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DEVELOPMENT AND EVALUATION OF METHODS FOR THE QUANTITATIVE ESTIMATION OF FUNGAL TREHALOSE WITH A CHARCOAL–GAS–LIQUID CHROMATOGRAPHIC-COUPLED ASSAY

KATHLEEN A. KILLICK

Boston Biomedical Research Institute, Department of Developmental Biology, 20 Staniford Street, Boston, Mass. 02114 (U.S.A.)

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ABSTRACT

Methods for the quantification of trehalose in extracts prepared from the cellular slime-mold, *Dictyostelium discoideum*, by a charcoal–gas–liquid chromatographic-coupled assay are described. After extraction of trehalose from cells with 80% (v/v) methanol, followed by evaporation of the samples, the preparations were subjected to reduction with sodium borohydride, methanol extraction and evaporation, and Dowex-(H^+) and charcoal-column chromatography, allowing the efficient preparation of the trimethylsilyl ether, which resulted in the complete chromatographic resolution of trehalose from other naturally occurring disaccharides (*e.g.*, maltose and cellobiose). The equivalence between results obtained with these methods and those resulting from enzymic analysis demonstrates that the chromatographic assay is a useful analytical tool affording a precise and sensitive method for the quantitative estimation of fungal trehalose.

INTRODUCTION

Quantitative or qualitative determinations of the nonreducing disaccharide trehalose (α-D-glucopyranosyl α-D-glucopyranoside) in extracts from the molds or the yeasts have been performed by several different methods. Routine procedures for α,α-trehalose analysis have usually involved either (a) the specific degradation of this sugar with the enzyme trehalase (α,α-trehalose glucohydrolase, EC 3.2.1.28) followed by the measurement of liberated D-glucose[1], or (b) colorimetric assays in which trehalose levels are measured after elimination of any contaminating reducing sugars[2]. Although the first method offers the critical advantage of specificity, its applicability is restricted by the level of D-glucose to be measured, the sensitivity of the D-glucose assay employed, and the availability of a purified trehalase preparation.

Since the development in 1963 by Sweeley *et al.*[3] of methods for the preparation of trimethylsilyl (Me_3Si) ethers of carbohydrates for analysis by gas–liquid chromatography, these procedures have been used for the assay of carbohydrates from a wide variety of biological materials. Application of these methods to the quantitative

estimation of trehalose in fungi depends upon the development of procedures that afford not only a sensitive and precise assay of this sugar, but that also result in the resolution of trehalose from other naturally occurring disaccharides, *e.g.*, maltose and cellobiose. Such a procedure has now been developed for the measurement of trehalose in extracts from the cellular slime-mold *Dictyostelium discoideum*. The uniqueness of the assay consists in the coupling of charcoal and gas–liquid chromatographies for the quantitative estimation of trehalose[4]. Such a procedure has so far never been reported for the assay of this disaccharide in any biological material. The equivalence of the results obtained by these chromatographic procedures with those from an enzymic analysis of trehalose demonstrates that the described method is a useful analytical tool for the precise and sensitive estimation of fungal trehalose.

EXPERIMENTAL

Reagents. — Carbohydrates for the preparation of standard curves were trehalose dihydrate from Sigma Chemical Co. (St. Louis, MO 63478), and maltose, glucose, sucrose, and cellobiose from Pfanstiehl Laboratories, Inc. (Waukegan, IL 60085). All of the carbohydrates were of analytical grade. *N,O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% of chlorotrimethylsilane (Me_3SiCl) was obtained from Pierce Chemical Co. (Rockford, IL 61105); pyridine (Baker Instra-analyzed), sodium borohydride (Baker grade), and hexane (nano-grade) from J. T. Baker Chemical Co. (Phillipsburg, NJ 08865) and Mallinckrodt Inc. (St. Louis, MO 63147), respectively; acid-washed Norit A charcoal from Fisher Scientific Co. (Pittsburgh, PA 15219); and pre-purified nitrogen and air (21.5% O_2) from Union Carbide Corp., Linde Div. (South Plainfield, NJ 07080).

Instrumentation. — Gas–liquid chromatography was performed with a Model 5831A Hewlett–Packard reporting gas chromatograph, equipped with a flame-ionization detector, GC terminal, and General Electric hydrogen generator. During an experiment, the terminal traced the chromatogram on heat-sensitive, z-fold paper, and printed the peak retention-time near the apex of each peak. The multifunction digital-processor accessory of the gas chromatograph was used to analyze integration data, which at the end of an experiment were automatically printed out by the terminal. Separations were carried out on a 0.3 × 180 cm stainless-steel column, packed with 3% of SE-30 and 80/100 Supelcoport from Supelco Inc. (Bellefonte, PA 16823). The column was conditioned, in the gas chromatograph, by use of a temperature program from 50 to 250° at 2°/min, with 20 mL/min of nitrogen flow. The column was then kept overnight at 250° at a constant nitrogen-flow (*i.e.*, 20 mL/min).

Operating conditions. — The injection-port temperature of the gas–liquid chromatograph was 240°, the flame-ionization detector (FID) temperature 280°, and the column temperature was programmed from temperature 1, 220° (time 0.1 min) to temperature 2, 260° (time 0) at a rate of 2°/min from the time of injection. Flow rates were as follows: carrier gas (N_2) at 25 cm^3/min at an output pressure of 4×10^5 Pa, and hydrogen and air at an output pressure of 1.35×10^5 Pa.

Organism and culture conditions. — *D. discoideum* (NC-4), ATCC 24697 was grown on nutrient agar with *Escherichia coli* as the bacterial associate, as previously described[5]. After consumption of the bacteria, myxamoebae were harvested with distilled ice–water, and any residual bacteria were removed by repeated centrifugation and washing. The cells were spread onto sheets of 2% non-nutrient agar and were incubated for 10 h at 22° (the aggregation stage of development), or for 24 h (the mature, sorocarp-stage of development).

Isolation of trehalose. — At the desired stage of development, the cells were harvested from the non-nutrient agar with cold, sterile distilled water, lyophilized to dryness, and stored in desiccated state at −12°. Trehalose was extracted[6] from samples of lyophilized cells with 80% (v/v) methanol at 23°. After removal of insoluble material by centrifugation, the pellet was extracted 3 or 4 additional times with 80% (v/v) methanol, and the combined extract was evaporated to dryness. The residue was solubilized with 70% (v/v) ethanol and centrifuged, and the supernatant liquid was stored at 6° in a screw-cap test tube.

Treatment of samples with sodium borohydride. — An aliquot of each sample (in 70%, v/v, ethanol) was evaporated to dryness *in vacuo* at 23° (EVAPOMIX), and the residue was solubilized in freshly prepared (distilled water), cold 0.25M sodium borohydride solution[7] (2 mL). After incubation overnight at 6°, the sample was transferred dropwise with a Pasteur pipette onto a layer of Dowex 50W-X12 (H^+) resin (1 mL) contained within a 12-mL, glass, conical centrifuge tube, while constant stirring of the Dowex–sugar mixture was maintained. After 30 min at 23°, the suspension was centrifuged, and the supernatant liquid was kept. The Dowex resin was washed 5 times with 5-mL aliquots of distilled water. The washings were pooled and evaporated to dryness. The boric acid was volatilized as its trimethyl ester by 7–10 additions of absolute methanol (8 mL), each followed by evaporation to dryness (EVAPOMIX). The residue was solubilized with distilled water (2.5 mL) and the solution was transferred to a small Pasteur pipette, plugged with glass wool, that contained a mixture of acid-washed charcoal (100 mg) and Celite[8] (100 mg). After the non-adsorbed material had passed through the column, the charcoal bed was washed with distilled water (2.5 mL) and the eluate was discarded. Trehalose was eluted from the column with 10% (v/v) 1-propanol (8–10 mL). After evaporation of the propanol, the samples were desiccated overnight in vacuum in the presence of phosphorus pentaoxide.

Preparation of per-O-trimethylsilyl derivatives. — Each sample was dissolved in anhydrous pyridine (1.0 mL), and an aliquot was transferred to a small glass vial. A solution of sucrose in pyridine was added as an internal marker, after which an equal volume of BSTFA was added to the mixture. The vial was sealed with a septum-lined screw-cap, and incubated overnight at 6°. After derivatization, the sample was evaporated to dryness under a stream of high-purity, dry nitrogen. The residue was redissolved in hexane (0.25–1.0 mL) and 3- or 4-μL aliquots were injected into the gas–liquid chromatograph.

Identification. — The peaks were identified by comparing the retention times

with those of standard derivatives of carbohydrates and by co-chromatography. The trehalose peak was confirmed by subjecting aliquots of the biological sample to be analyzed to an overnight incubation with purified *Dictyostelium* trehalase[9]. Since this enzyme is specific for trehalose, only the peak corresponding to authentic trehalose disappeared.

Enzymic quantitative determination of trehalose. — The lyophilized cells to be analyzed were extracted three times with 5-mL aliquots of 80% (v/v) methanol. After removal of cell debris by centrifugation (33 000*g*; 15 min), the extract and washes were pooled and evaporated to dryness. The residue was dissolved in 50mM potassium citrate (pH 5.5) buffer, and an aliquot (500 μL) was incubated overnight with 0.5 unit of trehalase (*i.e.*, 1 unit of trehalase activity produces 1 μmol of D-glucose per 30 min at 35°, under standard assay conditions) under a drop of toluene. The solution was then boiled for 3 min, and a 200-μL aliquot was incubated with the Glucostat Special reagent (500 μL). This reagent was prepared by dissolving the lyophilized enzyme and chromogen in 100mM potassium phosphate (pH 7.0) buffer (50 mL). After incubation for 30 min at 35°, the Glucostat reaction was terminated by the addition of 4M hydrochloric acid (50 μL). The incubation mixture was kept for 10 min at 23°, and the absorbancy at 400 nm was measured. The D-glucose concentration was determined from a standard D-glucose curve, prepared from a stock D-glucose solution incubated under the same conditions as just described. The calibration curve for D-glucose was linear up to a sugar concentration of 30 μg/mL.

RESULTS AND DISCUSSION

Identification and resolution of trehalose. — Prior to measuring the levels of trehalose in biological samples, the optimal conditions for the identification and resolution of trehalose in a mixture of known disaccharides were studied. Of a variety of disaccharides, the most likely to be present in a cell-free extract of *Dictyostelium* are maltose and cellobiose, these sugars presumably originating from turnover of glycogen and cellulose, respectively[10]. Thus, mixtures of trehalose, maltose, and cellobiose were per-*O*-trimethylsilylated with BSTFA, as described in the Experimental section, and were subsequently subjected to g.l.c. analysis. Sucrose was chosen as an internal marker for these and subsequent studies because (a) it is not synthesized by *Dictyostelium*, (b) it is a nonreducing sugar (as is trehalose) giving a single peak in the chromatogram as it does not undergo mutarotation, and (c) it has a retention time (R_t) distinct from, yet relatively close to, that of trehalose under the same conditions of temperature programming (*i.e.*, a linear increase of 2°/min from 220 to 260°). Sucrose was added to the samples either at the time of extracting trehalose from the slime mold or at the time of per-*O*-trimethylsilylation. Likewise, calibration curves were prepared in which: (a) sucrose (as an internal marker) and trehalose (as an internal standard) were processed through the entire extraction–determination scheme together, and (b) only trehalose (internal standard) was processed through the scheme and sucrose was added as an internal marker at the

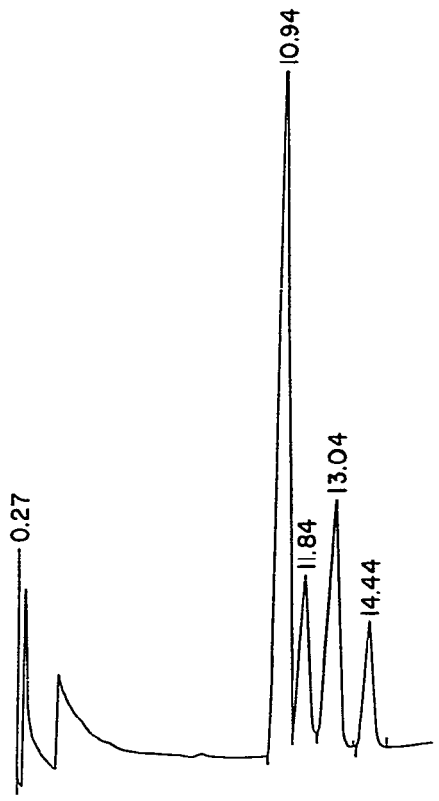

Fig. 1. Gas–liquid chromatogram of per-*O*-trimethylsilylated disaccharide standards. The sample (3 μL) injected contained derivatives of maltose, cellobiose, and trehalose (0.24 μg each) plus sucrose (0.72 μg) in hexane. Instrumental conditions and methods of sample preparation as described in the Experimental section.

time of per-*O*-trimethylsilylation. Plots of peak-area ratio *vs.* trehalose level for both processes were linear and had average slopes of 1.03–1.10 (*i.e.*, they were identical). This demonstrated that no differential loss of sucrose relative to trehalose occurred during the extraction–determination scheme, and hence that sucrose could be used either as an internal marker or as an internal standard.

It is apparent from Fig. 1, that, under the conditions employed, four peaks having values of R_t of 10.94, 11.84, 13.04, and 14.44 min were detectable in the chromatogram after injection of the Me_4Si derivatives of the standard disaccharide mixture just discussed. Since both cellobiose and maltose are reducing sugars, each of these disaccharides should give rise to at least two peaks in the chromatogram, corresponding to the α and β anomers. Thus, for complete resolution of sucrose, trehalose, cellobiose, and maltose, at least six peaks should have been observed. Although trehalose (R_t 13.09 min) was completely resolvable from sucrose (R_t 10.94 min), it was not well resolved from one of the maltose anomers (R_t 12.77 min). In addition, one cellobiose anomer (R_t 11.89 min) was poorly resolved from one of

TABLE I

RETENTION DATA AND RESOLUTION EFFICIENCY

Disaccharide	*Sample treatment*			
	None		*Reduction*	
	R_t[a] *(min)*	RR_t[b]	R_t *(min)*	RR_t
Sucrose	10.91	1.000	10.91	1.000
Trehalose	13.06	1.197	13.06	1.197
Maltose	11.77	1.079	15.86	1.454
	12.79	1.172		

[a] R_t, retention time. [b] RR_t, relative retention time.

the anomers of maltose (R_t 11.75 min). Resolution of trehalose from both maltose and cellobiose was achieved by subjecting the disaccharide-containing solution to reduction with sodium borohydride prior to preparation of the Me_4Si derivatives for chromatography. This treatment resulted in the conversion of maltose and cellobiose to maltitol and cellobitol, respectively, and the appearance in the chromatogram of a single peak for the Me_4Si derivative of each alcohol. After reduction, the retention times of the corresponding alditols of cellobiose and maltose were increased sufficiently to allow for complete resolution from trehalose (Table I).

As it was apparent that cell-free extracts prepared from *Dictyostelium* would have to be subjected to reduction with sodium borohydride (for the reasons just presented) prior to trehalose determination, both an effective chromatographic resolution of trehalose and an efficient preparation of the trimethylsilyl ether of this sugar were developed. As described in the Experimental section, the sodium borohydride left after overnight incubation of the samples was converted into boric acid by batchwise addition of Dowex-50 (H^+), and the boric acid was subsequently volatilized as its trimethyl ester by repeated additions and evaporations of absolute methanol. Samples of trehalose prepared in this manner, however, were inefficiently per-*O*-trimethylsilylated. Moreover, precise determination of trehalose following this procedure was difficult owing to the presence of numerous artifactual peaks at or near the trehalose peak (see Fig. 2A). Thus, a step that resulted in the removal of these interfering materials was introduced into the procedure. It consisted of the differential adsorption of trehalose onto small columns of acid-washed charcoal, followed by elution with 10% (v/v) 1-propanol[8]. Samples of trehalose prepared in this manner were per-*O*-trimethylsilylated 1.8-fold more efficiently (as deduced from peak-area ratio measurements) than those per-*O*-trimethylsilylated immediately after the Dowex-50 (H^+) treatment (see Table II), and resulted in clear, sharp chromatograms during g.l.c. analysis (Fig. 2B).

The trehalose peak in the chromatogram was routinely identified by co-chromatography and by comparison of the retention time of the unknown sample with that of a standard trehalose sample. As shown in Fig. 3, the presumptive trehalose peak

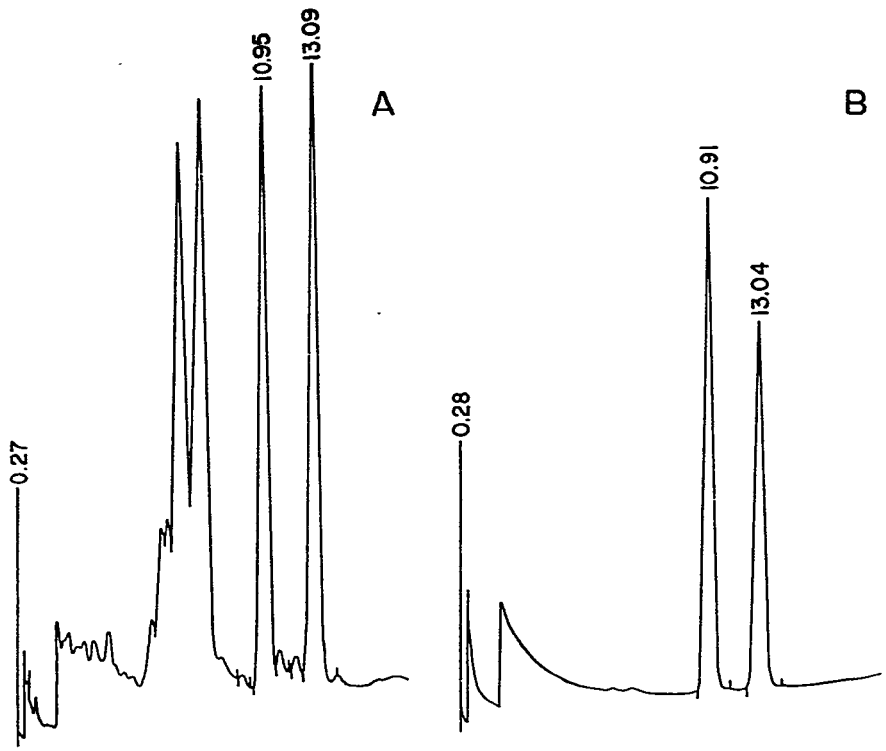

Fig. 2. Effect of charcoal-column chromatography as an adjunct to gas–liquid chromatography. Trehalose preparations were incubated with 0.25M sodium borohydride overnight at 6°. After Dowex-50 (H^+) inactivation of the excess of sodium borohydride, the samples were evaporated to dryness, and the per-*O*-trimethylsilylated derivatives were prepared before (A) or after (B) chromatography on charcoal columns as described in the Experimental section. After evaporation to dryness, the samples were solubilized with either 0.25 mL (A) or 1.0 mL (B) of hexane. The peaks with listed retention times in these chromatograms correspond, in order of increasing R_t value, to the solvent front, sucrose, and trehalose.

TABLE II

EFFICIENCY OF PER-*O*-TRIMETHYLSILYLATION AFTER CHARCOAL CHROMATOGRAPHY

Treatment	*Peak-area ratio (trehalose to sucrose)*[a]
Dowex-50	0.48
Dowex-50 + charcoal	0.88

[a]A trehalose sample (200 μg) was subjected to treatments with sodium borohydride and Dowex-50 (H^+) as described in the Experimental section. One aliquot of the resultant preparation was per-*O*-trimethylsilylated directly, while a second, equal aliquot was subjected to charcoal-column adsorption and elution prior to derivatization. Sucrose (100 μg) was added to each sample prior to the per-*O*-trimethylsilylation step. The values for the peak-area ratios have been corrected for the net losses of trehalose during these procedures.

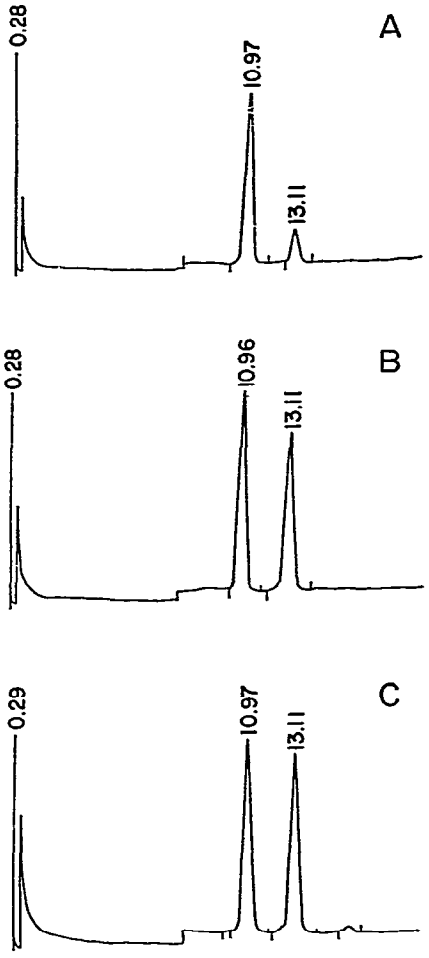

Fig. 3. Identification of the trehalose peak during gas–liquid chromatography. Trehalose was isolated from *Dictyostelium* and, after evaporation of the samples to dryness followed by overnight incubation with sodium borohydride, and Dowex-50 (H^+) and charcoal treatments, aliquots were mixed with (A) sucrose (50 μg), or (B) sucrose (50 μg) plus trehalose (50 μg), and per-*O*-trimethylsilylated overnight as described in the Experimental section. The control samples (C) consisted of trehalose (100 μg) and sucrose (100 μg), and were subjected to the same conditions of derivatization as samples (A) and (B). After incubation, the samples were evaporated to dryness under nitrogen, solubilized in either 0.5 mL (conditions A and B) or 1.0 mL (condition C) of hexane. The peaks correspond to the solvent front (0.28 min), sucrose (10.97 min), and trehalose (13.11 min).

(R_t 13.11 min) had the same retention time as that of authentic trehalose and, moreover, the two peaks coincided when aliquots of standard solutions of per-*O*-trimethylsilyl trehalose were chromatographed with those of the derivatized biological sample. In addition to confirming the identification of the presumptive trehalose peak that was obtained from the biological sample, the mixing experiment also served as a control for the detection of any material that might accompany trehalose during the isolation from *Dictyostelium*, and might subsequently interfere with the per-*O*-

trimethylsilylation reaction. No evidence for this was found, as the sum of the two peak-area ratios (trehalose to sucrose) for the trehalose regions in Figs. 3A and 3B was equal to the experimental value reported in Fig. 3C.

Precision and sensitivity of the method. — Prior to assay of prepared biological samples for trehalose content, calibration curves that related values of peak-area ratio (trehalose to sucrose) to trehalose level were constructed. In these studies, the sucrose concentration was maintained at 100 μg and the trehalose level was raised from 10 to 200 μg (*i.e.*, 0.08 to 1.6 nmol) per 1.0 mL of final volume of hexane. Aliquots (3 μL) were injected into the gas–liquid chromatograph, and the attenuation was set to 7. Other conditions were as described in the Experimental section. Plots of peak-area ratio *vs.* injected-trehalose level were linear under these conditions up to at least 1.6 nmol of trehalose.

In order to determine the lowest level of trehalose that could be detected, another calibration curve was prepared. In these studies, 10 μg of sucrose was added to each sample as an internal marker. The levels of trehalose examined ranged from 5.3 to 106 pmol of trehalose per 4 μL of injected sample. The attenuation and slope sensitivity changes were time-programmed to occur at specific intervals during the

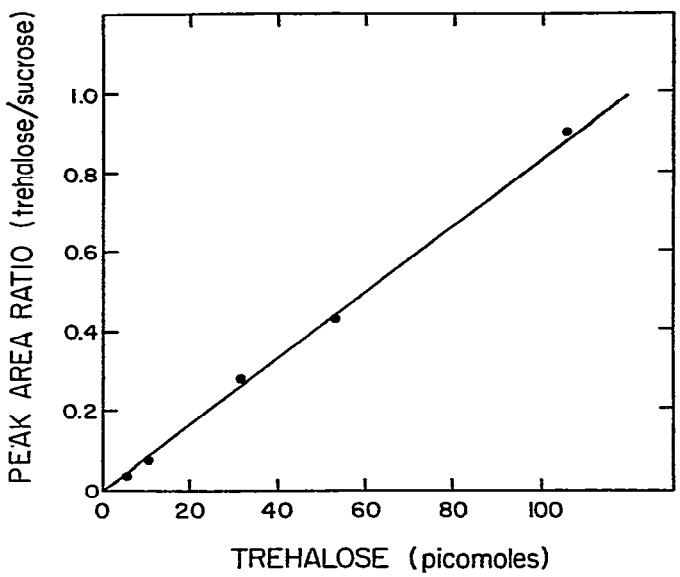

Fig. 4. Limits of detection of trehalose by g.l.c. analysis. After preparation of stock trehalose samples, sucrose (10 μg) was added to each sample, and the samples were subjected to overnight per-*O*-trimethylsilylation. Following evaporation to dryness under nitrogen, hexane (1.0 mL) was added to each sample, and aliquots were subjected to g.l.c. analysis. Attenuation and slope sensitivity changes were time-programmed to occur at specific times during the runs. The following programs were used for: (a) 5.3 pmol of trehalose, attenuation equalled 7 (2 min), 5 (9 min), and 4 (12 min) and the slope sensitivity was 0.02 (12 min); (b) 10.6 pmol of trehalose, attenuation equalled 7 (2 min) and 5 (9 min), and the slope sensitivity was 0.05 (12 min); (c) 31.7 pmol, attenuation equalled 7 (2 min) and 5 (9 min), and the slope sensitivity was 0.1 (12 min); and (d) 53 and 106 pmol of trehalose, attenuation equalled 7 (2 min) and 5 (9 min).

experiment. As shown in Fig. 4, a plot of peak-area ratio *vs.* trehalose level was linear over the range of ~6 to at least 100 pmol of trehalose. Thus, the two calibration curves just discussed demonstrate that g.l.c. analysis may be used to estimate quantitatively trehalose levels ranging from 0.006 to at least 1.6 nmol per 4 μL of injected sample.

As the samples prepared from *Dictyostelium* had to be subjected to sodium borohydride reduction, and Dowex-50 (H^+) and charcoal treatments prior to g.l.c. analysis in order to achieve efficient resolution of trehalose from other disaccharides, an additional standard curve was constructed. In this procedure, sucrose (70 μg) was added to each sample prior to per-*O*-trimethylsilylation. The final volume of all samples following derivatization was 1.0 mL in hexane, and 4-μL aliquots were subjected to g.l.c. analysis. As shown in Fig. 5, plots of peak-area ratio *vs.* injected-trehalose level were linear up to at least 2.5 nmol of trehalose, and comparison of the slope of the line obtained in Fig. 5 with that observed with standard trehalose solutions demonstrated that they were identical.

The precision and accuracy of the method was examined by subjecting, to the entire procedure, two different amounts of trehalose (50 and 100 μg) prepared from

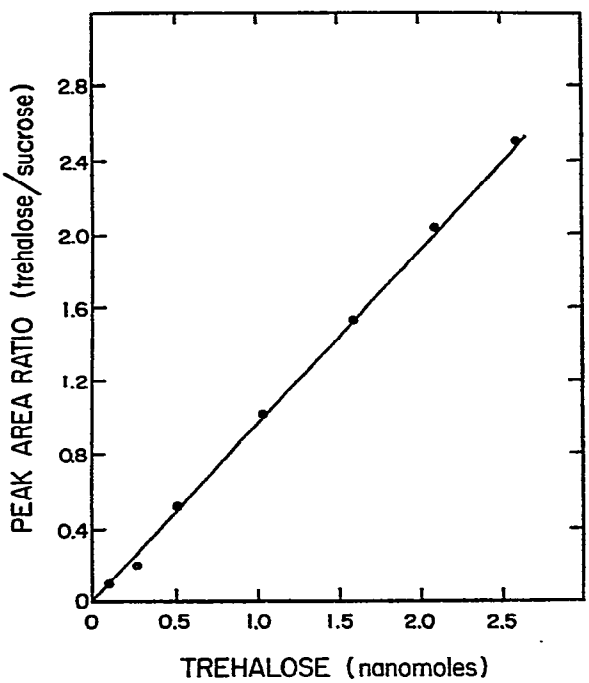

Fig. 5. Calibration curve for the quantitative estimation of trehalose. Trehalose samples were prepared for g.l.c. analysis by sodium borohydride, Dowex-50 (H^+), and charcoal treatments. Sucrose (70 μg) was added to each sample and the samples were derivatized. Following evaporation to dryness, hexane (1.0 mL) was added to each sample, and aliquots were subjected to g.l.c. analysis. The attenuation on the chromatograph was set at 6 for injected-trehalose levels of 0.1–1.6 nmol, and was increased to 8 for higher concentrations.

TABLE III

ACCURACY OF REPLICATE ANALYSES[a]

Sample	*Peak-area ratio (trehalose to sucrose) Cell preparation*	
	I	*II*
1	0.354	0.823
2	0.384	0.831
3	0.396	0.897
4	0.382	0.908
5	0.384	0.811
6	0.354	0.802
7	0.384	0.717
8	0.396	0.737
9	0.381	0.760
10	0.380	0.798
Mean	0.379	0.808
Standard deviation	0.014	0.059

[a]Replicate trehalose determinations were performed on cell samples from two different batches of lyophilized sorocarps. Slime-mold preparations utilized for these studies were equivalent to either 50 μg (I) or 100 μg (II) of trehalose in the cell preparation used as the starting material.

TABLE IV

RELATIVE SENSITIVITY OF METHODS FOR THE QUANTITATIVE ESTIMATION OF TREHALOSE

Method	*Lower limit of trehalose detection (nmol)*	*Sample volume (mL)*
Colorimetric[a]	7–14	0.5
Spectrophotometric[b]	0.20–0.40	1.0
G.l.c.	0.005	0.003

[a]Trehalose was measured enzymically with purified *Dictyostelium* trehalase and the Glucostat Special reagent as described in the Experimental section. [b]After hydrolysis with purified *Dictyostelium* trehalase, trehalose was estimated in D-glucose equivalents with a yeast hexokinase–D-glucose 6-phosphate dehydrogenase-coupled, spectrophotometric assay. The absorbance at 340 nm, as measured with a Gilford Model 2400 spectrophotometer, was amplified with a recorder attachment.

Dictyostelium. Replicate samples for each of these amounts were carried through the procedure, and the peak-area ratio for each was then determined. As shown in Table III, the accuracy achieved with ten replicate analyses of the trehalose content in two different preparations of slime-mold material was good. The sensitivity achieved with g.l.c. analysis was also compared to that of the enzymic estimation of trehalose employing spectrophotometric analysis. As shown in Table IV, the sensitivity of trehalose detection by g.l.c. analysis was about 40-fold higher than that achieved with spectrophotometric methods, and at least 250-fold greater than that obtained by use of the Glucostat Special reagent.

Quantitative estimation of trehalose during Dictyostelium *development.* — With each biological sample analyzed for trehalose content, known amounts of standard trehalose were processed under the same scheme. Based on the peak-area ratio measurements for the unknown biological samples relative to those for the known standard solutions, the trehalose concentrations in the slime mold were calculated. The same values for trehalose content were obtained, when they were determined from a trehalose calibration curve obtained from a trehalose–sucrose solution not processed through the entire scheme, after correction for net trehalose loss. Moreover, control experiments demonstrated that addition of a known amount of trehalose to the lyophilized cell-preparation, prior to methanol extraction and subsequent trehalose estimation, resulted in peak-area ratio values equal to the sum obtained from estimations for standard trehalose alone plus slime-mold trehalose. Trehalose recovery was determined from experiments using (a) standard trehalose solutions plus [^{14}C]trehalose, (b) [^{14}C]trehalose plus a slime-mold preparation, and (c) [^{14}C]trehalose, standard trehalose, plus a slime-mold preparation. In these studies, [^{14}C]trehalose (and standard trehalose, where indicated) was routinely added to the lyophilized mold-preparation prior to cell extraction. Recovery of standard solutions of both [^{14}C]sucrose and [^{14}C]trehalose processed through the entire g.l.c. preparatory procedure averaged ~84%.

Trehalose was routinely extracted from lyophilized cells with 80% (v/v) methanol, since previous work in this laboratory[6] had shown that not only is this solvent an excellent trehalose extractant, but also that at the mature-sorocarp stage of development, ~75–80% of the carbohydrate in a methanolic extract is trehalose. Samples of trehalose were prepared from cell populations at two different stages of development, aggregation (12 h) and sorocarp (24 h), as these periods of morphogenesis are representative of the minimal and maximal trehalose levels found in the developing organism[8]. Samples of trehalose from these cells were per-*O*-trimethylsilylated without exposure to sodium borohydride treatment, and subjected to g.l.c. analysis. Neither preparation (aggregation or sorocarp) contained detectable levels of either sucrose or cellobiose. Although maltose could not be detected in preparations from aggregate cells, maltose was evident at the sorocarp stage. Thus, it was possible to quantitatively estimate trehalose in extracts from cells during the early stages of development (aggregation) without prior exposure of samples to sodium borohydride reduction, and not encounter analytical difficulties associated with maltose-peak contamination of the trehalose-peak of the chromatogram.

Analysis of the trehalose concentration present at the aggregation and sorocarp stages of development using fungal preparations subjected to sodium borohydride reduction, and Dowex-50 (H^+) and charcoal treatments gave values equivalent to those previously reported (Table V). Comparison of the results from g.l.c. analysis with those from the enzymic assay of trehalose using purified trehalase indicates equivalence between the two methods. Since the trehalase preparation was specific for trehalose, this comparison demonstrates that g.l.c. analysis is as accurate and specific as the enzymic procedure. In addition, it is also apparent from Table IV that the greatest

TABLE V

QUANTITATIVE ESTIMATION OF TREHALOSE DURING DEVELOPMENT IN *Dictyostelium*

Developmental stage	*Trehalose (mmol of* D-*glucose equiv.)*[a]		
	Enzymic analysis	*G.l.c. analysis*	*Literature value*[1]
Aggregation	0.17	0.18	0.20
Sorocarp	9.9	9.6	10.

[a]The trehalose concentration is expressed as μmol of D-glucose equiv. per mL of packed-cell volume.

asset of the g.l.c. analysis of trehalose is its sensitivity. Hence, the described procedures should be of value for the measurement of cellular trehalose in *Dictyostelium* at those stages of the life cycle where either the absolute level of this sugar is low or the incremental changes in trehalose concentration are slight. Because of its sensitivity, precision, and wide range of applicability, the described method for trehalose estimation by g.l.c. analysis should prove useful to present studies in this laboratory, which are concerned with elucidating the *in vivo* regulatory mechanisms responsible for the activation of trehalase, and the co-incident catabolism of trehalose during spore germination in *Dictyostelium*.

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