# A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF CHOLINE BY USE OF A MUTANT OF NEUROSPORA

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Previous communications from this laboratory have described the production of biochemical mutants in the mold *Neurospora* by means of ultraviolet and x-rays (1, 2). Such mutants are characterized by the inability to carry out specific chemical syntheses which normally occur in the unmutated, or wild type, strain. In each case which has been genetically analyzed the failure of the synthesis has been found to be related to the mutation of a single gene. The strain to be described, known as No. 34486, or *cholineless*, arose from a culture of wild type *Neurospora crassa* which had been irradiated with ultraviolet light. It was found to be unable to grow in a medium containing only salts, sugar, and biotin, but it grew normally on the addition of a mixture of water-soluble vitamins. When the components of the mixture were tested singly, it was found that the addition of choline alone permitted normal growth.

Up to the present, no completely satisfactory method for the determinaton of choline in natural products and tissue extracts has been described. Chemical methods, such as precipitation of the reineckate, lack specificity, while the biological method of Fletcher, Best, and Solandt (3) is time-consuming and difficult, and "possesses many dangerous pitfalls for the chemist" (4). The whole subject has been critically reviewed by Best and Lucas (4). It was therefore of interest to determine whether the *Neurospora* mutant is a suitable test organism in a quantitative assay for choline. The experiments to be described show that this is the case and form the basis of a simple, sensitive, and specific method for the determination of choline in natural products. By this procedure it is possible to determine choline in a concentration of 0.02 mg. per liter; routine analyses can be run on 100 mg. samples of material.

## *Methods*

The basal medium used in these experiments has the following composition, in gm. per liter: ammonium tartrate 5, ammonium nitrate 1, monobasic potassium phosphate 1, magnesium sulfate ($7H_2O$) 0.5, sodium chloride 0.1, calcium chloride 0.1, sucrose 20, biotin $5 \times 10^{-6}$. In addition, it contains the following trace elements, added as salts, in mg. per liter: B 0.01, Mo 0.02, Fe 0.2, Cu 0.1, Mn 0.02, Zn 2.0. The medium is

made up without sucrose and biotin, is autoclaved, and is stored in 1 liter bottles. Sucrose and biotin are added before the medium is used. For convenience in storing, it may be made 3 times as concentrated as indicated and diluted before use.

Stock cultures of the mutant are maintained on agar slants composed of the basal medium plus the following: agar 1.5 per cent, Difco yeast extract 0.2 per cent, malt extract 0.2 per cent, choline 1 $\gamma$ per ml.

In the assay for choline, the mold is grown in 250 ml. Erlenmeyer flasks containing 25 ml. of medium. To inoculate, a spore suspension is made up in a few ml. of sterile distilled water, and 1 drop of the suspension is added to each flask. The flasks are incubated at 25° for 3 days, at the end of which time the pads are removed, pressed out on filter paper, and dried at 90°. They are then weighed to the nearest half mg.

## *Results*

*Growth Studies*—The growth rate of *cholineless* is a function of the concentration of choline in the medium. A typical growth response curve is shown in Fig. 1. The normal, *i.e.* wild type, growth rate is attained at a concentration of 50 to 60 $\gamma$ of choline per 25 ml. Under the conditions of these experiments the weight of the pads after a 3 day growth period is practically independent of the size of the inoculum. Thus, at a concentration of 2 $\gamma$ of choline per 25 ml. a 16-fold increase in inoculum size raised the dry weight from 12 to 16 mg.; at a level of 30 $\gamma$ of choline no increase in dry weight occurred.

The specificity of the response was tested with a wide variety of compounds. Of the eleven water-soluble vitamins and twenty-two amino acids tested only choline and methionine were found to be active. Methionine is approximately 0.002 as active as choline. The activity is not increased by the simultaneous addition of ethanolamine. Lecithin was the only other compound found to be active. Using a sample of pure lecithin, we found that 50 per cent of the potentially available choline was utilized in a 3 day period. The following substances related to choline were inactive: betaine, creatine, sarcosine, ethanolamine, dimethylamine, trimethylamine, and tetramethylammonium chloride.

The maximum growth obtainable with methionine after 3 days is considerably below that reached with choline. This is shown in Fig. 2, the data for which were obtained with analytically pure, synthetic *dl*-methionine. This result is interpreted as indicating a sparing action of methionine on the small amount of choline present in the inoculum. Such an interpretation is in accord with the known relationship between choline and methionine in the rat (5, 6). By providing an extra source of labile methyl groups, the addition of methionine permits choline to be used for other

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essential purposes, such as the synthesis of lecithin. On this basis, it is to be expected that the effect of the simultaneous addition of methionine and choline (at a suboptimal level) will be greater than the sum of the individual effects. This was found to be the case (Table I). The most striking evidence for this conclusion is furnished by the growth of *cholineless* on agar medium in horizontal tubes. In the presence of a given concentration of choline the rate of progression of the mycelial frontier, in mm. per hour, along the surface is a constant and continues at the constant rate to the end of the tube. If, instead of choline, methionine is supplied, the rate

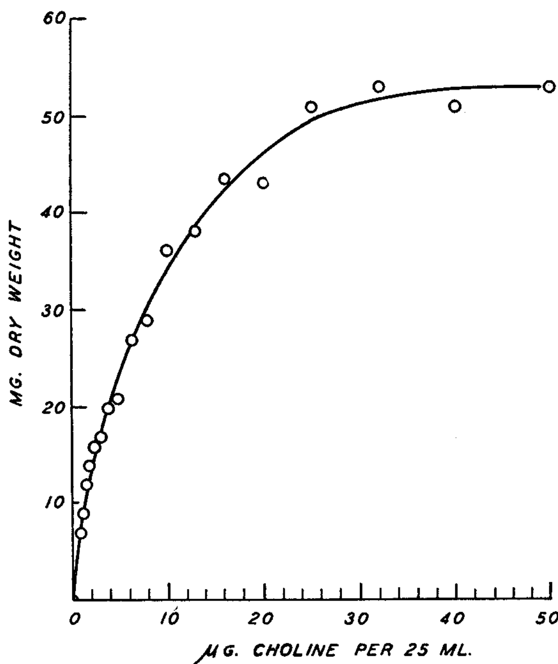


Fig. 1. Dry weight of *cholineless* after 3 days as a function of the concentration of choline in the medium.

first attains a characteristic value depending on the concentration of methionine and then falls off to zero before the end of the tube is reached. This shows that methionine can replace choline in some, but not all, of its functions; as soon as the store of available choline in the inoculum is exhausted, growth ceases. The methyl group of methionine is apparently not used for choline synthesis by the mutant.

*Preparation of Materials for Assay*—To assay natural products with *cholineless*, the sample is first autoclaved with 3 per cent sulfuric acid for 2 hours at 15 pounds. This treatment liberates choline from lecithin which,

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possibly because of its low solubility, is but slowly utilized by the mutant. Table II shows that, in the materials tested, hydrolysis is complete within 2 hours and may be continued without loss of choline for at least 3.5 hours. Refluxing with 3 per cent sulfuric acid for 7 hours gave essentially the same

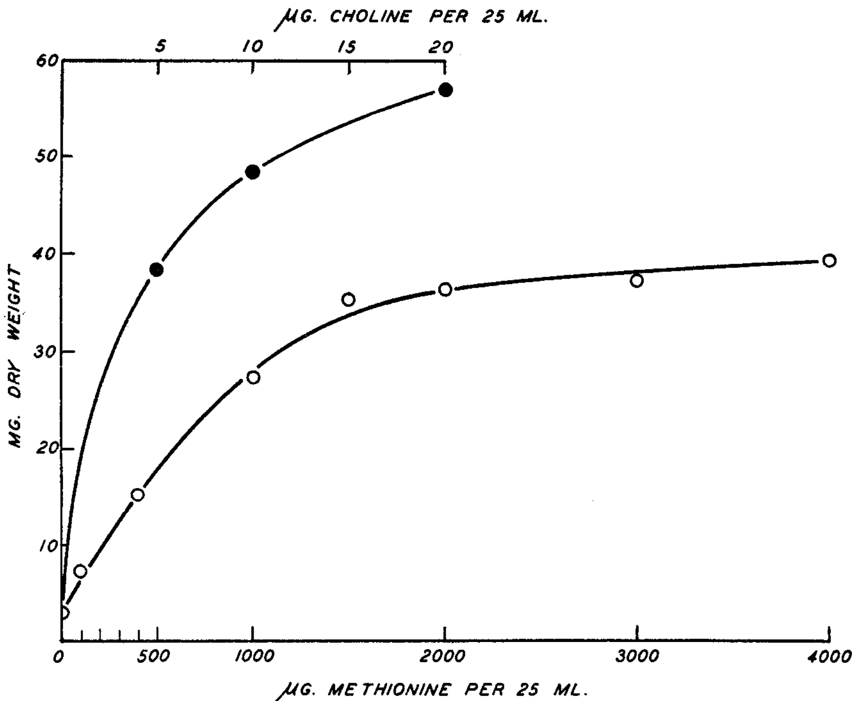


FIG. 2. Growth of *cholineless* on methionine after 3 days (open circles). The solid circles show the growth curve on choline obtained simultaneously.

TABLE I

*Effect of Simultaneous Addition of Choline and Methionine on Growth of Cholineless*

Quantities are expressed in mg. of dry weight of mold after 70 hours at 25°.

| Choline per 25 ml. | Mg. *dl*-methionine per 25 ml. | | | |
|---|---|---|---|---|
| | 0 | 0.50 | 1.00 | 2.50 |
| *mg.* | | | | |
| 0 | 0.5 | 7.0 | 10.5 | 30 |
| 0.002 | 17 | 34.5 | 46 | 62 |
| 0.030 | 60 | 61.5 | 64.5 | 75 |

result as autoclaving. Following neutralization with barium hydroxide, the solution is treated with permutit[1] in order to separate choline from

[1] Permutit (according to Folin), obtained from The Coleman and Bell Company, Norwood, Ohio.

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methionine. *dl*-Methionine interferes with the choline assay when present in excess of 0.1 mg. per 25 ml. of culture medium. Although this concentration is fairly high, considering the conditions of mild hydrolysis and high dilution in the assay, it may be exceeded in those cases in which the material being tested contains much protein and little choline. In any case, the permutit treatment is recommended for the reason that it eliminates all non-basic substances which may inhibit or stimulate the growth of the mold.

TABLE II

*Liberation of Choline from Natural Products by Various Treatments*

100 mg. samples were hydrolyzed with 10 ml. of 3 per cent sulfuric acid by the procedures indicated. Choline content is expressed in micrograms per 100 mg.

| Material | No treatment | Autoclaving | | | | | Refluxed 7 hrs.* |
|---|---|---|---|---|---|---|---|
| | | 1 hr. | 1.75 hrs. | 2.0 hrs. | 2.5 hrs. | 3.5 hrs. | |
| Dried brewers' yeast | 48 | | | 262 | | 258 | 266 |
| " milk | 60 | | | 81 | | 83 | 88 |
| White flour | 16 | 120 | 125 | 119 | 132 | 130 | 138 |
| Corn-meal | 10 | 31 | 35 | 34 | 28 | 37 | 37 |

* The hydrolysates were not treated with permutit.

TABLE III

*Elution of Choline from Permutit*

5 ml. of a solution containing 20 $\gamma$ of choline per ml. were passed through permutit columns, followed by 5 ml. of 0.3 per cent sodium chloride. Sodium chloride solutions in the amounts and concentrations indicated were then passed through and the filtrates assayed with *cholineless*.

| Sodium chloride solution | | Choline eluted |
|---|---|---|
| Concentration | Amount | |
| *per cent* | *ml.* | *per cent* |
| 0.3 | 5 | 0 |
| 1.0 | 10 | 55 |
| 2.0 | 10 | 84 |
| 3.0 | 5 | 80 |
| 3.0 | 10 | 90 |
| 5.0 | 5 | 80 |
| 5.0 | 10 | 100 |

The adsorption is carried out in columns measuring 110 × 0.6 mm., containing approximately 1 gm. of permutit. The design of these columns is given by Dubnoff and Borsook (7). The best conditions for adsorption and elution of choline were determined by running known solutions through

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the columns and testing the filtrates with the mutant. It was found that a permutit column of the above dimensions completely removes the choline from 5 ml. of a solution containing up to 0.5 mg. of choline per ml. Repeated tests have shown that adsorbed choline is quantitatively eluted with 10 ml. of 5 per cent sodium chloride. The results of eluting with various sodium chloride solutions are shown in Table III.

Methionine is not adsorbed by permutit. Formol titrations made on solutions of methionine before and after passing through a permutit column, followed by washing with 0.3 per cent sodium chloride, gave quantitative recoveries.

*Procedure*

Details of the procedure which has been used in assaying for choline are as follows:

100 mg. of the dry material to be analyzed are weighed into a 50 ml. Erlenmeyer flask, followed by 10 ml. of 3 per cent sulfuric acid. The flask is plugged with cotton and autoclaved at 15 to 17 pounds for 2 hours.

After cooling, the contents are transferred quantitatively to a 50 ml. Pyrex centrifuge tube and neutralized to Congo red with saturated barium hydroxide. The barium sulfate, together with the undissolved residue remaining from the previous step, is centrifuged down, and the supernatant is filtered through a Whatman No. 50 paper. 3 ml. of distilled water are added to the precipitate in the centrifuge tube and the contents brought to a boil, with stirring. After cooling and centrifuging, the washing is added to the previous supernatant. The clear filtrate is neutralized to litmus with M sodium hydroxide. It is then brought to a convenient volume, usually 30 ml., with distilled water.

5 ml. of the neutralized solution are run through a column of permutit of the dimensions described above. If the solution is known to contain less than 3 $\gamma$ of choline per ml., 10 ml. are usually run through. The column is then washed with 5 ml. of 0.3 per cent sodium chloride. The filtrate and washing are discarded. A test-tube marked at 10 ml. is now placed under the column, and the choline is eluted with 10 ml. of 5 per cent sodium chloride. The filtrate is brought to 10 ml. with distilled water. It is usually convenient to adsorb two or more portions of the solution simultaneously, in separate columns; in this way, sufficient filtrate is provided for an orienting assay in case the choline content is completely unknown.

The solution is distributed among 250 ml. Erlenmeyer flasks, and the volume in each flask is made up to 25 ml. with basal medium. Usually not more than 5 ml. of the solution being tested is added to a flask. For best accuracy, the final concentration of choline should lie between 0.5

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and 20 γ per 25 ml. Each concentration of unknown is made in duplicate. At the same time a standard series is set up containing pure choline in a range of concentrations from 0 to 20 γ per flask. The flasks are autoclaved at 15 pounds for 5 to 10 minutes. After cooling, they are inoculated, placed in the incubator, and the dry weight of the mycelium determined at the end of 3 days. The choline values are calculated in the usual way from a plot of the standard series.

Table IV

*Within-Series Reproducibility of Choline Assays and Recoveries of Added Choline*

| Material | Filtrate | Choline added | Duplicate dry weight of mold | Choline found | | Recovery of added choline |
|---|---|---|---|---|---|---|
| | | | | Per flask | Per 100 mg. material | |
| | *ml.* | γ | *mg.* | γ | γ | γ |
| Dried brewers' yeast | 0.5 | 0 | 22, 23 | 2.2 | 264 | |
| " " " | 1.0 | 0 | 31, 31 | 4.3 | 258 | |
| " " " | 2.5 | 0 | 43, 44.5 | 11.0 | 264 | |
| " " " | 0.5 | 5.0 | 38, 40 | 7.2 | | 5.0 |
| " " " | 2.5 | 5.0 | 47, 48 | 16.4 | | 5.4 |
| White flour | 0.5 | 0 | 20.5, 22 | 2.0 | 120 | |
| " " | 1.0 | 0 | 28.5, 30.5 | 3.8 | 114 | |
| " " | 2.5 | 0 | 43, 43 | 10.2 | 122 | |
| " " | 0.5 | 5.0 | 38.5, 39 | 7.0 | | 5.0 |
| " " | 2.0 | 5.0 | 46.5, 46.5 | 15.0 | | 6.8 |
| Corn-meal | 2.0 | 0 | 22.5, 23.5 | 2.3 | 34.5 | |
| " | 3.0 | 0 | 27.5, 28 | 3.4 | 34 | |
| " | 6.0 | 0 | 34.5, 35.5 | 5.5 | 27.5 | |
| " | 2.0 | 5.0 | 39.5, 40 | 7.5 | | 5.2 |
| " | 6.0 | 5.0 | 41.5, 40.5 | 8.5 | | 3.0 |
| Dried milk | 1.0 | 0 | 24.5, 26.5 | 2.8 | 84 | |
| " " | 2.0 | 0 | 35.5, 36 | 5.8 | 87 | |
| " " | 5.0 | 0 | 43.5, 45.5 | 12.2 | 73 | |
| " " | 1.0 | 5.0 | 39.5, 40.5 | 7.6 | | 4.8 |
| " " | 4.0 | 5.0 | 46, 47.5 | 15.4 | | 4.6 |

By the above procedure, choline assays on ten different samples have been carried out simultaneously by one worker.

*Reproducibility*—Dry weights from duplicate flasks agree within 5 per cent, on the average. Choline values determined on different amounts of the same solution generally agree within 10 per cent. Recoveries of added choline are usually within 90 to 110 per cent of theoretical. Table IV shows the results obtained on four different products, all regular commercial samples.

It has been found that standard curves obtained on different days are

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not, in general, superimposable. The variation is apparently not related to inoculum size, but possibly to the age and condition of the spores. This does not affect assay values, which show good day to day reproducibility. It is necessary, however, to run a standard series each time a new spore suspension is used.

In Table V is shown the choline content of a number of different natural products, as determined with *cholineless*. Where the results are expressed on both a dry and a wet weight basis, the samples were first dried to constant weight at 90°. In all other cases regular commercial products were used without further drying.

TABLE V

*Choline Content of Some Natural Products*

| Material | Choline content per 100 mg. | | Remarks |
|---|---|---|---|
| | Wet weight | Dry weight | |
| | γ | γ | |
| Bacon fat | 6.5 | | |
| " lean | 97 | | |
| Beet | 7.9 | 47 | Fresh; root only |
| Butter | 7.9 | | |
| Carrot | 6.7 | 45 | Fresh; root only |
| Coconut milk | 0.03 | | |
| Yellow corn-meal | | 34 | |
| Corn steep water concentrate | 254 | 381 | |
| " germ | | 67 | |
| Egg white | 0.3 | 2.2 | Hard boiled |
| " yolk | 1130 | 2170 | " " |
| Dried whole milk | | 81 | Commercial brand |
| *Neurospora* | 64 | 243 | Wild type |
| Potato | 19.5 | 67 | New potato; skin included |
| Polished rice | | 89 | |
| Rye flour | | 104 | |
| White wheat flour | | 119 | |
| Dried brewers' yeast | | 262 | Commercial brand |

## *Genetic Analysis*

*Cholineless* was crossed with the sex-linked character *albino*. The eight ascospores from each of twenty-one of the resulting asci were isolated in order and germinated on basal medium supplemented with choline. The cultures were then transferred to unsupplemented basal medium. Of each set of eight ascospores, four failed to grow in the absence of choline, showing that the mutation involved a single gene. In eleven sets the *cholineless*

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gene segregated in the first division, while ten sets showed second division segregation. This corresponds to a map distance of approximately 25 units from the centromere. The gene is not linked with sex or with *albino*.

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## SUMMARY

A microbiological method for the determination of choline, by use of an artificially produced mutant of *Neurospora crassa*, has been described. Of more than forty compounds tested, only choline, lecithin, and methionine were found to support growth of the mutant. A rapid procedure for the separation of choline from methionine in tissue extracts is given. The *Neurospora* method is simple, sensitive, and specific.

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