The Isolation of [^{35}S]Homocystine from *Neurospora*

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SUMMARY

Evidence is presented inferring accumulation of [^{35}S]homocystine in methionineless mutants of *Neurospora crassa*. Certain mutants formed five times as much homocystine as did the wild type; [^{35}S]homocystine was identified as homocystine and homocysteic acid.

INTRODUCTION

Extensive studies have shown the physiological activity of homocysteine in replacing methionine (Butz & Du Vigneaud, 1932; Horowitz, 1947, 1950; Shapiro, 1955; Schlenk & DePalma, 1955; Durell, Anderson & Cantoni, 1957). Thus the role of homocysteine as an intermediate in methionine biosynthesis has become accepted although it has never been isolated from biological material. Because homocysteine usually occurs only as a transitory intermediate in methionine biosynthesis it was felt that methionineless mutants of *Neurospora crassa* might accumulate homocysteine and would be particularly useful in attempts to show directly the occurrence of this compound. The experiments described below show that homocysteine occurs in *N. crassa* and in greater concentration in these mutants than in wild type, as reported in a preliminary note (Leinweber & Liverman, 1959). After this work was completed the authors learned of a communication on the isolation of homocystine from the human adrenal gland (Biserte, 1957); quantitative data, however, were not reported.

METHODS

Wild type strain 5297a and mutants 38706Ra and 80702Ra of *Neurospora crassa* were obtained from Dr M. Fling, Division of Biology, California Institute of Technology, Pasadena, California, U.S.A. Mutants T-27 and T-112 originated in the Genetics Laboratory of the University of Texas and were obtained from Dr Robert A. McRorie, University of Georgia, Athens, Georgia, U.S.A. Mutants 38706Ra, T-27 and T-112 do not grow on any precursor of methionine biosynthesis (Horowitz, 1947, 1950) but require methionine for growth. They are believed to lack an enzyme necessary for the biosynthesis of methionine from homocysteine. With regard to mutants T-27 and 38706Ra, at least two differences are known. Growth of mutant T-27 on methionine is enhanced by augmenting sulphate in the medium; in mutant 38706Ra, sulphate has no effect on growth in the presence of methionine. S-methyl-

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L-cysteine can serve as sole sulphur source for mutant T-27 but not for 38706Ra (Ragland & Liverman, private communication). Mutant 80702Ra does not grow on sulphate or sulphite and was proposed to have a genetic block between sulphite and thiosulphate (Horowitz, 1950).

The various strains of *Neurospora crassa* were grown on 20 ml. of sulphur-free minimal medium contained in 125 ml. Erlenmeyer flasks incubated at 25°. The medium was prepared by replacing the sulphates of the Difco Choline Assay Medium (*Difco Manual*, 1953) by the chloride salts in equimolar amounts with respect to the cation. Methionine was supplemented to all strains in a concentration of $5 \times 10^{-4} \text{M}$ which was found optimal for growth and was added aseptically after filter-sterilization. Radiosulphate, purchased from the Oak Ridge National Laboratories, was added either carrier-free or with non-radioactive potassium sulphate. The final concentration of radiosulphate in the medium was 8–15 $\mu\text{c./ml.}$, the specific radioactivity 150–174 $\mu\text{c./}\mu\text{mole}$ unless otherwise stated.

At the end of the growth period, the mycelium was harvested by filtration, freed from culture medium by suction, and repeatedly washed with distilled water. The filtrate was discarded unless otherwise stated. The mycelium was ground in a mortar with sand and 10 ml. of cold 10% (w/v) trichloroacetic acid (TCA), and the homogenate centrifuged. The pellet was twice resuspended and washed with 10 ml. of 5% (w/v) TCA and again centrifuged. The supernatant fluids were pooled and accurately weighed amounts of homocysteine (e.g. 1.5 m-mole; purchased from Nutritional Biochemicals Corporation, 21010 Miles Ave., Cleveland, Ohio, U.S.A.) + homocystine (0.75 m-mole; purchased from California Corporation for Biochemical Research, 3625 Medford St., Los Angeles 63, California, U.S.A.) were added in order to isolate [^{35}S]homocysteine formed in the mycelium. The homocysteine in the TCA extract was then oxidized to the disulphide by the following procedure which was developed in this laboratory. The extract was adjusted to pH 8–9 with sodium hydroxide solution. One drop 0.3M solution of ferric chloride was added per m-mole of homocysteine-carrier, and oxygen bubbled through the solution until oxidation was complete, as indicated by fading of the violet colour of the iron-sulphydryl complex. The solution was then adjusted to pH 6 to precipitate homocystine. Any contaminating protein was removed by dissolving the precipitated homocystine in 7 ml. of 2N-hydrochloric acid and dialyzing against 50 ml. of 0.5N-hydrochloric acid. The cellophane sack and its remaining contents were discarded. The homocystine in the diffusate was precipitated by adjusting the solution to pH 6. After dissolving the homocystine in a minimum amount of dilute hydrochloric acid it was further purified by subjecting to paper electrophoresis, using a Spinco Continuous Flow Electrophoresis Apparatus, Model CP, at 5°–10°. The homocystine separated as a narrow band when the electrophoresis was carried out at 900 V. in 0.25M-acetic acid. The homocystine samples thus obtained were recrystallized until the specific radioactivity and the decomposition temperature became constant. Chromatographic assays of the re-isolated homocystine were performed, using four different solvent systems, in one and in two dimensions. The solvent systems used were: (1) methyl ethylketone + *n*-butanol + distilled water + dicyclohexylamine (10 + 10 + 5 + 2); (2) 3-butanol + 90% (w/v) formic acid + distilled water (70 + 15 + 15); (3) phenol + de-ionized water + 0.88 N-ammonia (160 + 40 + 1); (4) *n*-butanol + distilled water + pyridine (1 + 1 + 1).

RESULTS

In a preliminary experiment it was found that *Neurospora crassa* methionineless mutants 38706Ra and T-27 formed [^{35}S]homocystine. The concentrations of [^{35}S]homocystine were rather low. It was, therefore, attempted to enhance the formation of [^{35}S]homocystine by adding non-radioactive sulphate to the medium. In this experiment homocystine was re-isolated from the combined TCA extract and the filtered culture fluid after 9 days of incubation. At a concentration of $8.6 \times 10^{-5}\text{M}$ -carrier-sulphate the [^{35}S]homocystine concentration, expressed as amount [^{35}S]homocystine/g. dry mycelium, approximately doubled as compared to that obtained in the absence of potassium sulphate, as shown in Fig. 1. Further increasing the concentration of carrier-sulphate does not give rise to higher concentrations of [^{35}S]homocystine; since, however, a 3.3-fold increase in carrier-

Fig. 1

Fig. 1. [^{35}S]homocystine formation in *Neurospora crassa* as function of sulphate concentration in the culture medium. Left scale refers to wild type strain 5297a, right scale to mutant 38706Ra. The mycelium was incubated for 9 days. Specific radioactivity of sulphate at $8.6 \times 10^{-5}\text{M} = 98 \mu\text{c./}\mu\text{M}$.

Fig. 2

Fig. 2. [^{35}S]homocystine formation in *Neurospora crassa* as function of time of incubation.

sulphate concentration resulted in a decrease in [^{35}S]homocystine of only *c.* 25 %, the optimal sulphate concentration for homocystine formation is apparently higher than $8.6 \times 10^{-5}\text{M}$.

The data suggest that some optimal concentration of sulphate is required for homocystine formation. As mentioned under Methods and as shown below, sulphate alone does not promote any growth in mutant 38706Ra unless incubated for longer than 2 weeks. Growth, under the conditions employed, therefore, is a function of methionine concentration. Mutant 38706Ra accumulated five times as much [^{35}S]homocystine as did wild-type strain 5297a.

It was expected that increases in incubation time of the mycelia would enhance [^{35}S]homocystine accumulation. Wild-type and mutant 38706Ra were, therefore, incubated for 4, 8, 12 and 16 days in the presence of $5 \times 10^{-4}\text{M}$ -methionine, $8.6 \times 10^{-5}\text{M}$ -non-radioactive sulphate and $15 \mu\text{c.}$ of [^{35}S]sulphate/ml. medium. As shown in Fig. 2, the maximum accumulation of [^{35}S]homocystine, as measured in the TCA

extract, was noted after four days of growth. Prolonging the incubation period resulted in lower [^{35}S]homocystine concentrations. It is of interest to note that between the 4th and 8th day of incubation the [^{35}S]homocystine concentration in mutant 38706Ra decreased while the mycelial dry weight increased by 58 %, indicating that the decrease in [^{35}S]homocystine concentration is not related to depletion of methionine in the culture fluid. Considering the difference in the ordinate scale of a factor of 4 for the plot of mutant and wild type, it can be seen in both figures that the mutant formed 5–11 times as much [^{35}S]homocystine as did the wild type. Measurements of [^{35}S]methionine formation were not made; the results suggest, though, that the block preventing methionine biosynthesis from homocysteine is shown to be incomplete if the incubation period is extended beyond 4 days. In an experiment to test this postulate mutant 38706Ra was grown in parallel on $2 \times 10^{-4}\text{M}$ -methionine or $2 \times 10^{-4}\text{M}$ -potassium sulphate for 4, 12, 17 and 22 days. After 4 days there was no growth on sulphate but after 12, 17 and 22 days the growth was 8, 50 and 86 % respectively of that on methionine. This experiment strongly supports the above postulate. In a mutant of *Neurospora crassa* which is blocked between cystathionine and homocysteine and which grows only on homocysteine or methionine it was demonstrated that methionine biosynthesis from [^{35}S]sulphate takes place after prolonged incubation (Strauss & Minagawa, 1959); [^{35}S]cystathionine first accumulated, and later disappeared as [^{35}S]methionine was formed.

Homocystine formation in mycelia of mutant T-112 and 80702Ra and wild-type strain 5297a was compared. It was found that mutant T-112 formed 4.8 times as much [^{35}S]homocystine as did wild type, whereas mutant 80702Ra formed only 0.58 times as much. Such a result might be expected since mutant 80702Ra is blocked before homocysteine in the biosynthetic pathway. The observation that homocystine re-isolated from this mutant was slightly radioactive might indicate that the metabolic block is not complete.

A chromatographic study confirmed that the radioactivity measured in mutants 38706Ra, T-27 and T-112 was due to [^{35}S]homocystine. The ninhydrin-positive material always coincided in size, shape and location with the black spot on the radioautograms. The nitroprusside test for disulphide compounds (Toennies & Kolb, 1951) also supported the identity of the radioactive material as homocystine. Furthermore, when homocystine was oxidized to homocysteic acid with 30 % (w/v) H_2O_2 before developing the chromatograms, only one black spot appeared on the film which coincided with the ninhydrin-positive material on the paper. Because the specific radioactivity of homocystine from wild type was too low we were unable to obtain radioautograms in this case. It seems, however, reasonable to attribute the radioactivity measured in homocystine from wild type to the formation of some [^{35}S]homocystine.

The chromatographic techniques used permit differentiation of homocystine from other sulphur amino acids, e.g. methionine, cysteine, cystine, cystathionine, lanthionine, djenkolic acid. Although homocysteine was oxidized during the isolation procedures it is probable that the [^{35}S]homocystine finally identified was due to formation of [^{35}S]homocystine in the mycelium.

Since varying the growth period or the sulphate concentration never resulted in [^{35}S]homocystine yields larger than 0.3 % in terms of ^{35}S initially present in the

medium, no attempts were made to isolate homocysteine without employing non-radioactive homocysteine-carrier.

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