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Sensitive Enzymatic Assay for Glucose Determination in Natural Waters[1]

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A new enzymatic method for glucose determination is described. It allows measurement of glucose concentration as low as 10^{-7} M. Such sensitivity makes this method particularly appropriate for estimation of glucose in natural-water bodies, generally without prior concentration or extraction. The method is based on the reaction between glucose and adenosine 5'-triphosphate, catalyzed by hexokinase to form glucose-6-phosphate. The amount of adenosine 5'-triphosphate consumed in this reaction, which is directly proportional to the amount of glucose present in the sample, is measured by the luciferin-luciferase assay. The optimal conditions for glucose determination by this method have been defined as follows: 20 min of incubation at 30°C, magnesium concentration of 10^{-3} M, and pH in the range of 7.5 to 10.5. The specificity of the assay to different carbohydrates has also been studied. Recovery of known amounts of glucose added to Lake Kinneret water was in the range of 80 to 114%. Application of this method is demonstrated in eight monthly profiles of the glucose content in Lake Kinneret.

During the past 10 years, considerable effort has been devoted to understanding the uptake of inorganic nutrients by microorganisms. Conversely, much less information is available concerning the flux of dissolved organic compounds in the ecosystems. This is due, among other reasons, to technical difficulties in measuring the concentration of simple organic compounds in natural-water bodies.

Several attempts have been made to determine the concentration of organic compounds in natural water. Rigler (9) developed a tracer bioassay which estimated only the maximum substrate concentration. Another method, the dilution bioassay, was first proposed by Hamilton and Austin (6) and used by Allen (1, 2). A similar bioassay was reported by Hobbie and Wright (8, 12, 13). Enzymatic determination of various sugars in natural waters has been described by Ruchti and Kunkler (10). Andrews and Williams (3) determined glucose in the sea by glucose oxidase assay after filtration of the water through charcoal followed by evaporation and lyophilization. Hicks and Carey (7) increased the sensitivity of an enzymatic assay for glucose and used it for the direct determination of 10^{-8} M glucose in natural waters without prior concentration or extraction. The disadvantage of their method is that the blank value was as much as 80% of the experimental value.

Since glucose is a common sugar in natural water and is generally assimilated by most heterotrophic microorganisms, new techniques allowing greater precision and lower detection limits seem to be a prerequisite for further work on the flux of these compounds into the various populations in natural waters. In this paper, we present a new enzymatic assay for determination of glucose and its application to glucose determination in Lake Kinneret.

Existing methods for determination of hexoses are inappropriate for the low concentrations found in natural waters. The present method is based on the transformation of glucose to glucose-6-phosphate catalyzed by hexokinase. There is a stoichiometric relationship between the amount of adenosine 5'-triphosphate (ATP) cleaved in this reaction and the amount of glucose transformed into glucose-6-phosphate according to equation: ATP + D-hexose → adenosine 5'-diphosphate + D-hexose-6-phosphate.

According to the above equation, the difference in the ATP content at the beginning and at the end of the incubation is then related to the amount of the hexoses present in the sample. Assays for ATP determination are very sensitive and are also very accurate.

The ATP is determined by the ATP-dependent luciferin-luciferase enzyme, which gives rise to bioluminescence as described by Strehler

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and Totter (11) and modified by Cavari (4). The amount of ATP in the sample is proportional to the total amount of light emitted in the enzymatic reaction in a finite time. The bioluminescence is measured by a liquid scintillation spectrometer, and the number of photons produced over a definite time interval after adding the enzyme to the vial is counted.

MATERIALS AND METHODS

The hexokinase reaction was run as follows: a 1-ml sample containing between 0 and 200 μg of glucose per liter was mixed with 1 ml of a solution containing ATP (10^{-6} M), $MgSO_4$ (10^{-3} M), and tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5 (3.4×10^{-2} M). Samples and standards were placed in a water bath at 30°C, and 0.1 ml of hexokinase containing 20 U was added at a rate of one sample per 30 s. ATP was then determined exactly 20 min after the addition of hexokinase, as follows: 0.5 ml of the reaction mixture was added to a glass scintillation vial containing 0.1 ml of glycylglycine buffer, pH 7.5 (0.75 M), and 0.1 ml of $MgSO_4$ (2.0 M); then 0.1 ml of luciferin-luciferase preparation was rapidly injected into the vial, which was placed into the scintillation counter and read for 0.1 min, as previously described (4).

Luciferin-luciferase enzyme mixture was prepared 24 h before use by grinding 10 mg of desiccated firefly tails with 5 ml of sodium arsenate, pH 7.4 (0.1 M). This homogenate was then centrifuged at 15,000 $\times g$ for 20 min, and 10 mg of $MgSO_4$ was added to the supernatant, which was then kept at 4°C.

Glucose content of lake water was determined as follows: 2.5 ml of lake water was filtered (984 H Reeve-Angel filters) into side-arm test tubes. The filtered water was immediately lyophilized in these test tubes and then reconstituted with 1 ml of distilled water. The enzymatic reaction was then carried out in the same test tubes as above. It must be noted that all glassware was previously cleaned by dichromate cleaning solution and then rinsed with distilled water and oven-dried at 240°C for 12 h.

RESULTS

Effect of the time of incubation with hexokinase. At glucose concentrations of 45 and 90 μg/liter, the decrease in counts, i.e., in ATP content, was a simple inverse logarithmic function of the time of incubation during the first 30 min of the experiment (Fig. 1). At concentrations of 180 μg/liter, we observed a break in the curve, which took place after 25 min of incubation. To be able to measure glucose in a wide range of concentrations, we chose a period of incubation of 20 min, for which all the curves show a logarithmic decrease.

Effect of temperature. The hexokinase reaction was run at different temperatures. Without glucose added, there was no change in the ATP content (Fig. 2). With the addition of 90 μg of glucose per liter, there was a decrease in the ATP content. The largest decrease, which indicated the highest activity, was observed at 30°C. At 15, 20, and 40°C, there was an inhibition of about 12%, whereas at 50°C the enzyme underwent complete inactivation.

Effect of pH. The hexokinase reaction was completely inhibited at pH 5.2 (Fig. 3). The enzymatic activity continuously increased up to

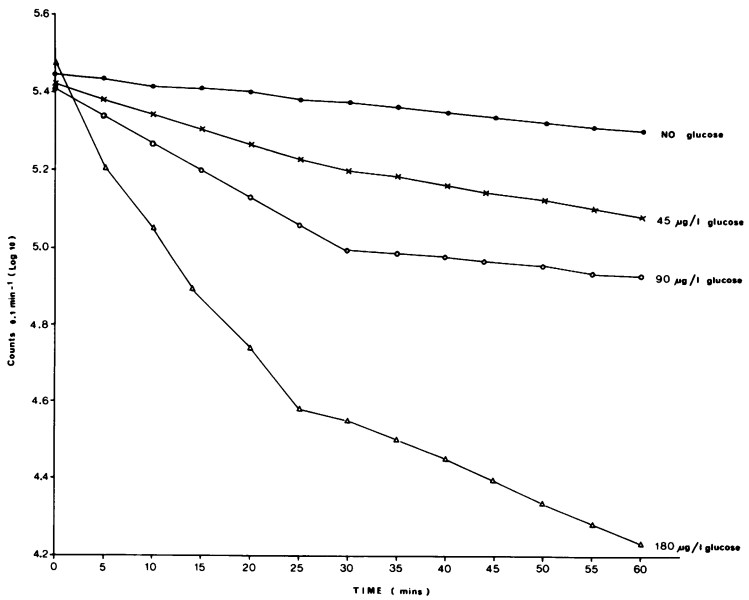

FIG. 1. *Time of incubation with hexokinase with three different concentrations of glucose.*

pH 7.2. A further increase of pH up to 10.5 did not affect the enzymatic activity.

The buffer used for the range 8.5 to 10.5 was Tris-maleate. This buffer inhibited the enzymatic activity to some extent (Fig. 3).

Effect of magnesium concentration. The hexokinase reaction has an absolute requirement for Mg^{2+}. Fromm and Zewe (5) found that the optimal ratio of Mg^{2+} to ATP is 2.5:1. In our experiments, we found that the optimal Mg^{2+} concentration was 10^{-3} M (Fig. 4), which is a ratio of 1,000:1 of Mg^{2+} to ATP. Decreasing the magnesium concentration to 10^{-6} M caused a decrease in the enzymatic activity by 73%. Increasing the magnesium concentration to 10^{-2} M also caused inhibition of about 27%.

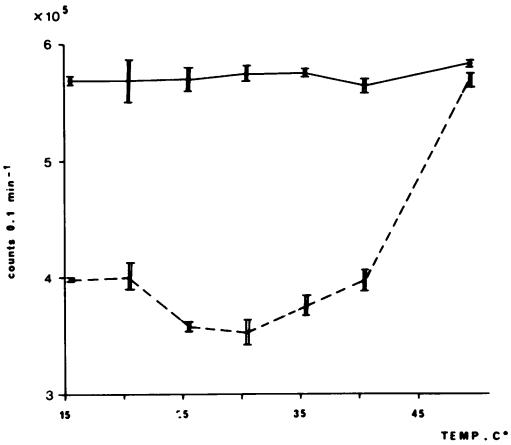

FIG. 2. *Effect of temperature on glucose determination. Symbols:* (●——●) *no glucose added;* (●- - -●) *90 μg of glucose per liter. The bars represent the distribution of counts from triplicate measurements.*

Effect of hexokinase amount. The effect of different amounts of hexokinase at different glucose concentrations is shown in Fig. 5. The higher the amount of the enzyme in the reaction mixture, the higher the rate of activity. However, since the enzyme preparation contains some glucose as an impurity, the higher the amount of enzyme in the reaction mixture, the lower the count obtained at zero glucose. With no enzyme and no glucose, we obtained 340,000 counts in comparison to 290,000 counts

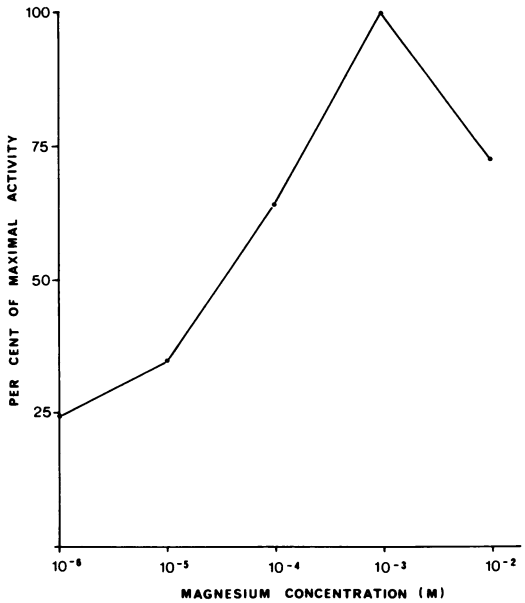

FIG. 4. *Effect of magnesium concentration on glucose determination.*

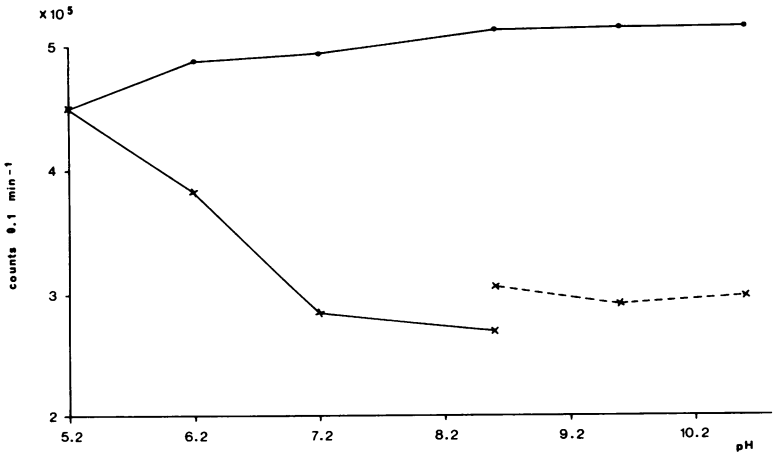

FIG. 3. *Effect of pH on glucose determination. Symbols:* (●——●) *no glucose added;* (×——×) *90 μg of glucose per liter (buffer: Tris);* (×- - -×) *90 μg of glucose per liter (buffer: Tris-maleate).*

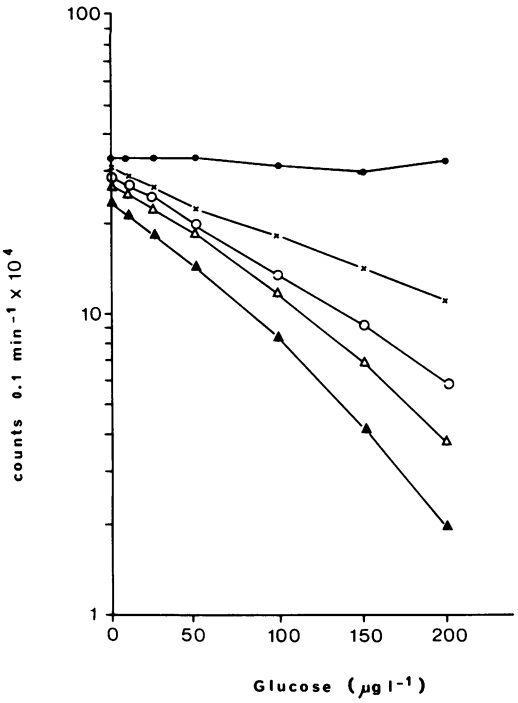

FIG. 5. *Effect of hexokinase amount on glucose determination. Symbols:* (●) *no hexokinase;* (×) *10 U of hexokinase;* (○) *20 U of hexokinase;* (▽) *30 U of hexokinase;* (▼) *40 U of hexokinase.*

for 20 U of enzyme and 240,000 counts for 40 U of enzyme. We decided to run this enzymatic reaction with 20 U of the enzyme, which, in the 25- to 200-μg/liter range of glucose concentration, gives a large enough difference in counts and remains in the acceptable economic range.

Specificity of the enzyme. Since this new method of glucose determination is aimed at measuring the available organic carbon in natural waters, it was important to check the specificity of the enzyme to different carbohydrates.

Figure 6 represents a calibration curve of glucose. In Table 1, the relative activity of different substrates to glucose is presented. As can be seen from this table, fructose showed about 50% of the activity of glucose. Mannitol showed only 20% of the activity of glucose, and other sugars showed even lower activity.

Internal standardization. The method of glucose determination described above was developed for our needs in glucose estimation in Lake Kinneret. To check its efficiency using lake water, known amounts of glucose were added to 1 ml of lake water. Routine measurements of glucose content in lake water were usually done by lyophilization of 2.5 ml of water; therefore, we also added known amounts of glucose to samples after lyophilization. The results of these two treatments are summarized in Table 2. As can be seen, the recovery of the glucose from the lake water was between 85 and 114%. The counts received for the amount of glucose added showed a coefficient of correlation of 0.990, 0.995, and 0.981 for distilled water, 1 ml of lake water, and 2.5 ml of lyophilized lake water, respectively.

An additional experiment was run to check whether there was any influence of lyophilization on the glucose determination. In this experiment, glucose was added to 2.5 ml of lake water before lyophilization. The recovery of the glucose added was between 80 and 100% (Table 3).

Glucose content of Lake Kinneret water. Lake water from five different depths was fil-

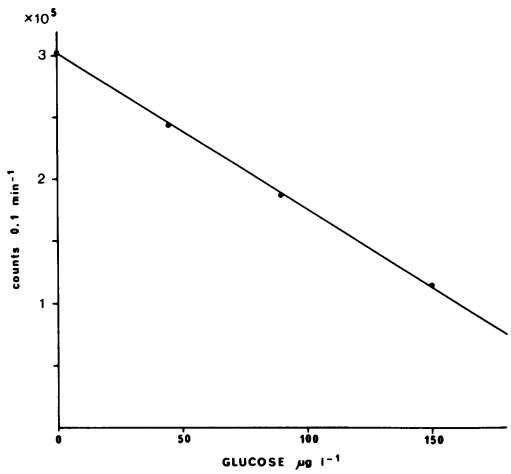

FIG. 6. *Calibration curve of glucose concentration.*

TABLE 1. *Relative hexokinase activity with different carbohydrates*

Carbohydrate	Concn (μg/liter)	Counts/0.1 min (×10⁻⁵)	Concn as glucose equivalent[a] (μg/liter)
None		3.03	0
Ribose	75	3.00	0
Galactose	90	2.91	7
Cellobiose	170	2.85	8
Mannitol	90	2.76	18
Sucrose	170	2.75	18
Fructose	90	2.45	43
Glucose	90	1.87	90
Cellulose	300	2.97	5
Glycogen	300	2.82	12

[a] Calculated from the glucose standard curve (Fig. 6).

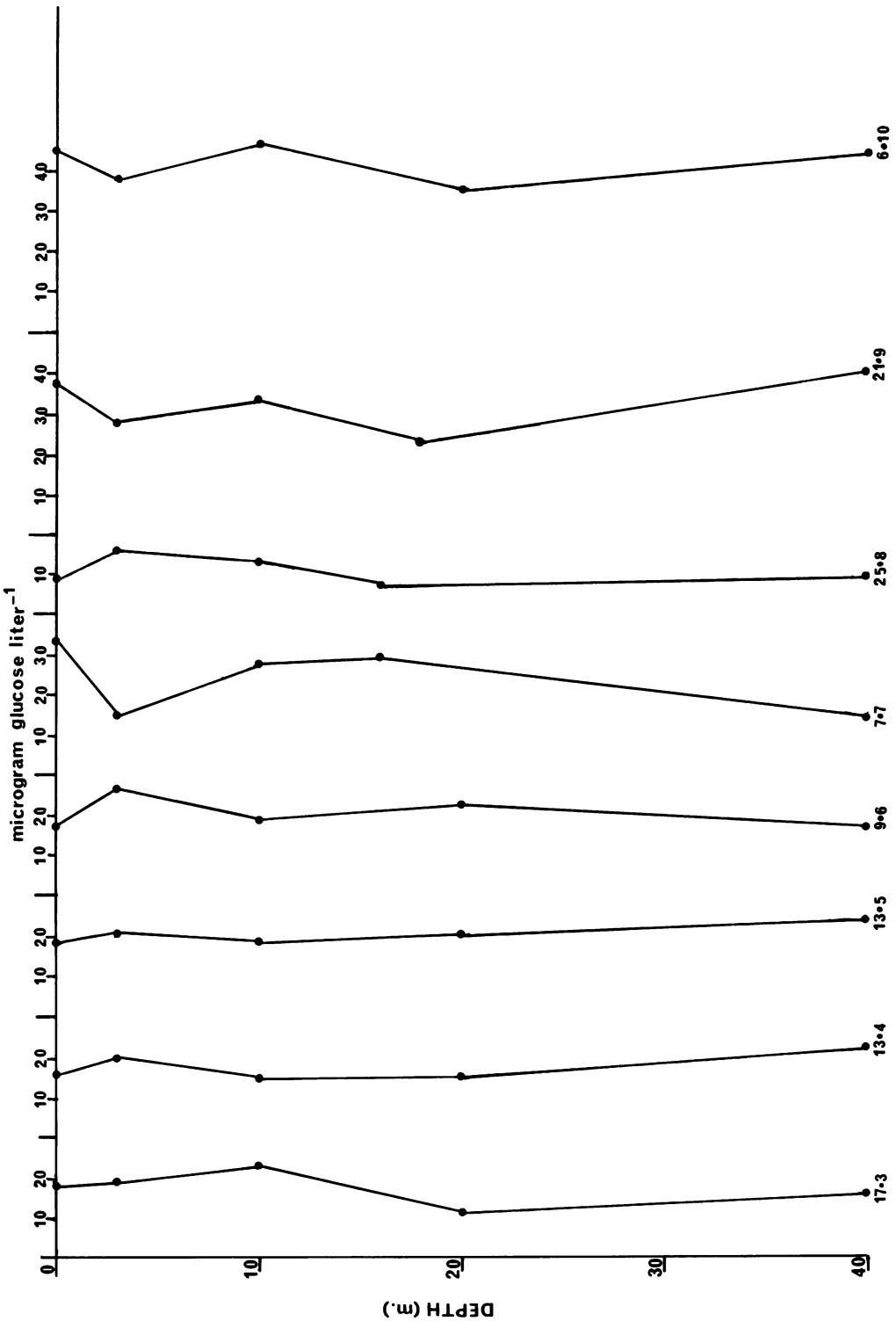

FIG. 7. *Vertical distribution of glucose, in micrograms per liter, from March to October 1976.*

TABLE 2. *Recovery of added glucose from lake water after lyophilization*

Glucose added (ng/ml)	Distilled water counts (0.1 min) × 10^{-5}	1 ml of lake water				2.5 ml of lake water			
		Counts (0.1 min) × 10^{-5}	Glucose amt (ng/ml)		Recovery (%)	Counts (0.1 min) × 10^{-5}	Glucose amt (ng/ml)		Recovery (%)
			Total	Total minus lake water content			Total	Total minus lake water content	
0	4.78	4.36	16.5			3.85	43		
50	3.66	3.34	70.5	54	108	2.98	100	57	114
100	2.71	2.57	111.5	95	95	1.96	143.5	100.5	100.5
150	1.75					1.44	171	128	85
200	1.06	1.04	192.5	176	88				

TABLE 3. *Recovery of added glucose from lake water before lyophilization*

Glucose added (ng/ml)	Distilled water counts (0.1 min) × 10^{-5}	Glucose added (ng)	Counts (0.1 min) × 10^{-5}	Glucose amt (ng)		Recovery (%)
				Total	Total minus lake water content	
0	3.09	0	2.43	34		
50	2.17	25	2.04	54	20	80
100	1.40	50	1.54	84	50	100
200	0.51	75	1.33	104	70	93
		100	1.15	114	80	80
		150	0.72	164	130	87

tered on the boat immediately after sampling, and glucose content was determined as described in Materials and Methods. The depth distribution showed small variations, with an average of about 20 μg/liter during the period March–July (Fig. 7). In August, the concentration decreased a little, followed by an increase in September and a continuous increase during October.

DISCUSSION

The new method for determination of glucose presented in this paper was developed to determine glucose in natural-water bodies. The dilution bioassay used by Allen (1, 2) for determination of glucose involves determination of glucose uptake kinetics at several glucose concentrations for each measurement of glucose content of a water sample. It is therefore very complicated and time-consuming when routine determinations of glucose content in lakes is required.

The enzymatic assay of Hicks and Carey (7) was tested in our laboratory, and it was found that with lake water, the lowest glucose concentration that could be determined was 10^{-6} M. In the present method, we lyophilized 2.5 ml of lake water before determining the glucose content, since the lowest detection limit using this method is about 20 μg/liter, and sometimes we found concentrations of 8 μg of glucose per liter in the lake. The internal standardization of this method with lake water showed that lake water did not interfere with the determination of glucose, and the recovery of the glucose added was never below 80%. Lyophilization of the water also had no deleterious effect on the glucose determination.

By using this method, we are able to estimate the heterotrophic activity of lake water, and results of such determinations will be published soon.

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