

Automated Method for the Determination of D-Glucose-Isomerase Activity

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ABSTRACT

An automated procedure for the determination of the glucose-isomerase activity of extracts of microorganisms is described. Activities of whole cells and sonicates of whole cells can also be determined, provided they are sufficiently well dispersed. In the procedure, the sample is incubated with buffered D-glucose solution, and the fructose produced is determined colorimetrically with cysteine-carbazole-sulfuric acid reagent by Technicon AutoAnalyzer equipment. Samples are analyzed in pairs, and one sample of the pair provides a blank to correct for substances which may interfere with the colorimetric fructose determination. The standard deviation of single determinations is $\pm 2.5\%$ relative. The preparation of a stable glucose-isomerase standard used for calibration of the automated procedure is also described.

Numerous studies have been conducted on enzymes from various microorganisms that convert D-glucose to D-fructose (1,2,3), and intense interest has been generated in this class of enzymes owing to its large-scale application for the commercial production of fructose-containing syrups (4). Hence, a need for a rapid and facile method for determination of glucose-isomerizing activity arose. The usual procedure has been to incubate the enzyme preparation with a solution of D-glucose and then determine the amount of fructose produced colorimetrically (2,3). In an effort to simplify this procedure, some workers have automated the colorimetric procedure for fructose determination (5,6,7). This paper describes an entirely automated procedure employing AutoAnalyzer equipment in which the only manual operation required is that involved in sample preparation. Provision is made for the determination of blank values so that compensation can be made for the effects of any interfering sugars in the sample. A manual method involving use of a recording polarimeter is also described for determining the activity of standard enzyme preparations used for calibrating the automated procedure.

MATERIALS AND METHODS

Polarimetric measurements were carried out with a Bendix NPL automatic polarimeter equipped with a 20-mm. jacketed cell and a 589-nm. filter (sodium D line). The output of the polarimeter was placed in series with a 50-ohm precision resistor, and the current through the resistor measured with a Sargent Model MR recorder. One-milliamp (mamp.) change in output was equivalent to 62.5 mdegrees change in rotation. Temperature within the cell was maintained by circulating water through the jacket from a water bath (Precision Scientific). Temperature of the circulating water was adjusted to 61.1°C. Owing to the heat loss in the circulation lines, the temperature of the sample within the cell was $60 \pm 0.1^\circ\text{C}$., as determined with a thermistor bead thermometer.

pH was determined throughout with a Leeds and Northrup meter (No. 7405, expanded scale) standardized with pH 6.87 phosphate buffer (Leeds and Northrup Part No. 103-1-0-2). AutoAnalyzer (Technicon Instruments Corp.) equipment was

used throughout for the automated assay except where noted. Sonication of cell preparations was carried out with a Branson Sonifier, Model J 17V.

Enzymes displaying glucose-isomerase activity are elaborated intracellularly and, in the absence of extensive lysis, whole fermentor-broth samples and cell isolates were sonicated before assay to release the cell contents. For most fresh preparations of *Streptomyces* mycelia, a sample sufficient to yield 20 to 100 units of activity was diluted to 25 ml. with water in a 50-ml. tube and was sonicated at 20 Kc. with full power. Complete release of activity was achieved usually within 40 sec. For each type of sample, it was necessary to establish the proper sonication time for maximum activity release. After sonication the samples were diluted to an appropriate volume, centrifuged 5 min. at 31,000 X g, and the supernates analyzed. Sonicates could be assayed without clarification, but this was not done routinely. Also, small microorganisms in suspension containing glucose-isomerase activity could be analyzed intact by aspirating directly into the analytical stream without sonication provided that the cells were well dispersed. Whenever samples contained suspended matter, the vibratory sample mixing device was used to resuspend any settled solids in the sample cups just before analysis.

D-Fructose from Pfanstiehl Laboratories, Inc. (C.P. Special) was used without further purification. No contaminants were found on checking the fructose by paper chromatography (3-mg. sample, Schleicher and Schuell No. 2043-B paper, irrigated with 8:2:1 ethyl acetate:pyridine:water, sprayed with ammoniacal silver nitrate solution). D-Glucose used for calibration of the polarimeter-recorder was Standard Reference Material 41a from the National Bureau of Standards. Glucose used in the automated procedure was commercial glucose monohydrate (Clinton Corn Processing Company). Triton X-100 is an alkylphenoxypolyethoxy ethanol from Rohm and Haas. All other chemicals were of analytical reagent quality.

Glucose-isomerase standard enzyme preparation was made by extracting freshly harvested cells of a *Streptomyces* sp. by stirring in 0.001M cobaltous chloride (CoCl_2) solution overnight at pH 6.7 and 45°C. The extract was separated from the cell debris by filtration, concentrated, and 1 g. of diatomaceous earth per 200 units of activity added, followed by two volumes of cold acetone to precipitate the isomerase. The resulting preparation, after being air-dried and blended, was stored at 4°C., under which conditions it was found to be stable indefinitely. This provided standard reference material for the AutoAnalyzer method with a nominal potency of 200 units per g. Thorough study of the end products of the action of this type of glucose-isomerase preparation on D-glucose and D-fructose under the conditions of the assay showed that the sole action on these two sugars was their interconversion.

Composition of the six different solutions used in the automated procedure for glucose-isomerase potency determination is shown in the following table:

<i>Solution</i>	<i>Composition</i>
Substrate	0.22M D-glucose, 0.06M magnesium sulfate
pH 6.7 buffer	0.4M sodium maleate, maleic acid
pH 4.0 buffer	0.1M sodium acetate, acetic acid
Triton	0.1% Triton X-100
Acid-carbazole	80% sulfuric acid (by weight) containing 22 p.p.m. carbazole
Cysteine	1% cysteine hydrochloride

The acid-carbazole reagent was made by diluting sulfuric acid to 80% with water. The carbazole was added to the diluted acid as a 0.5% solution in ethanol. Reagent-grade sulfuric acid from Mallinckrodt Chemical Works was preferred, since it did not produce appreciable colored products when combined with the carbazole. Cysteine hydrochloride solution was made up daily.

RESULTS AND DISCUSSION

Polarimetric Procedure for Determining Potency of Isomerase Standards

To set up a reliable automated assay for glucose-isomerase activity, it was necessary to provide a stable enzyme preparation of known potency which could be used as a reference material. Determination of the potency of standard enzyme preparations was accomplished by measuring reaction rate as a function of rate of change in optical rotation while a reaction mixture was contained in the cell of a recording polarimeter. The difference in the specific rotations of glucose and fructose is large (about 112° at 60°C .), so that a sensitive and accurate procedure could be set up.

The optical rotations of D-glucose and D-fructose are known with considerable precision. We preferred, nevertheless, to calibrate the polarimeter-recorder to accurately reflect the transformation of glucose to fructose under the exact conditions of usage. Standard solutions were made up, in the one case, to contain exactly 2M glucose; and in another, 1.9M glucose-0.1M fructose. Both solutions contained 0.02M magnesium sulfate, 0.001M CoCl_2 and 0.2M, pH 7.0 sodium maleate buffer. Rotations of both solutions were determined at 60°C ., and from the results a recorder response (R) was calculated (-69.7 mamp. per molar change in glucose to fructose).

A unit of activity was defined as that amount of enzyme which catalyzes the transformation of D-glucose to D-fructose at the rate of $1\ \mu\text{mole}$ per min. under the specified conditions (pH 7.0, 60°C ., 2.0M D-glucose, 0.02M Mg^{++} , 0.001M Co^{++} , 0.2M sodium maleate buffer).

Maleate was chosen as the buffer after considerable search. Other buffers were available in this pH range but either inhibited the isomerase, complexed with the cobalt, or formed a precipitate with the magnesium. Figure 1 shows the pH-activity curve for the standard isomerase in maleate buffer. The pH chosen for the assay was a compromise between the activity optimum of the isomerase at pH 7.4 and the maximum buffering capacity of the buffer at pH 6.1.

To determine activity of the standard enzyme preparation, a 100-ml. solution containing enough enzyme preparation to provide about 200 units was made up to contain the above-mentioned concentrations of glucose and salts. The solution was made up at 25°C . and adjusted to pH 6.84 and then made to final volume. It was immediately placed in the thermostated polarimeter cell. Warming to 60°C . occurred within 3 min., and the pH increased from 6.84 to 7.00 ± 0.02 . The rate of change in rotation was recorded for a 15-min. period thereafter. Figure 2 shows a typical recorder trace.

The isomerization reaction is reversible, reaching equilibrium at 60°C . when about half the glucose is converted to fructose (8). The velocity of the reaction decreases markedly as conversion proceeds and approaches zero at equilibrium. However, the first portion of the conversion is nearly linear. In this polarimetric procedure for determining activity, less than 2% of the glucose initially present was

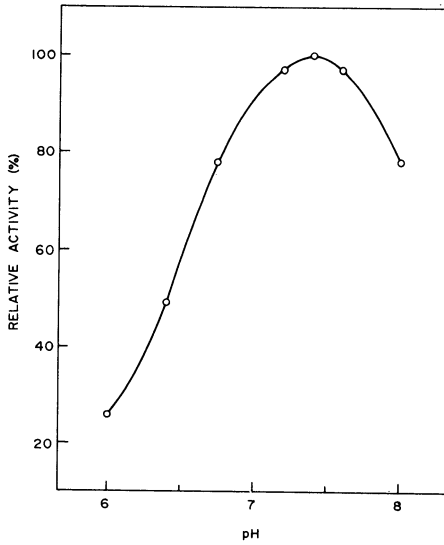


Fig. 1. pH-activity relationship for standard glucose isomerase in sodium maleate-maleic and buffer.

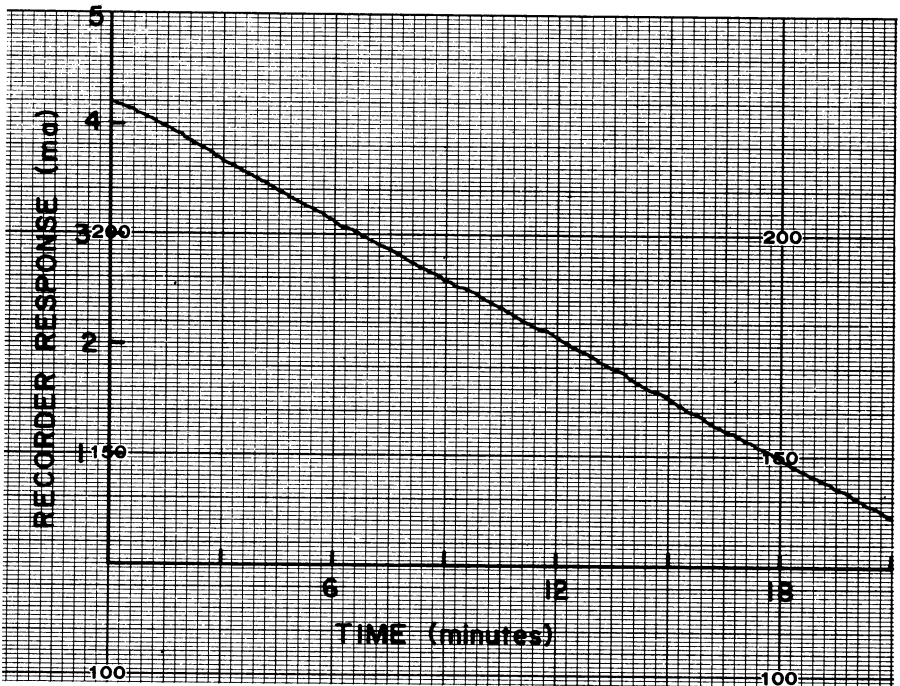


Fig. 2. Recorder trace of polarimeter output. Horizontal and vertical axes and notations were drawn after the recorder tracing was scribed.

converted to fructose, so that the rate determined was essentially the initial reaction rate and the reaction was linear with time.

The activity of the standard preparation was calculated from the slope of the recorder tracing by the following formula.

$$P = \frac{S \times 10^5}{R \times W}$$

where P = potency ($\mu\text{moles min.}^{-1} \text{g}^{-1}$); S = slope (mamp. min.^{-1}); R = recorder response constant (mamp. $\text{mole}^{-1} \text{liter}$); and W = weight (g.) of enzyme sample per 100 ml. of reaction mixture.

There was a possibility that mutarotation was a rate-limiting factor which could alter the velocity of the reaction when carried out within the polarimeter cell. This was checked by running reactions under the conditions of the assay as stated, but using a lower enzyme-substrate ratio so that reaction time was extended to 3 hr. for the same degree of reaction ordinarily accomplished in 15 min. Also, these reactions were run outside of the polarimeter cell, the isomerase inactivated by acidulation, and repeated determinations of rotation made to ensure full mutarotation. There was no significant change in the apparent potency of the enzyme preparation when carried out in this manner, and it was concluded that mutarotation did not affect the overall rate of the reaction as run within the polarimeter cell.

Precision of a single determination was $\pm 1.5\%$ relative and the average of at least eight determinations was taken in establishing the activity of standard enzyme preparations.

Automated Procedure

The objective in automating the analysis of glucose-isomerase activity was to provide a rapid and accurate method which could be used in the laboratory development of new sources of isomerase and for monitoring the activity in large production fermentors. A colorimetric procedure was automated involving incubation of the isomerase with glucose solution and measurement of the fructose produced by the cysteine-carbazole-sulfuric acid reagent of Dische and Borenfreund (9). Since fermentor broths ordinarily contained keto sugars which interfered with determination of the analytically produced fructose, the method was designed to also provide for blank corrections.

Figure 3 shows a schematic diagram of the arrangement of the AutoAnalyzer equipment. The sampler was operated at a rate of 20 per hr. and the turntable was loaded with samples in pairs. One sample of each pair was used for the activity determination and the other as a blank correction. Wash water was used between each sample at a ratio of 1 volume of water per volume of sample. The sample was mixed with substrate solution and with one of two buffers. For determination of activity, pH 6.7 buffer was used; and for the blank determination, pH 4.0 buffer was used. In the latter case the isomerase displayed no enzymatic activity. The flow of buffers was controlled by an automatic valve described previously (10), the action of which was synchronized with the advance of the turntable by means of a microswitch actuated by an extra cam attached to the timer cam of the sampler module. Figure 4 shows the construction of the cam and Fig. 5 illustrates the arrangement of the cam and microswitch on the sampler module.

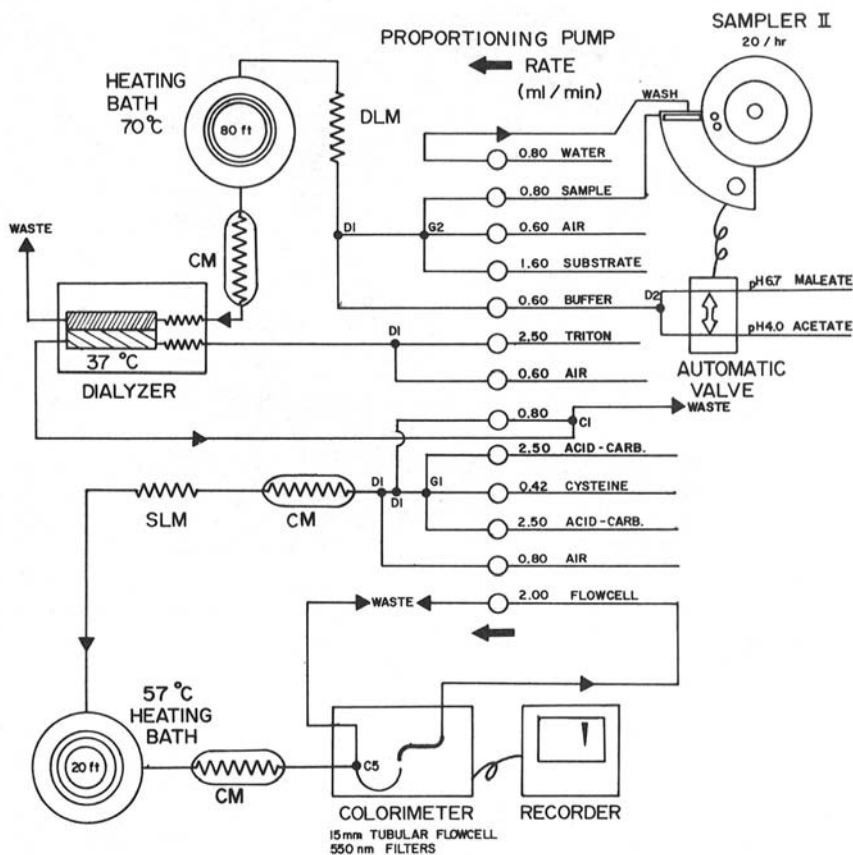


Fig. 3. Schematic flow diagram for automated glucose-isomerase assay. D1, D2, G1, G2, C1, and C5 are glass connectors; SLM, DLM, and CM are single-length, double-length, and cooled mixing coils, respectively.

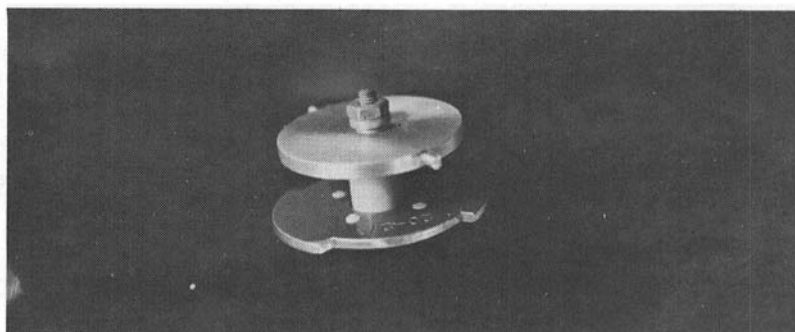


Fig. 4. Construction of cams. The bottom cam controls the action of the sampler module in the usual manner. The top cam has two spurs on its periphery and controls the switching of the automatic valve.



Fig. 5. Arrangement of cams and microswitch on sampler module. The top cam controls the automatic valve and is positioned relative to the bottom cam underneath (not shown) so that the change in buffers occurs at their point of mixing with the substrate (see Fig. 3) while the wash water aspirated between samples is combined with the substrate solution.

The mixture of sample, substrate, and buffer solution passed through a heating bath at 70°C. Retention time in the bath was about 11 min., and for the sample at pH 6.7, partial isomerization of glucose to fructose occurred in this bath. No isomerization occurred in the mixture at pH 4. The stream was then cooled and passed through a dialyzer, and a stream of water containing Triton X-100 picked up the glucose-fructose mixture that passed through the membrane. The function of the Triton X-100 was to help maintain a uniform bubble pattern in the stream passing through the dialyzer. This stream was debubbled, and part of it combined with sulfuric acid-carbazole solution and then with cysteine solution and heated to 57°C. in a color-development bath with a 20-ft. coil. Residence time in the bath was about 2 min. The solution was cooled, its absorbance determined at 550 nm. in a 15-mm. flow cell, and the result recorded. Total elapsed time between introduction of sample until appearance of a readout on the recorder was 22 min. Ten samples per hr. could be analyzed.

Conditions for development of color in the 57°C. water bath were chosen to give maximum sensitivity and yet maintain a favorable selectivity for the detection of fructose. The cysteine-carbazole-sulfuric acid reagent produces a small amount of colored product with glucose (9). As used in this assay procedure, the intensity produced by fructose was 360 times as great as that given by glucose.

Figure 6 shows typical recorder tracings given by standard enzyme solutions and a standard curve is shown. The response was linear with enzyme concentration and the range of the assay was 0 to 2 units per ml. The readings for blank values in the spaces marked with arrows are not identifiable as peaks, owing to the fact that the standard solutions had very little impurity and, consequently, produced almost no

detectable increase in absorbance with the cysteine-carbazole reagent. Nevertheless, absorbance at this position on the chart was subtracted from the peak value for the corresponding assay sample in calculating responses for construction of standard curves.

Acid-resistant pump tubing was not used to proportion the color reagent containing the 80% sulfuric acid, as the clear standard tubing was found more reliable, provided that new tubing was installed after every 100 hr. of use. Owing to the effects of the acid on the resiliency of the tubing, pumping rate changed slowly with time. A single sample of standard isomerase solution was run about every hour after the initial standardization, to check for drift.

Figure 7 shows recorder tracings for samples of sonicated and clarified whole fermentor broths. The small peaks showing the blank absorbance values are quite prominent.

In the production of glucose isomerase by fermentation, cobaltous salts may be added to the culture media as a stimulant for enzyme production (2,3). Hence, cobalt may appear in certain samples where it can act as an interfering substance in the automated assay, since it is an activator at low concentrations (3,11). For the isomerase from *Streptomyces* sp. it can also act as an inhibitor at high concentrations. Figure 8 shows the relative effect of the concentration of cobaltous ion in samples analyzed by the automated method. Maximum activity was displayed when the concentration in the sample at the point of aspiration was

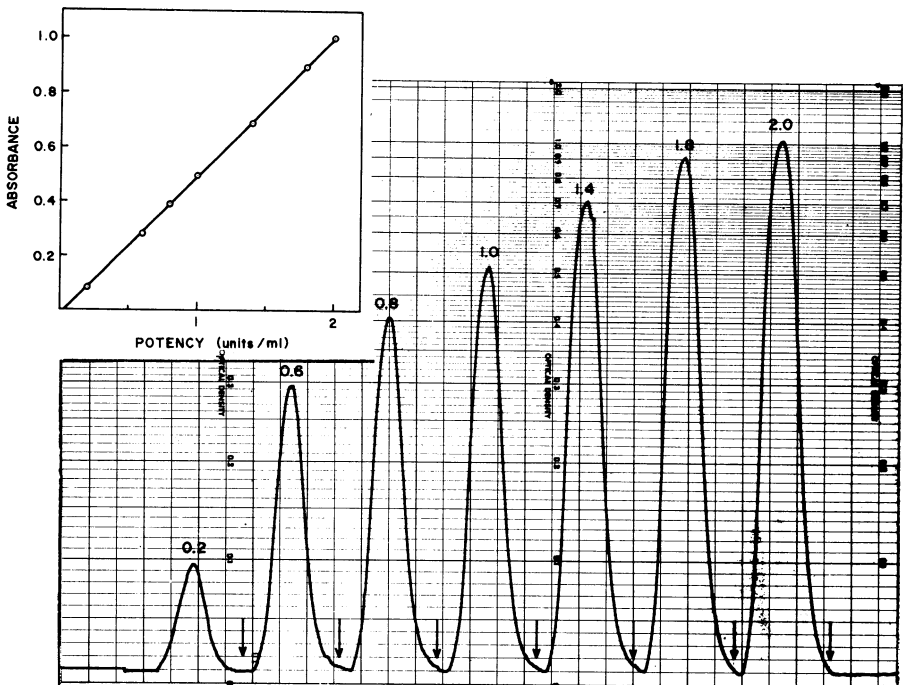


Fig. 6. Recorder tracings for a series of standard enzyme solutions. Arrows show the positions of blank absorbance values. Notations at tops of peaks give the glucose-isomerase concentrations in units per ml. Plot in upper left-hand corner shows linearity of response.

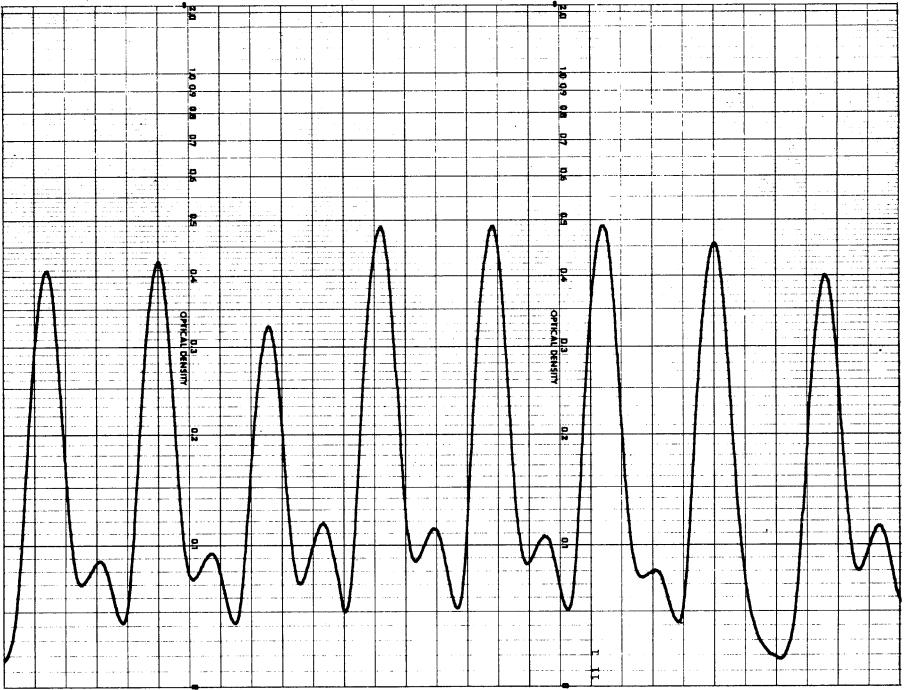


Fig. 7. Typical recorder tracing for the analysis of sonicated, clarified fermentor broths. The small peaks to the immediate right of the major peaks give the blank corrections.

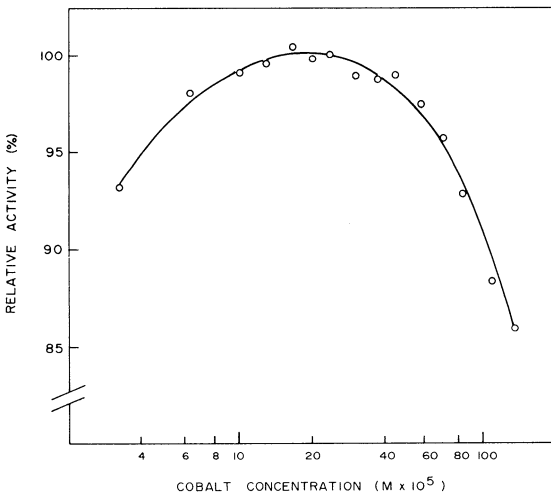


Fig. 8. Influence of the concentration of cobaltous ion in the sample at the point of aspiration on the relative activity.

0.0001 to 0.0004M. To obviate interfering effects of cobaltous ions in the samples, it was usually sufficient to dilute them for assay with 0.0001M CoCl₂ solution, so that any cobalt contributed by the sample itself (usually 0.0001M or less) had a negligible effect.

Standard deviation for the analysis of single samples was $\pm 2.5\%$ relative. The procedure has been in use in these laboratories for the past 5 yrs. for the analysis of over 80,000 samples, with a high degree of reliability. It is used routinely in the control of isomerase-production facilities.

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