ANALYTICAL BIOCHEMISTRY 47, 90–101 (1972)

Apparatus for Continuous Measurement of Active Uptake of Radioactive Substances

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Received August 4, 1971

To obtain kinetics for the active uptake of substances into cells, the most commonly employed method is to take samples, at various times, of the cells or of the surrounding medium and to determine the content of the respective compound. The separation of the cells from the medium is achieved either by centrifugation or by filtration through membrane filters. In order to have a convenient and more direct way to follow active uptake and also efflux, an apparatus based on the following principle has been developed: With a membrane, the cells are kept near a radioactivity-sensing device, e.g., a scintillation crystal in conjunction with a photomultiplier. Above this is a larger volume of liquid containing the labeled compound that is taken up. Of the total radioactivity in the medium only a small fraction is detected, as most of the radiation is relatively far from the crystal and is absorbed by the water. As the labeled compound is taken up by the cells, the radioactivity is concentrated on the scintillation crystal and can be registered. The medium above the membrane has to be stirred in order to minimize concentration gradients developing during the uptake process.

DESCRIPTION OF THE APPARATUS

The cells are confined in a round flat compartment. Its bottom is a scintillator (stilbene) over which an aluminized polyester foil (6 μm) is stretched by use of an O-ring to prevent contamination (Fig. 1). The scintillator is cemented to a supporting Plexiglas base. The height of the compartment is 0.1 mm, determined by a ring-shaped polyethylene spacer, and the top is formed by a Sartorius nitrocellulose membrane filter, pore width 3 μm. The filter is drawn with an O-ring over one end of a Plexiglas cylinder the inner diameter of which matches that of the spacer. To apply cells to the apparatus, a small drop of suspension is set on the coated scintillator; the spacer is put on the membrane filter

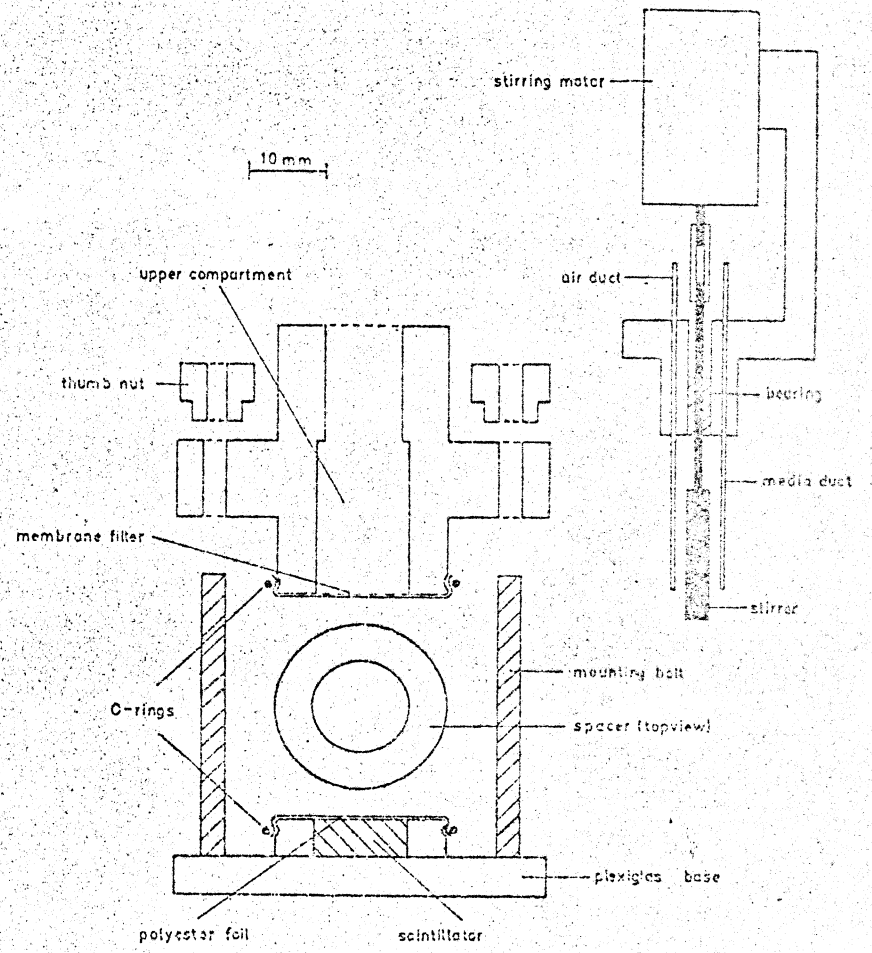

FIG. 1. Setup for measuring uptake of radioactive substances by cells. Vent in plug-in unit (top right) not shown for clarity.

(already mounted to the Plexiglas cylinder), adhering to it because it is moist. Since the middle part of the cylinder wall is shaped as a protruding ring with holes that fit corresponding bolts from the Plexiglas base, it is centered and screwed down with thumb nuts. Any excess cell suspension is thus squeezed out. In the top of the cylinder a unit with a motor-driven stirrer, a vent, and two stainless-steel tubes to feed in gases and liquids is plugged in. The whole assembly is placed on the window of an EMI 9536 end-on photomultiplier with a silicon oil immersion between the window and the Plexiglas base and is set under a light-tight cover. Liquids can be added or withdrawn through 0.5 mm

i.d. black plastic tubing with a syringe outside the cover. The photo multiplier is connected to a Frieseke FH 49 counter, which provides an outlet to record the level of radioactivity.

APPLICATIONS

For the method outlined to be useful, it is essential that the rate of diffusion through the membrane filter does not limit the rate of uptake by the cells. Therefore first experiments were carried out without cells to discover the kinetics of mere diffusion.

Diffusion experiments. Into the space above the membrane filter (upper space or compartment) was pipetted 0.2 ml 0.08 *M* sodium phosphate buffer, pH 6.5. The space beneath the membrane filter (lower space or compartment) was also filled with buffer; its volume was only 11 μl. Then 1 ml of solution with ^{14}C-labeled D-glucose or a D-glucose analog containing 1–2 μCi was injected into the upper compartment and the increase in radioactivity registered. Figure 2 shows an example of the kinetics obtained in this way. After about 2–3 min the radioactive substance had equilibrated between the two compartments. The measured radioactivity was proportional to the concentration of radioactivity in the medium; in the equilibrated state 1140 cpm corresponded to 1 μCi/ml.

Rate constants were calculated from the maximum change in radioactivity after injection and from the final amplitude. A series of five experiments with α-methyl-D-glucoside gave an average value of 1.1×10^{-2} sec^{-1}, the maximum deviations being +25% and −20%. Similar values were obtained with 3-*O*-methyl-D-glucose and D-glucose. The rate constant was used to estimate the maximum diffusion rate for later

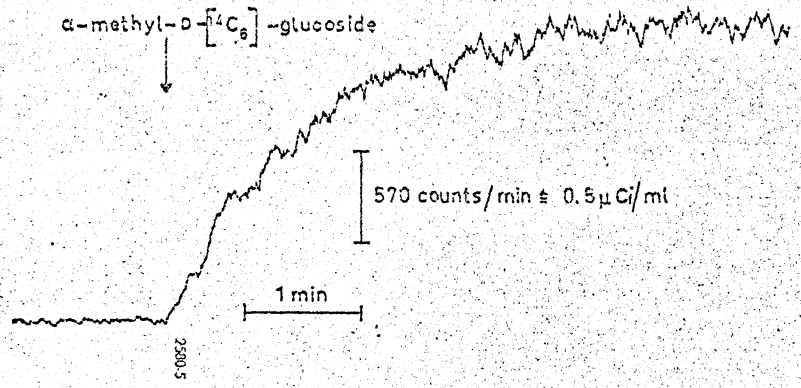

FIG. 2. Increase in measured radioactivity with time on addition of α-methyl-D-[$^{14}C_6$]-glucoside. No cells present.

experiments with cells, where the low level of radioactivity used obliterated a direct measurement of the diffusion rate.

By faster stirring with a propeller-shaped stirrer it was possible to obtain rate constants up to 2×10^{-2} sec^{-1}. Probably this condition makes possible a complete mixing in the upper compartment almost immediately, whereas with low-speed stirring and a rectangular stirrer (Fig. 1) a zone of less turbulence forms somewhat above the membrane filter. This may lead to a longer equilibration time in the upper compartment itself and, by inference, also between the two compartments. The rapid stirring was not employed further, as with algae in the lower compartment pressure changes or turbulences transduced through the membrane filter caused the cells to distribute unevenly.

3-O-Methyl-D-glucose uptake by Chlorella vulgaris. Unicellular algae, e.g., *Chlorella,* are able actively to take up sugar analogs that have accumulated more than 100-fold without being further metabolized (1,2). For the experiments reported here, *Chlorella* was grown as described previously (3). Since the uptake system for D-glucose and D-glucose analogs in *Chlorella vulgaris* is inducible (1–3), the cells (40 μl packed cells/ml suspension) were preincubated with 7×10^{-5} *M* D-glucose for 2–3 hr, after which time all D-glucose had been taken up and metabolized (1).

All experiments were carried out in 0.08 *M* sodium phosphate buffer, pH 6.5; the upper compartment was aerated to prevent oxygen depletion in the algal layer. Because of the manifold accumulation of the label in the cells, a much smaller amount of radioactivity could be employed (0.1 μCi) than was used in the diffusion experiments.

Kinetics. Figure 3a shows an example of uptake and efflux kinetics of 3-*O*-methyl-D-glucose. The increase in measured radioactivity to be expected upon equilibration in the absence of algae is indicated by the dotted lines. The dashed line shows the corresponding initial rate of diffusion calculated with the rate constant mentioned above.

The efflux of 3-*O*-methyl-D-glucose out of the algae was made apparent by adding a small volume of nonlabeled D-glucose solution. This sort of efflux (countertransport) with *Chlorella* was found previously (4). Surprisingly, efflux rates could be several-fold higher than the calculated maximum diffusion rate. Obviously, as soon as the labeled substance is extruded from the layer of algae in almost immediate contact with the scintillator, it is no longer detected even without having passed through the membrane filter into the upper space.

This explanation has been substantiated by determining the absorptivity of thin layers of water for ^{14}C-β-radiation. In this case, the upper compartment of the apparatus was closed with a 3.5 μm thick polyester

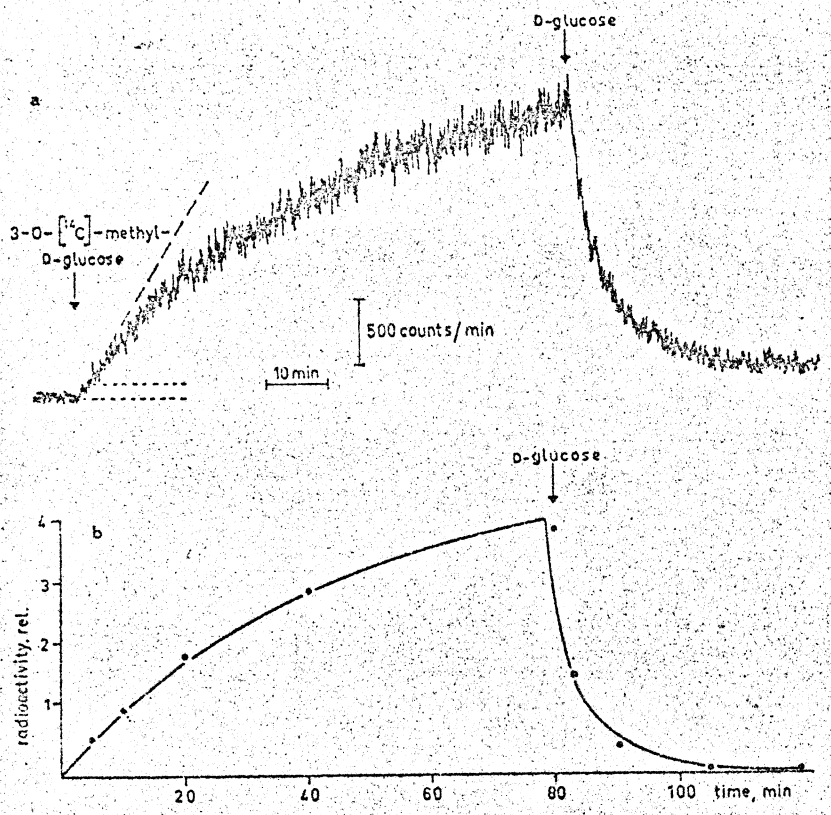

FIG. 3. (a) Uptake and efflux of 3-*O*-[^{14}C]-methyl-D-glucose with *Chlorella vulgaris*. Initial concentration 6.25×10^{-6} *M* corresponding to 0.1 μCi/ml; increase in radioactivity to be seen without algae is indicated by dotted lines, the calculated maximum diffusion rate by the dashed line. A suspension with 70 μl packed cells/ml was applied to the lower compartment; liquid volume in upper space 1.0 ml. Efflux was initiated by adding 0.05 ml 0.25 *M* D-glucose solution. (b) Kinetics of 3-*O*-[^{14}C]-methyl-D-glucose uptake and efflux obtained with the membrane filter technique (points) and with our apparatus (full line, taken from Fig. 3a).

foil impervious to water and filled with a solution of labeled α-methyl-D-glucoside (1–2 μCi/ml). Between this radiation source and the scintillator different amounts of water were brought with the help of spacers of various thicknesses. It became evident (Table 1) that about 30% of the β-radiation was absorbed by a layer of water only 25 μm thick. Therefore the high efflux rates may reflect a quick upward diffusion of the labeled substance into a space 25 μm and more above the scintillator yet under the membrane filter.

The actual uptake (efflux) kinetics one may assume to show up about

TABLE 1
Absorption of ^{14}C-β-Radiation by Layers of Water of Different Thicknesses

Thickness of water (spacer), mm	Measured radioactivity, % of maximum
0.10[a]	5
0.05	7
0.025	18
0.0	100

[a] Spacer normally used in the experiments described.

2 min after injection of 3-*O*-methyl-D-glucose (D-glucose), this time being necessary for the substances to diffuse to the algae. This is depicted in Fig. 3b (full line), where only the part beginning 2 min after injection has been redrawn from the trace in Fig. 3a.

Comparison with another method. To check the validity of the results obtained with our apparatus, comparisons were made with a standard method for measuring uptake processes. With the standard method, 6 ml of algal suspension (2–3 μl packed cells/ml) was shaken in an Erlenmeyer flask in the presence of labeled 3-*O*-methyl-D-glucose (0.15 μCi) of the same concentration as used in the apparatus. Aliquots were withdrawn at intervals and the cells extracted after membrane filtration as described previously (4). The radioactivity of the extract was determined in a liquid scintillation counter.

A parallel experiment of the kinetics of Fig. 3a (respectively, 3b, full line) was performed with algae of the same batch using the conventional filter technique. The result is shown by the points in Fig. 3b, which satisfactorily fit the curve obtained with the new method. Note that the arrow indicating addition of glucose refers to the experiment with the standard method. In the curve from the apparatus, glucose was added 2 min earlier; however, efflux then starts with only 0.5 min delay, probably because it is already fully initiated after minute amounts of glucose have reached the algae.

In further experiments, the K_m for the uptake of 3-*O*-methyl-4-glucose was determined with both techniques, always using the same algal preparations. With the new method, initial uptake rates were obtained from the tangents through the points 3 min after injection of 3-*O*-methyl-D-glucose (compare Fig. 4c). With the standard technique, the 2.5 min values were used. A typical result is shown in Fig. 4. Both methods gave a K_m of about 6×10^{-4} *M*.

Calibration. In view of the fair agreement with the standard technique

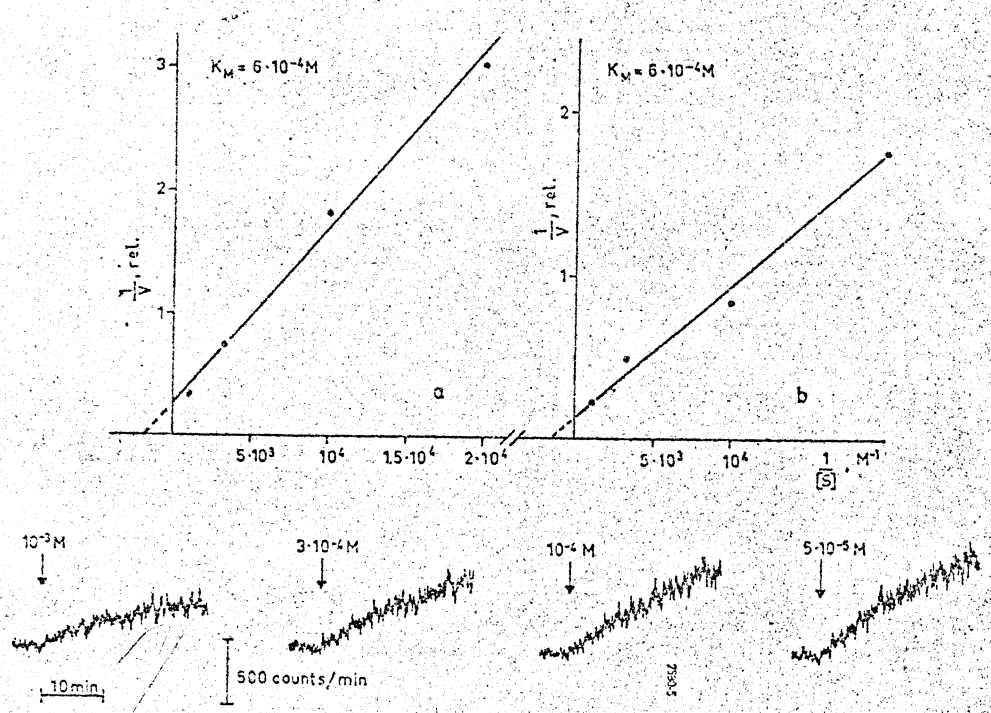

FIG. 4. (a) Lineweaver-Burk plot of uptake rates (V) for 3-*O*-methyl-D-glucose obtained from kinetics shown in Fig. 4c. (b) Lineweaver-Burk plot of uptake rates (2.5 min values) obtained with standard membrane filter technique. (c) Original traces produced with the apparatus. Arrows indicate injection of 3-*O*-[^{14}C]-methyl-D-glucose solution containing 0.1 μCi in all cases; a suspension with 50 μl packed cells/ml was applied to the lower compartment.

an attempt was made to calibrate the relative uptake rates obtained with our apparatus. For that purpose the cell density in the lower compartment was kept to about 50 μl packed cells/ml and the increase in radioactivity (counts/min²) was measured for the interval between 3 and 8 min after injection of labeled 3-*O*-methyl-D-glucose. These rates, representing about the first 5 min of uptake by the algae, were further corrected, assuming a standard specific activity of 1.0 Ci/mole had been employed. Comparison was then made with the rates measured for the first 5 min in parallel experiments using the standard technique. As an average from eight experiments involving five batches of algae an uptake rate of 1 μmole/hr·ml packed cells was found to correspond to an increase in radioactivity of 3.44 counts/min², the maximum deviations being +47% and −26%. From that relation one can derive:

$$1\ \mu\text{mole}/\mu\text{l packed cells} \triangleq 2.06 \times 10^5\ \text{cpm}$$

This expression can be used to estimate from the additional radioactivity in the presence of algae the molarity of 3-*O*-methyl-D-glucose inside the cells (assuming the 3-*O*-methyl-D-glucose is uniformly distributed in the cell). Knowing the 3-*O*-methyl-D-glucose concentration in the medium, one can further estimate the ratio 3-*O*-methyl-D-glucose concentration inside/outside the cell. For instance, in the experiment shown in Fig. 3a, 80 min after addition of 3-*O*-[^{14}C]-methyl-glucose the algae gave 2140 cpm; the specific activity was 16 Ci/mole—therefore with 1 Ci/mole (the specific activity for which the calibration is valid) one would have observed 134 cpm. With the expression given above one calculates the concentration of 3-*O*-methyl-D-glucose inside the cells as

$$134/2.06 \times 10^5 = 6.5 \times 10^{-4}\ M$$

The initial outside concentration of 3-*O*-methyl-D-glucose was $6.25 \times 10^{-6}\ M$ in a total volume of 1 ml. This concentration had changed during the uptake process: The lower compartment contained approximately 0.75 μl packed cells, which after 80 min had reached a 3-*O*-methyl-D-glucose concentration of $6.5 \times 10^{-4}\ M$; the outside concentration then must have come to

$$6.25 \times 10^{-6} - 6.5 \times 10^{-4} \times 0.75/1000 = 5.77 \times 10^{-6}\ M$$

The ratio inside/outside concentration is, then, $6.5 \times 10^{-4}/5.77 \times 10^{-6} = 113$.

In addition, a different approach was made to calibrate the system. As is known from experiments without algae (compare Fig. 2), a shift in measured radioactivity of 1140 cpm is generated by a change in ^{14}C concentration of 1 μCi/ml in the medium; having a specific activity of 1 Ci/mole, a change by 1 μCi/ml corresponds further to a concentration change of $10^{-3}\ M$. When one observes in the presence of algae an increase in radioactivity of 1140 cpm (specific activity again 1 Ci/mole), the concentration inside the algae has increased by $10^{-3}\ M$ also if the following conditions are fulfilled:

(*a*) above the scintillator the space whose radioactivity is registered is completely filled with algae;
(*b*) the algae do not change the counting efficiency.

The first condition probably is not met and one can reason as follows: If the algae occupy only an effective fraction f of the space whose radioactivity is monitored, then a molarity change of $10^{-3}\ M$ in the algae would yield only a change by $f \times 1140$ cpm (specific activity again 1 Ci/mole). A molarity change in the cells of $1\ M = 1\ \mu$mole/μl packed cells would correspond to $f \times 1.14 \times 10^6$ cpm.

We tried to determine f in the following way: A labeled compound that cannot penetrate algal cells is brought into the medium. If no algae are present, the measured radioactivity has a certain level; with algae in the lower compartment, less radioactivity will be seen, as the cells—impervious to the labeled compound—sort of dilute the radioactivity. The fraction by which the algae diminish the original radioactivity level should then be equal to the effective fraction f which the algae occupy from the relevant space above the scintillator.

A suitable substance that does not enter *Chlorella* cells is α-methyl-D-glucoside. This has been shown experimentally: To 2 ml of a thick *Chlorella* suspension with 300 µl packed cells/ml (value corrected for intercellular water according to (5)), 0.05 ml α-methyl-D-[$^{14}C_6$]-glucoside solution was added. After 5, 15, and 90 min, samples were withdrawn and centrifuged, and aliquots of the supernatant checked for their radioactivity. In the control, instead of algal suspension, the same volume of buffer was mixed with the α-methyl-D-glucoside solution. After 5 and 15 min, the α-methyl-D-[$^{14}C_6$]-glucoside concentration in the medium of the *Chlorella* suspension was found to be 1.43 times as high as in the control, as would be expected if no α-methyl-D-[$^{14}C_6$]-glucoside had penetrated the cells. After 90 min, the concentrations differed still by a factor 1.24.

Figure 5 shows which fraction f of the radioactivity due to α-methyl-D-[$^{14}C_6$]-glucoside is screened off with different cell concentrations in the lower compartment. It is evident that even thick suspensions do not completely occupy the space detectable by the scintillator. Further, it can

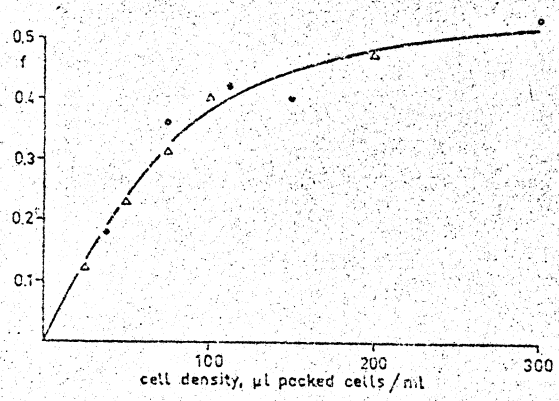

FIG. 5. Fraction f by which the measured radioactivity due to α-methyl-D-[$^{14}C_6$]-glucoside is diminished in dependence on cell density in the lower compartment. Triangles and points belong to experiments with different algal preparations.

be seen from Fig. 5 that cell concentrations of a certain percentage cause an even higher percentage of weakening of radioactivity, e.g., with a suspension of 50 µl packed cells/ml (= 5%) a weakening of already 23% is achieved. This may be explained by a settling of the cells, so that the cells are more densely packed in the layer the radioactivity of which is detected by the scintillator.

Taking $f = 0.23$ for 50 µl packed cells/ml, our calibration comes out with 1 µmole/µl packed cells $\hat{=} 0.23 \times 1.14 \times 10^6 = 2.62 \times 10^5$ cpm. From comparison with the membrane filter technique, an actual concentration change by 1 µmole/µl packed cells was found to yield the somewhat smaller radioactivity change of 2.06×10^5 cpm. This suggests the radioactivity in the cells is counted less efficiently than that in the

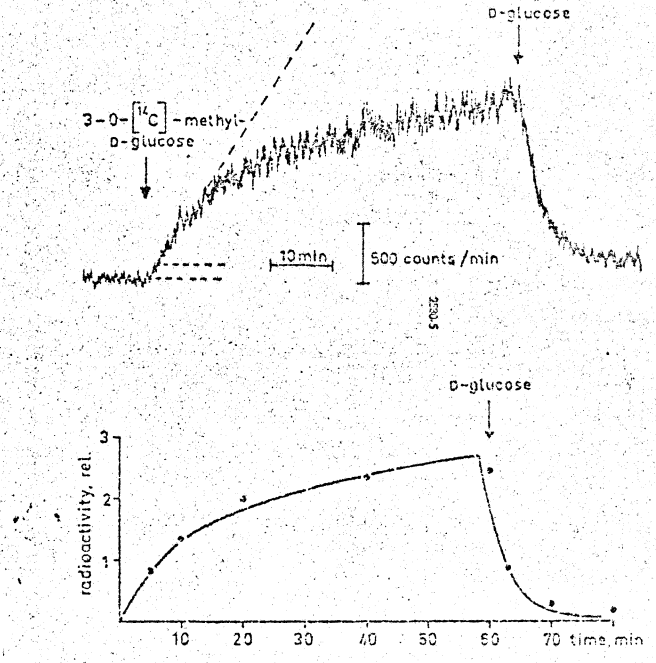

FIG. 6. (a) Uptake and efflux of 3-*O*-[^{14}C]-methyl-D-glucose with conidia of *Neurospora crassa*. Concentration 10^{-4} *M* corresponding to 0.1 µCi/ml; increase in radioactivity to be seen without conidia is indicated by dotted lines, the calculated maximum diffusion rate by the broken line. A suspension with 10 µl packed cells/ml was applied to the lower compartment; liquid volume in upper space 1.0 ml. Efflux was initiated by adding 0.02 ml 5×10^{-2} *M* D-glucose solution. (b) Kinetics of 3-*O*-[^{14}C]-methyl-D-glucose takeup and efflux obtained with the membrane filter technique (points) and with the apparatus (full line, taken from Fig. 6a).

outside medium. This might be due to an uneven distribution of the label in the cell and/or a stronger absorption of β-radiation by the cell wall than by water.

3-O-Methyl-D-glucose uptake by Neurospora. *Neurospora* conidia can accumulate 3-*O*-methyl-D-glucose in the cell manifold above the concentration in the medium; furthermore, a countertransport has been found to occur on addition of glucose (6). These processes seemed promising for further testing of the applicability of the apparatus.

Neurospora crassa strain 74-OR 23-1A De Serres was grown as described elsewhere (7); conidia were harvested in Fries minimal medium (8) and for the experiments transferred into 0.08 *M* sodium phosphate buffer, pH 6.5. An original trace of the kinetics obtained is depicted in Fig. 6a. Comparison was also made with the membrane filter technique. Cell concentration was, in these experiments, approximately 0.8 μl packed cells/ml; otherwise the procedure was the same as with *Chlorella*. As shown in Fig. 6b, both methods gave about the same kinetics.

DISCUSSION

When comparing the merits of the two methods used in this work for uptake measurement, the membrane filter technique seems better suited for short-term kinetics, e.g., uptake during the first 2 min after addition of substrate. If, however, one is concerned with uptake phenomena taking place in a time range of 10 min or longer, the new method seems to be competitive; it also is more time-saving.

Comparison with another method should always be made when working with a new organism. It has been tried, for instance, to measure α-methyl-D-glucoside uptake with *Salmonella typhimurium*, but only qualitative agreement of the kinetics has been found as compared with the filter technique, although accumulation of radioactivity could be clearly demonstrated with the apparatus.

Improvements in the response time of the apparatus might be achieved with membrane filters allowing faster diffusion between the two compartments. One could even omit the membrane filter if thin thalli or filamentous algae were held to the scintillator only at its circumference.

SUMMARY

An apparatus is described allowing the registration of uptake kinetics of radioactive compounds by cells. Cells are held in a thin layer close to a scintillation crystal so that uptake of a labeled compound is seen as an increase in measured radioactivity. As a demonstration of the method, accumulation and countertransport kinetics are shown with 3-*O*-[^{14}C]-methyl-D-glucose for *Chlorella vulgaris* and for conidia of *Neurospora crassa*; for *Chlorella* a K_m of 6×10^{-4} *M* is found for the uptake. Parallel experiments with the membrane filter technique show good agreement and can be used to calibrate the apparatus.

ACKNOWLEDGMENTS

I am greatly indebted to Dr. Drexler from Gesellschaft für Strahlenforschung for the use of the photomultiplier and the scintillation crystal, and to Dr. Tanner for his stimulating interest, Dr. Klingmüller for providing conidia of *Neurospora*, and Miss H. Meyer for skillful technical assistance.

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