THE CHEMICAL ISOLATION OF VITAMINES.

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The existence of at least three vitamines, namely the anti-scorbutic, the so called fat-soluble, and the antineuritic, is known at the present time. This paper deals principally with the antineuritic vitamine.

The important stage in the chemical investigation of this substance began with the classical research of Eijkman in 1897, who was able to show that rice polishings contain the antineuritic vitamine and that this substance is dialyzable and not precipitated from its solution by alcohol. Eijkman's work stimulated Funk to attempt the chemical isolation of the substance. In a series of researches he established the following facts. First of all he found that the curative substance is of a simple nature, as yeast can be hydrolyzed for 24 hours with 25 per cent sulfuric acid without causing the destruction of its vitamine. The active substance is precipitated by phosphotungstic acid and is mainly found in the silver nitrate-baryta fraction, when subjected to silver precipitation. This fraction contains three classes of biological products; namely, the histidine, pyrimidine, and nicotinic acid groups. On working up these fractions from yeast Funk isolated three substances which on analysis yielded the following formulas: \( \text{C}_{24}\text{H}_{19}\text{O}_{9}\text{N}_5 \), \( \text{C}_{26}\text{H}_{23}\text{O}_{9}\text{N}_5 \), and \( \text{C}_6\text{H}_5\text{O}_2\text{N} \) (nicotinic acid). By using the same or similar methods, Suzuki, Shimamura, and Odake, Vedder and Williams, and Edie, Evans, Moore, Simpson, and Webster were also able to isolate more or less active vitamine fractions from active raw material. Similar fractions were obtained by Funk from milk, brain, lime juice, rice polishings, and cod liver oil.
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The fact that the antineuritic vitamine is precipitated from its solution by reagents, which as a rule precipitate basic substances, and the observation that the active, purified fraction contained nitrogen led Funk to the adoption of the term vitamine, a terminology which future work may perhaps prove to be incorrect from the standpoint of chemical nomenclature.

The work of the chemical isolation of vitamine up to 1913 had thus led to a considerable increase in our knowledge of the chemical character of the antineuritic vitamine, but satisfactory methods for the preparation of the substance in a pure form and in large amounts were still lacking. In 1913 the United States Public Health Service extended its investigations of the etiology of pellagra on account of the menacing increase in this disease in the southern states. One of us (Voegtlin) began a study of the nutritional conditions in these districts, followed early in 1914 by a chemical study of the possible etiological factors of this disease. That the disease might be due to the deficiency of certain factors of the vitamine type as suggested by Funk had been early recognized by Voegtlin, who undertook extensive animal experiments in this connection. With the same purpose in view, work was also begun in this laboratory on new methods for the preparation of highly active vitamine preparation in relatively large quantities for the treatment of pellagra. As the first result of this work, Seidell succeeded in obtaining a very active preparation by treating filtered autolyzed yeast with fullers' earth. This active preparation can be obtained easily on a large scale and was used for the treatment of pellagra patients by Voegtlin, Neill, and Hunter.

In the experimental work, part of which the authors of the present paper are about to describe and which covered a period of several years, brewer’s yeast was also made use of as a raw material for the antineuritic vitamine. Nearby breweries furnished an ample supply of bottom yeast, which was pressed into a cake by means of a filter press before additional fermentation had taken place.

The yeast was then placed in a hot room (40°C.), in alcohol barrels as containers, and allowed to autolyze. 200 cc. of chloroform were used for every 100 pounds of yeast. Autolysis was usually complete in about 36 hours, at which time the thin liquid
was run through the filter press yielding a clear dark liquid with a specific gravity of about 1.01. This liquid was used as the source of material in the early work, even though it was finally found to be very unsatisfactory on account of its chemical complexity.

*Extraction with Olive Oil.*

When autolyzed yeast filtrate is treated with concentrated hydrochloric acid, a heavy, flocculent precipitate is obtained. It was found in connection with the yeast filtrates used in this laboratory that 40 cc. of concentrated acid were necessary for complete precipitation of 1 liter of filtrate. This material is easily filtered off and amounts to about 4.35 per cent of glue-like material, giving the common protein reactions. In all the work which is described in the following pages, the yeast filtrate was treated in this manner. It was found that this filtrate could be kept for a period of at least 2 years without losing its physiological activity, that it can be used for oral administration in the treatment of polyneuritis by neutralizing the acid with sodium hydroxide, and that it represents a suitable raw material for adsorption isolation experiments when the hydrogen ion concentration is adjusted by means of sodium borate.

In view of the fact that in nature the antineuritic vitamin is closely associated with lipoids, the idea suggested itself that this vitamin might be soluble in fats.

Autolyzed yeast filtrate was shaken with olive oil on a shaking machine until an emulsion was formed, with 1 cc. of olive oil for each 4 cc. of yeast filtrate. The emulsion was allowed to stand until two distinct layers were formed and by means of a separatory funnel the oil layer was separated. The oil was filtered to remove a small amount of sediment and then taken up in eight or ten volumes of ether. 0.1 per cent hydrochloric acid was used for the extraction of the ether solution. The acid extract was slightly pigmented and was concentrated in vacuum. A heavy precipitate was obtained with phosphotungstic acid, giving a deep blue color after the addition of sodium carbonate. An insoluble precipitate is formed with picric acid. The biuret
test is negative. The extract promptly relieves the polyneuritic symptoms of pigeons.

The same results are obtained by the use of oleic acid instead of olive oil.

**Dried Yeast.**

After several unsuccessful attempts to remove impurities from the products obtained by extraction, the following method was used with a considerable degree of success. Dried brewer's yeast was selected as the source of material. The yeast as it was obtained from the brewery was pressed until it crumbled easily between the fingers. It was broken into small particles and dried with a current of air at ordinary temperature, which required 36 to 48 hours. This dried product retains its activity for many months if stored in a dry place.

Dried yeast prepared in this way was ground in a mill repeatedly until a very fine powder was obtained. This was placed in a balloon flask of suitable size with reflux condenser, and extracted with 95 per cent methyl alcohol in the proportion of 2 cc. of alcohol for each gm. of yeast. 1 cc. of concentrated hydrochloric acid was added for each liter of alcohol used in the extraction. The contents of the flask were heated to boiling on the water bath for 3 hours and the soluble part was filtered off by means of suction. The residue was washed once with 1 cc. of hot methyl alcohol per gm. of yeast: The yeast was then extracted a second time using the same proportions of solvent. The extracts were combined and the alcohol was removed in vacuum at 35°C. The wax-like residue was repeatedly extracted with ether and 0.1 per cent hydrochloric acid, the volume being kept as small as possible. The final acid extract should never exceed 2 cc. for each gm. of yeast. The acid aqueous extract is always tested on polyneuritic birds for its activity and then it is purified as described below.

Hot aqueous silver acetate is added to the extract until precipitation is complete by testing a small portion in a test-tube. This purine precipitate, which also contains AgCl, is carefully washed with distilled water. Only a small amount of the active material passes over into this purine fraction.
A large excess of silver acetate was then added to the filtrate from the purine precipitate, followed by saturated barium hydroxide solution until the mixture was distinctly alkaline to litmus. The precipitate was filtered and carefully washed with cold distilled water. Considerable precipitate is produced in this manner, and additional precipitate may be obtained by further addition of baryta.

The silver-baryta precipitate containing the vitamine and some other extraneous material was suspended in water and made distinctly acid with sulfuric acid. The precipitate was then decomposed with hydrogen sulfide with occasional stirring in order to break up any lumps. A slightly pigmented filtrate was obtained after removing the silver sulfide. The excess hydrogen sulfide was removed in vacuum and the filtrate was then treated with a slight excess of lead acetate to remove the sulfuric acid. The lead was removed by hydrogen sulfide and the filtrate concentrated in vacuum at 35°C, with a small amount of ethyl alcohol to aid the distillation. Up to this stage practically none of the activity is lost. This concentrated solution of vitamine and impurities was then treated with mercuric sulfate prepared according to the directions of Kossel and Patten. A pale yellow precipitate was formed upon the addition of the mercuric sulfate, which was filtered off and washed with a small amount of ice water. This precipitate represents the histidine fraction and does not contain active material. The filtrate containing an excess of mercuric sulfate was treated with absolute ethyl alcohol until precipitation was complete. This precipitate was pale yellow and contained the bulk of the active material. The degree of separation in this last procedure is influenced by two factors, (1) the concentration of excess mercuric sulfate present and (2) the final concentration of ethyl alcohol. If these adjustments are right, none of the active material remains in the alcoholic filtrate. The alcohol-insoluble precipitate was suspended in water and the mercury was removed by means of hydrogen sulfide. The filtrate from the mercuric sulfide was freed of hydrogen sulfide in vacuum. The sulfuric acid was removed by means of lead acetate, and the excess lead by hydrogen sulfide.

By eliminating the first lead treatment the process might be shortened, but it was found that this would lead to considerable pigment passing over into this fraction.
This solution was concentrated in vacuum at a low temperature. This fraction gives a purple solution when tested with ninhydrin. Phosphotungstic acid gives a heavy precipitate, slightly soluble in an excess of the reagent, with only a slight blue color upon the addition of saturated sodium carbonate. With diazotized sulfanilic acid and sodium carbonate a reddish brown color was obtained. The biuret test was negative. The solution does not yield a precipitate with picric acid. The solution was highly active when tested on polyneuritic birds. If the solution is concentrated in vacuum, over soda lime, a definitely crystalline product is obtained which shows activity as long as the crystals are surrounded with the mother liquor. The material was soluble in methyl alcohol and yielded a soluble hydrochloride. As soon as the crystals are washed with absolute ethyl alcohol and dried, the physiological activity is lost and the crystal form changes from spindles to prisms. When the prisms are redissolved in a relatively large volume of water and again allowed to crystallize, the spindle-shaped crystals are reformed. It seems probable that there are at least two substances in the final solution both of a distinctly basic character. Work is being continued on this subject, the material being tested as a protective product. It is realized that the activity may be in the non-crystallizable mother liquor as well as in the crystals.

One of the impurities is a histamine-like substance, as shown by the positive Pauly reaction. Voegtlí and Myers have recently shown that the active fractions possess physiological activity when introduced intravenously into dogs. There is a fall of blood pressure and a stimulation of pancreatic and bile secretion following the injection of this material. It is possible that this stimulating action on pancreatic secretion and the fall in blood pressure are actually due to the presence of traces of histamine or a histamine-like substance in these fractions, as according to Abel and Kubota histamine is very widely distributed in animal tissues and probably also in vegetable cells.

**SUMMARY.**

Autolyzed yeast filtrate on account of its complexity represents an unsatisfactory material for the chemical isolation of the antineuritic vitamine. Mastic, Lloyd's reagent, and ferric phos-
phate, in experiments which, because of their negative outcome, it has not seemed desirable to detail, have been found unsatisfactory adsorbing reagents because they lack specificity. These reagents remove also inactive basic material which cannot be separated by our present methods from the active material. Olive oil and oleic acid remove the antineuritic substance from autolyzed yeast filtrate, thus showing that it is fat-soluble as well as water-soluble in the form of a crude extract. Stachydrine, trigonelline, and allied betaines show no antineuritic activity. Histidine and its esters are likewise inactive.

The active material is readily extracted from dried yeast by means of acid methyl alcohol. It can be purified by use of the Funk silver method and the mercuric sulfate procedure, yielding an apparently crystalline active substance. This substance becomes inactive upon drying and it is believed that impurities still remain which can be removed with additional modifications of the method described in this paper. The present method eliminates purines, histidine, proteins, and albumoses, leaving a liquid that can be crystallized and probably contains histamine or histamine-like substances. The physiological action of the active fractions resembles that of extracts obtained from the mucosa of the small intestine, when the intestinal and yeast extracts are purified in the same manner.

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