIVED JUNE 8, 1938

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF OREGON STATE COLLEGE]

A Study of Reduction with Hydriodic Acid: Use in Micro Determinations of Hydroxyl Groups

BY HERSCHEL K. MITCHELL AND ROGER J. WILLIAMS

The work described in this paper was undertaken primarily for the purpose of establishment of a new method for the micro determination of hydroxyl groups in amorphous, hydrophilic compounds such as pantothenic acid.[1] It was also desirable to develop a method for the quantitative estimation of a given type of hydroxyl as well as the total number per molecule.

A semi-micro method depending on acetylation with acetic anhydride in pyridine, has been described by Freed and Wynne.[2] Recently the description of a similar micro method has been published by Stodola.[3] A more or less standard procedure for the micro determination of hydroxyl groups in conjunction with other groups containing active hydrogens is that given by H. Roth.[4] This is based upon the Zerewitinoff (Grignard) reaction. A gravimetric method for accomplishing the same purpose, based upon the replacement of hydrogen with deuterium, has been proposed by Williams.[5]

None of the above methods proved suitable for the determination of hydroxyls in highly hygroscopic amorphous materials. In any case the adhering water interferes seriously and in the case of the Zerewitinoff method no solvent (lacking active hydrogen) could be found to dissolve the hygroscopic material.

(1) Williams, Weinstock, Rohrmann, Lyman, Truesdail and McBurney, THIS JOURNAL, **60**, 2719 (1938).

(2) Freed and Wynne, *Ind. Eng. Chem., Anal. Ed.*, **8**, 278 (1936).

(3) Stodola, *Mikrochemie*, **21**, 180 (1937).

(4) F. Pregl, "Quantitative Organic Micro Analysis," Third English Edition, P. Blakiston, Philadelphia, Penna., 1937, pp. 156–166.

(5) Williams, THIS JOURNAL, **58**, 1819 (1936).

It was believed probable that the following normal reactions of hydriodic acid with alcohols would be analytically useful

$$(1)\quad ROH + HI \longrightarrow RI + H_2O$$
$$(2)\quad RI + HI \longrightarrow RH + I_2$$

Hydroxyl groups, if completely reduced, must pass through both of the steps above. Complete reduction is difficult, however, and the second step may take place only partially. It was assumed that conditions might be found in which at least the first reaction would take place quantitatively.

If the first reaction only, takes place

Milliequiv. of OH groups = Milliequiv. of HI used up

If an additional amount of hydriodic acid is used to complete the second reaction, this amount (which is equivalent to the millimoles of iodine liberated) is subtracted from the total and

Milliequiv. of OH = Milliequiv. HI used up − Millimoles of I_2 liberated

The milliequivalents of OH groups reduced are equal to the millimoles of I_2 liberated and the milliequivalents of OH groups merely replaced are equal to the total hydroxyl groups minus those reduced.

It seemed probable that the stage of completion of reaction (2) above would give information concerning the type of grouping being reacted upon. This was based on the statement[6] given that the reactivity of alkyl halides toward hydrogen iodide is in the decreasing order; tertiary, secondary and primary.

Other Reactive Groups.—There are several types of functional groups that are readily at-

(6) Ogg, *ibid.*, **56**, 526 (1934).