Cellular Components Specifically Labeled during Sorbose Stimulation of Sugar Transport in Neurospora

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(Z. Naturforsch. 26 b, 409—413 [1971]; received January 13, 1971)

A number of independent systems for sugar transport in Neurospora have been postulated. An approach to identification of specific components of the sorbose transport system in ungerminated conidia pretreated with sorbose is described here. The fact that sorbose stimulates sorbose-uptake without causing germination was exploited in differential labeling experiments. Sorbose treated and untreated conidia were incubated with ($^3$H)-leucine and ($^{14}$C)-leucine respectively, and then mixed extracts fractionated on CM-cellulose at pH 4.75. Changes in the $^3$H/$^{14}$C-ratio indicated sorbose-induced or -repressed substances. In selecting labeling conditions, care was taken to avoid errors that might arise from any differences in the internal amino acid pool size.

The investigation revealed a buffer-soluble component repressed by sorbose and a Triton-soluble component induced by sorbose. The buffer-soluble, sorbose repressible protein is discussed in connection with the phenomenon of sorbose toxicity in Neurospora. The Triton-soluble, sorbose stimulated component, probably of carotenoid nature, is considered as a possible constituent of the sorbose transport system investigated here.

Conditions for sorbose uptake into cells of Neurospora have been described in a series of publications from this laboratory. In conidia pregerminated with fructose, uptake was shown to be energy dependent, to work against a concentration gradient up to 600 fold, and to follow saturation kinetics. This, together with substrate specificity and the existence of mutants with a decreased rate of sorbose uptake, was considered as indicative of an active transport mechanism, functioning by means of a "permease" 1–3.

Detailed genetic studies have then shown that sorbose uptake is a complex process, since at least four separate genes govern the transport rate 4–8. One of these genes seems to be of the regulator type 9. The others, in lack of evidence to the contrary, are considered as structural genes. From current physiological investigations it follows that at least 3 separate systems for active uptake of sorbose exist in Neurospora. They are used by the cell in different situations 10, 11 and can be named accordingly

1. the system in conidia pregerminated with fructose (as mentioned above),
2. the constitutive system of ungerminated conidia
3. the system in ungerminated conidia pretreated with sorbose.

The fact that these systems cause the uptake of sorbose does not exclude that they may in addition or even primarily serve the uptake of quite different sugars.

Because of the multiple systems existing for sorbose uptake and the separate genes governing it, it is to be supposed that several components are involved in addition to the "permease" cited. Detailed knowledge of them seems crucial for the understanding of the functioning of sugar uptake. We have therefore begun to work out methods for the identification and isolation of those components.

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The approach used here was adapted from Kolber and Stein and others. It consists of measuring the $^3$H/$^{14}$C ratio in extracts from induced cells grown in the presence of a $^3$H-labeled amino acid mixed with non-induced cells grown in the presence of the corresponding $^{14}$C-labeled amino acid. An increase in the ratio is indicative of a substance produced only during induction.

In order to decrease the likelihood of contamination with proteins synthesized during germination, the system observed in ungerminated conidia pretreated with sorbose (No. 3) was chosen. This system seemed exceptionally well suited for such purposes since during pretreatment of the conidia with sorbose no germination occurs during the first 24 hours. In contrast, most conidia pretreated with fructose germinate within 4 hours. It was therefore expected that any proteins needed for germination of conidia or growth of hyphae would be synthesized in small amounts, if at all, during sorbose pretreatment of the cells, and hence would probably not disturb the assay. Since induction dependent changes in amino acid pools might influence this type of experiment, a prior study of pool sizes was considered necessary.

Details of our experimental approach and some of the results obtained are communicated here.

Material and Methods

Strain and cultural conditions: Neurospora crassa wildtype 74-OR 23-1A De Serres was grown on glycerol complete at 25°C. Conidia of 7 day old cultures were harvested as dry powder, suspended in water, filtered through cotton to remove mycelial fragments and counted in a haemocytometer. The suspension was adjusted to $1 \times 10^7$ conidia/ml and kept at 25°C for 2 hours.

Pretreatment with sorbose: $4 \times 10^6$ conidia of the above suspension were filtered onto millipore filter disks, washed twice with water, and resuspended in 1 l 0.037 M citrate-phosphate buffer of pH 4.75 which contained 1% filtersterilized sorbose. This pH is optimal for the transport system in ungerminated conidia pretreated with sorbose. The suspension was distributed in 100 ml portions into 200 ml Erlenmeyer flasks and incubated on a shaker at 60 cycles per minute. $4 \times 10^6$ conidia taken from the same suspension and set up in buffer in identical fashion but without sorbose served as untreated reference material.

Labeling of proteins: 3½ hours after resuspending the conidia in the buffer with or without sorbose, 0.2 ml solutions of ($^3$H)leucine or ($^{14}$C)leucine were added to respective flasks. The ($^3$H)leucine was applied as a solution of 0.05 mc/ml (final radioactivity was 0.1 μc/ml, final concentration of leucine $10^{-4}$ M). The ($^{14}$C) leucine was applied as a solution of 0.01 mc/ml (final radioactivity was 0.02 μc/ml, final concentration of leucine $0.64 \times 10^{-4}$ M). After addition of the label the cultures were shaken for further 30 min., then filtered off onto millipore filter disks, washed twice with water and stored at 0°C.

Extraction: Sorbose-treated, $^3$H-labeled and untreated, $^{14}$C-labeled conidia were mixed, suspended in 10 ml ice-cold 6.7 mM citrate-phosphate buffer of pH 4.75 and disrupted by shaking with glass beads in a Bühler homogenizer. The raw extract, decanted from the beads, was divided by 60 min. centrifugation at 105 000 g into supernatant, containing the buffer soluble material, and sediment, containing cell debris plus buffer insoluble components. The supernatant was used directly in column chromatography. The sediment was washed twice with buffer and extracted with 10 ml buffer plus 2.5% Triton X-100. Triton-soluble material was then separated from the residual particles by 60 min. centrifugation at 70 000 g.

Controls: Experiments, where both halves of the cellular suspension were free of sorbose, but one half received the $^3$H-label, the other the $^{14}$C-label as above, served as controls. They were set up from the same conidial suspension as above and, apart from the omission of sorbose, were carried through in identical fashion.

Protein determinations: Protein was determined by the biuret method, as described by Schneider et al., or by a semiquantitative method derived from Reindel and Hoppe. For protein determinations in the Triton material, acetone was used as a precipitant.

Counting procedure: The liquid scintillation spectrometer used was a Packard 3300, the channels of which in the double label experiments were set as follows: $^3$H-channel 29.3% of $^{14}$C-activity; $^{14}$C-channel less than 0.1% of $^3$H-activity. Samples were measured in 3 parts toluene plus 1 part Triton X-100 plus 4 g BBOT per liter. Since quench was constant in all samples from the same column, and nearly so between columns, absolute activities were not calculated.

References:

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Special substances: Radiochemicals, obtained from the Radiochemical Center, Amersham, England were (U-14C)sorbose, specific activity 3.0 mc/mM; L-leucine-4,5-T, specific activity 1000 mc/mM; and (U-14C)L-leucine, specific activity 312 mc/mM. BBOT was the CIBA product. Triton X-100 was obtained from Serva-Entwicklungslabor, Heidelberg.

Results

1. Stimulation of sorbose uptake by sorbose pretreatment: Cells pretreated with buffer or buffer + 1% sorbose for 3½ hours were washed and resuspended in buffer + (14C) sorbose. Sorbose uptake of both types of cells was then followed by the millipore filter technique (Fig. 1). It can be seen that the uptake of sorbose is increased considerably as result of the sorbose treatment. Details of this effect have been worked out and will be published in two separate papers. Here it may be enough to say that only a small part of this effect is caused by countertransport, the rest is considered as due to induction of an active transport system (No. 3 of the introduction).

2. Studies on the amino acid pool: Information on pool sizes for different amino acids in cells pretreated with sorbose or not was gained as follows: Cells obtained as stated in methods and either treated with buffer alone or with buffer + 1% sorbose for 3½ hours were filtered off, resuspended in water and boiled. The aqueous extract was cleared by 10 min. centrifugation at 27,000 g. The content of individual amino acids in the supernatant was then measured by means of an amino acid analyzer. Results are given in Table I. It was found that the concentration of certain amino acids is increased by the sorbose treatment (lysine, arginine, valine, tryptophane, phenylalanine) whereas that of others (glycine and alanine) is decreased. Only histidine, isoleucine and leucine concentrations were not affected by sorbose. The latter was chosen for the labelling procedure. The magnitude of leucine uptake is not influenced by sorbose pretreatment.

3. Buffer-soluble material: If buffer-soluble material either from controls or from induced versus uninduced cultures is separated on CM-cellulose at pH 4.75, optical density tracing in the eluate at 280 m/J- reveals 5 peaks. Samples representing peak 3, on thin layer chromatography contain at least 7 different components, one of which has the same

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\begin{array}{|c|c|c|}
\hline
\text{amino acid} & \text{material treated with} & \\
 & \text{buffer} & \text{buffer + sorbose} \\
\hline
\text{Lys} & 0.0210 & 0.0251 \\
\text{His} & 0.0100 & 0.0102 \\
\text{Arg} & 0.0196 & 0.0359 \\
\text{Gly} & 0.0517 & 0.0389 \\
\text{Ala} & 0.0804 & 0.0730 \\
\text{Val} & 0.0268 & 0.0303 \\
\text{Ile} & 0.0125 & 0.0138 \\
\text{Leu} & 0.0175 & 0.0179 \\
\text{Tyr} & + & 0.0109 \\
\text{Phe} & + & 0.0117 \\
\hline
\end{array}
\]

Table I. Effect of sorbose on amino acid pool sizes. Conidia were treated with buffer or with buffer + 1% sorbose for 3½ hours. Treatment was stopped by transferring the cells into boiling water. The supernatant was cleared by centrifugation and aliquots of it, taken up in 0.2 mM Na-citrate buffer of pH 2.2 were run through a Beckman amino acid analyzer Unicrom A 1011 V. Figures from Lys to Arg in μmoles per 0.5 ml, the rest in μmoles per 2.5 ml. Statistical deviations are less than ±3 per cent.
mobility as free leucine, and constitutes the bulk of radioactivity. It is considered to represent free \(^{(3}H)\)- and \(^{(14}C)\)-leucine, which has been taken up by the conidia without becoming incorporated into protein during the 30 min. labeling period.

The radioactivity profile from scintillation countings in fractions from peaks 4 and 5 of the o.d. tracing is given in Figs. 2 a and b. Results are shown for the control run, e.g. untreated versus untreated material (Fig. 2 a) and for a run with material half of which had been treated with sorbose, the other half not (Fig. 2 b). It will be seen that peak 4 of the o.d. tracing represents substances not labeled and therefore not synthesized during the labeling period, no matter whether cells were treated with sorbose or not. In contrast, peak 5 contains both labels, but in different relative amounts for the treated versus untreated run (Fig. 2 b) as compared to the untreated versus untreated control (Fig. 2 a).

Since the \(^{(3}H)\)-activity is unexpectedly low in peak 5 material from conidia treated with sorbose, it can be ascertained that synthesis of certain buffer soluble substances contained in the conidia and depending on the presence of leucine is repressed under conditions of sorbose treatment.

4. Triton-soluble material: When the Triton-extract from controls, in which untreated cultures were mixed with untreated cultures, was fractionated on CM-cellulose at pH 4.75, the radioactivity profile (Fig. 3 a) revealed 3 peaks. One peak has a maximum of activity in fraction 15 and is correlated with an orange colour during elution from the
column. Two others, not clearly separated, have maxima in fractions 40 and 45. All peaks were equally labelled by $^3$H and $^{14}$C, a fact born out by a constant ratio of $^3$H/$^{14}$C-activity for the individual fractions, as plotted in the same figure.

In contrast, in the extract from treated versus untreated cells (Fig. 3 b) $^3$H-activity but not $^{14}$C-activity in peak one is strongly increased, as corroborated in the plot of the $^3$H/$^{14}$C-ratios. Due to several washings of the sediment before extraction with the detergent, no free leucine label or other amino acids were present in the Triton extract. Also, proteins or peptides could not be found in measurable quantities. It was realized that leucine via conversion to mevalonate and isopentenylpyrophosphate could label compounds such as sterols, carotenoids, terpenes and others. The colour in peak one indicates a carotenoid. It was not separable from the zone of $^3$H-enrichment by disc-electrophoresis on polyacrylamide. The $^3$H-enrichment in peak one of the fractionated Triton extract is therefore possibly due to carotenoids, synthesized preferentially during sorbose treatment of the conidia. Whether this suggestion is true, and whether these substances play a role in sorbose stimulated sugar transport in Neurospora, will be the subject of further studies.

**Diskussion**

It has been found that certain buffer-soluble substances of Neurospora, which are synthesized in resting conidia in the absence of sorbose, are no longer made when sorbose is added. This finding may explain in part the toxic and paramorphogenic effects of sorbose in Neurospora, two probably related phenomena, which are now poorly understood. Appealing candidates for the reproducible substances were the enzymes G-6-PDH and PGM. Mutational loss of these enzymes causes the compact growth observed in so-called colonial mutants, and an identical phenotype is produced by sorbose in the wildtype. The possibility existed that here sorbose was repressing one or both of these enzymes. This hypothesis found little experimental support, however, since the two enzyme activities mentioned were not present in the fraction containing the repressible substance.

It has been found in addition that certain Triton-soluble substances, probably of carotenoid nature, are synthesized in the presence of sorbose. These may conceivably be part of the transport system, since they apparently originate from the disrupted cells, are synthesized preferentially when transport is enhanced, and are synthesized in amounts which are roughly proportional to the increase in sorbose uptake (see Fig. 1, 3 a and b). Our findings thus parallel those of other authors, who reported several lipids to be involved in active transport processes. Following part of the interpretation advanced by Fox for the role of unsaturated fatty acids in galactoside transport of E. coli it can tentatively be suggested here, that some of the Triton-soluble substances of probably carotenoid nature, synthesized in Neurospora in the presence of sorbose, may play an essential structural role in the transportation of sugar transport sites on the cellular membrane.

Thanks are due to Prof. G. BRAUNITZER and Mr. A. STANGEL of the Max-Planck-Institut für Biochemie, München, for the amino acid determination (Table I) and to Dr. G. NEUMANN of the same institute for scanning the thin layer chromatograms. The investigations were supported by the Deutsche Forschungsgemeinschaft and the Bundesministerium für Wissenschaftliche Forschung. I am grateful to Mrs. G. SCHNEIDER for excellent technical assistance, to Prof. F. KAUFMANN who provided the laboratory space and the facilities needed, as well as to Dr. W. ORME for help with the english manuscript.

21 Optical density tracings are not feasible in the eluate, since Triton itself absorbs strongly in the UV-range.


31 A. WEISS (manuscript in preparation).


