THE ISOLATION OF p-AMINOBENZOIC ACID FROM YEAST

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The present investigation is primarily concerned with the isolation of p-aminobenzoic acid from yeast. The presence of this substance in yeast was adumbrated by Woods (1) who made the important discovery that this aromatic amino acid possesses the unique property of antagonizing the bacteriostatic action of sulfanilamide and that the component of yeast extracts which exerts a similar antisulfanilamide action has properties consonant with those of p-aminobenzoic acid. Subsequently Rubbo and Gillespie (2), utilizing a procedure involving the benzylation of a suitably prepared yeast extract, succeeded in isolating from 30 kilos of yeast 2 mg. of p-benzoylaminobenzoic acid whose identity was established only by its melting point.

Determinations of the antisulfanilamide activity of yeast extracts conducted by Woods (1) indicate that if such activity is due solely to p-aminobenzoic acid the concentration of this substance in yeast must be very considerably greater than that indicated by the isolation mentioned above. In agreement with this we have found upon colorimetric analysis that commercial pressed bakers' yeast contains a quantity of diazotizable aromatic amine equivalent to approximately 0.5 mg. per cent when calculated as p-aminobenzoic acid. In consequence it appeared desirable to undertake the present investigation in order to establish definitely the nature of the aromatic amine present in yeast and to gain a more precise estimate of the quantity of p-aminobenzoic acid present.

At the outset it appeared that the isolation procedure might be materially facilitated by starting with a commercial yeast extract of the type used as a source of the vitamin B complex. Subse-
p-Aminobenzoic Acid from Yeast

Consequently, by a modified procedure involving the formation of the slightly soluble acetyl derivative of p-aminobenzoic acid, it was found that this substance may be isolated with relative ease from commercial pressed yeast. Nevertheless, the isolation from the yeast concentrate will be described, since it led to the isolation of p-aminobenzoic acid as such and to its unambiguous identification.

In connection with our experiments with yeast it has been observed that yeast contains not only p-aminobenzoic acid as such but also some substance, possibly a peptide, from which this aromatic amino acid is liberated during autolysis. This is indicated by the observation that upon autolysis the quantity of diazotizable amine present in cytolyzed yeast increases and that following such autolysis p-aminobenzoic acid may be isolated from yeast, as the acetyl derivative, in greater amount than is possible before such enzymatic degradation.

**EXPERIMENTAL**

*Isolation of p-Aminobenzoic Acid from Yeast Extract*—The yeast extract used in the present experiment was Fleischmann’s Type III. For a generous supply of this material we are indebted to Dr. Charles N. Frey of The Fleischmann Laboratories, New York, who informed us that this extract is prepared by the extraction of fresh yeast with aqueous alcohol and subsequent evaporation of the solvent in such a way that each gm. of the extract represents the extractives of approximately 9 gm. of yeast.

Analysis of this extract showed it to contain about 3.8 mg. per cent of arylamine calculated as p-aminobenzoic acid. This analysis, and all others of a similar nature referred to in this paper, was conducted by the method of Bratton and Marshall (3) originally devised for the determination of sulfanilamide and related drugs in body fluids but equally applicable to the determination of all diazotizable amines.

As a result of a number of preliminary experiments whose details will be omitted, it was found that the arylamine present in the yeast extract mentioned above could be most readily separated from the bulk of other substances in the extract in the fashion described below, which eliminates the relatively large losses of material which inevitably accompany more complex techniques of isolation.
1 liter of 95 per cent alcohol was added to 1000 gm. of the finely powdered yeast extract contained in a 5 liter flask. The mixture was shaken to form a homogeneous paste, made definitely acid to litmus by the addition of 10 N sulfuric acid; then 1 liter of ether was added immediately and the mixture vigorously shaken for 15 minutes, with care that all lumps of material were broken up. The resulting mixture was allowed to stand 3 hours with occasional shaking and then filtered with suction. The marc was returned to the flask and vigorously shaken for 10 minutes with 750 ml. of ether and again filtered. This operation was repeated once more with a like volume of ether and the marc finally washed on the filter with two 50 ml. portions of ether. The combined filtrates from this extraction were turbid, owing to the dilution of the initial clear filtrate with ether. This turbidity was readily removed by admixture with a small quantity of Filter Cel and subsequent filtration. A second 1000 gm. portion of the yeast extract was treated in precisely the same fashion and the alcohol-ether solutions resulting from the two extractions were combined and the solvents distilled off with the aid of a water bath.

The resulting residue was taken up in 500 ml. of water, ammonia added in quantity sufficient to bring the pH to approximately 8, and the mixture placed in an ice box overnight to permit congelation of some insoluble material which appeared to be of lipid nature, somewhat fluid at room temperature. Following removal of this material by filtration there remained a straw-colored solution having a distinct fluorescence. Analysis of an aliquot of this solution by the method (3) previously mentioned showed it to contain 65.3 mg. of arylamine calculated as p-aminobenzoic acid or 86 per cent of the amount estimated to be initially present in the 2000 gm. of yeast extract. A determination of the total solids present showed that the solution contained 4.26 gm. or 0.21 per cent of the original solids.

The solution was then treated with a slight excess of a saturated solution of basic lead acetate. The resulting precipitate was removed by centrifugation and the supernatant fluid neutralized with sulfuric acid and filtered, a procedure which did not involve the loss of any of the arylamine present. The solution was evaporated on the steam bath to a volume of approximately 100 ml. The resulting solution, which had darkened considerably during
this evaporation, was brought to pH 3.8, the isoelectric point of p-aminobenzoic acid (4), and extracted in a separatory funnel with five 100 ml. portions of ether.

The combined ether extracts were distilled down to a volume of approximately 50 ml. when 25 ml. of water were added and the remainder of the ether distilled off. The resulting solution was mahogany-colored. Upon analysis it was found to contain but 59 mg. of p-aminobenzoic acid. Since the aqueous solution following extraction with ether as described above was found upon analysis to be free of arylamine, it appears that a portion of the arylamine present was destroyed during the process of evaporation.

The solution was made alkaline to litmus with ammonia, clarified with a slight excess of basic lead acetate, filtered, and the pH brought to 3.8 by the addition of sulfuric acid. Following removal of the precipitated lead sulfate, the solution was extracted five times with a like volume of ether. In order to minimize the further production of colored substances, the ether extracts were evaporated at a temperature not exceeding 35° in a small flask containing 5 ml. of water.

Following the removal of the ether the solution was brought to pH 3.8 and allowed to stand overnight, when crystals appeared. The contents of the flask were transferred to a centrifuge tube, chilled in ice water, and the crystals separated by centrifugation. The crystals were dissolved in a minimum volume of hot water and the solution transferred to a weighed Emich cone. After crystallization had taken place, the crystals were centrifuged off, washed with a few drops of water, and the cone and its contents were dried at 100° and weighed.

The recrystallized product weighed 7 mg., melted at 186.4° (corrected), and caused no depression in the melting point of an authentic sample of p-aminobenzoic acid. A portion of the recrystallized product (4 mg.) was converted to the picryl derivative by interaction with picryl chloride in the conventional manner (5). After recrystallization from hot glacial acetic acid this derivative melted at 300–301° (corrected, 287–288° uncorrected) and caused no depression in the melting point of an authentic sample of p-picrylaminobenzoic acid.

The mother liquors, from which the crude p-aminobenzoic acid
K. C. Blanchard

initially separated, were combined with the mother liquors resulting from the recrystallization of this substance, evaporated to a volume of 3 to 4 ml., and shaken with a few crystals of sodium acetate and 0.2 ml. of acetic anhydride until the latter passed into solution. When the solution had stood overnight in the ice box, crystals separated. These were centrifuged off, washed with a few drops of cold alcohol which removed the adherent brown mother liquors, and recrystallized from a minimum of boiling water. This recrystallization involved the loss of considerable material but was necessary in order to obtain a colorless product. In this way 11 mg. of $p$-acetylaminobenzoic acid (equivalent to 8.4 mg. of $p$-aminobenzoic acid) were obtained. The product melted with decomposition at 259.5° (corrected) and caused no depression in the melting point of an authentic sample of $p$-acetylaminobenzoic acid.

**Isolation of $p$-Aminobenzoic Acid from Yeast before and after Autolysis**—Rubbo and Gillespie (2) claim that $p$-aminobenzoic acid is a growth factor for *Clostridium acetobutylicum* and related anaerobes. Previously, Weizmann and Rosenfeld (6), while attempting to isolate such a factor from yeast, had observed that autolysis of yeast led to an increase in the content of the *Clostridium* growth factor. Taken together these observations suggested that possibly yeast contains $p$-aminobenzoic acid in a combined form as well as in the free state and that the latter is liberated from the former during the course of autolysis. Such proved to be the case, as is shown in the following experiment wherein this increase in the content of $p$-aminobenzoic acid was followed both colorimetrically and by isolation.

Fresh commercial pressed bakers’ yeast (1200 gm.) was plasmolyzed with 120 ml. of ethyl acetate. When the mass had become fluid, it was allowed to stand at room temperature for $\frac{1}{2}$ hour and then during the next 2 hours a 10 per cent solution of trisodium phosphate was added from time to time at a rate such that the mixture remained substantially neutral to litmus. This required 248 ml. of the phosphate solution. The resulting mixture was divided into two equal portions by weight. One portion was placed in an incubator at 37° and the other was heated to 80° for approximately 10 minutes to inactivate the enzymes present.

For colorimetric analysis 10 ml. portions were withdrawn and
precipitated with 4 ml. of 12.5 per cent trichloroacetic acid and
the analysis conducted by the method previously specified. These
analyses disclosed that the initial mixture had an arylamine con-
tent, calculated as p-aminobenzoic acid, of 0.47 mg. per 100 gm.
of yeast. After 4 days autolysis the corresponding value had
become 0.79 mg. per 100 gm., while the arylamine content of the
heated mixture remained unchanged. No further increase in
the arylamine content occurred during a further 3 day incubation.

While it is a reasonable assumption that the increment in aryla-
mine content occasioned by autolysis is due to the liberation of
p-aminobenzoic acid from some precursor, it appeared desirable
to confirm this by direct isolation of this substance. In the
absence of any specific precipitant, suitable for the isolation of
p-aminobenzoic acid in a quantitative fashion, the two prepara-
tions of plasmolyzed yeast described above were subjected to the
same treatment on the assumption that the manipulative losses
involved would be essentially the same in both cases. In the
procedure utilized for this purpose, which was first tried out on
another sample of yeast, the p-aminobenzoic acid was isolated as
the acetyl derivative, not as the free acid whose isolation as such
is capricious when very small quantities are involved. Both of
the yeast preparations were treated as described below.

The mixture was made acid to Congo red by the addition of 10
N sulfuric acid, 1.5 liters of alcohol added, and the mixture vigor-
ously shaken for 10 minutes and allowed to stand overnight.
The supernatant liquid was decanted, and the residue was trans-
ferr ed to centrifuge bottles with the aid of 100 ml. of alcohol, and
centrifuged. The residue was then shaken up with 200 ml. of
a 50:50 alcohol-ether mixture and again centrifuged. This process
was repeated twice more, with 175 ml. of the alcohol-ether mix-
ture each time.

The ether was distilled off and the extract concentrated in
vacuo to a volume of 75 ml. This was made faintly alkaline
with ammonia chilled in ice and centrifuged free of solids which
had separated. The deep yellow supernatant fluid was brought
to pH 3.8 with sulfuric acid and extracted four times with an
equal volume of ether. The ether extracts were evaporated in a
small flask containing 10 ml. of water. This solution, which upon
cooling deposited some lipid, was clarified with a slight excess of
basic lead acetate, the resulting precipitate removed by centrifugation, and the supernatant fluid extracted with ether as before after adjustment of the pH to 3.8.

The ether extract obtained at this point was evaporated in a previously weighed 3 ml. Emich cone containing 0.7 ml. of water. The resulting solution was acetylated by being stirred with 2 drops of acetic anhydride. Upon scratching, crystals separated; the tube was placed in crushed ice for an hour, then centrifuged. The sedimtnent crystals were twice washed with 2 drops of 50 per cent alcohol and the tube and contents dried at 100° and weighed.

In this fashion 2.1 mg. of p-acetylaminobenzoic acid were obtained from 600 gm. of the plasmolyzed and heated yeast, while 3.7 mg. of the same substance were obtained from a like quantity of the autolyzed yeast. These quantities are equivalent respectively to 1.6 and 2.8 mg. of p-aminobenzoic acid and account for 57 and 60 per cent of the amounts of p-aminobenzoic acid initially estimated by the colorimetric reaction to be present in the respective yeast preparations. In both instances the identity of the acetylated product was established by means of a mixed melting point with authentic p-acetylaminobenzoic acid.

DISCUSSION

The experiments described above show that yeast contains p-aminobenzoic acid in both a free and a combined form. As yet we have no information concerning the precise nature of the latter, but since this substance yields p-aminobenzoic acid during the course of autolysis it would appear to be a peptide. If such is the case, the aromatic amino group must be involved in the peptide link, since this substance is not diazotizable prior to autolysis. Recently, Loomis, Hubbard, and Neter (7) have obtained from yeast a fraction containing an entity which inhibits the bacteriostatic action of sulfanilamide but which is insoluble in ether, is not diazotizable, and is not inactivated by acetylation. Such behavior would be expected of a peptide derived from p-aminobenzoic acid and it may be that the antisulfanilamide factor encountered by Loomis et al., is identical with the substance which gives rise to p-aminobenzoic acid during the autolysis of yeast.
926

p-Aminobenzoic Acid from Yeast

SUMMARY

1. p-Aminobenzoic acid has been isolated from yeast and definitely characterized.

2. Evidence has been presented which indicates that yeast also contains a combined form of p-aminobenzoic acid from which this substance is liberated during autolysis.

The writer is indebted to Dr. E. K. Marshall, Jr., for suggesting this investigation, for frequent advice, and for the hospitality of his laboratory during a leave of absence from New York University.

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THE ISOLATION OF p-AMINOBENZOIC ACID FROM YEAST
Kenneth C. Blanchard


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