Short-Term Radiorespirometry of Cell Suspensions

By WILFRID G. DUNCOMBE

The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

(Received 26 June 1974)

1. An apparatus and method are described with which the oxidation of labelled substrates to $^{14}\text{CO}_2$ by cell suspensions may be examined. 2. The use of high-specific-radioactivity substrates at low concentration together with frequent quantitative collection of CO_2 permit a more detailed analysis of the appearance of various substrate carbon atoms as CO_2 than is possible by existing techniques. 3. Typical experiments with various cell types are reported, in which pathways of glucose oxidation are examined.

The occurrence and relative importance of various pathways of glucose catabolism in different tissues and organisms have been extensively investigated by the use of glucose and metabolic intermediates specifically labelled with ^{14}C . Bloom & Stetten (1953) pioneered the use of C-1- and C-6-labelled glucose with tissue preparations *in vitro*. A comparison of the yields of $^{14}\text{CO}_2$ from these two glucose carbon atoms provided some information about catabolic pathways, since the two carbon atoms would be expected to be oxidized equally via the Embden-Meyerhof-Parnas glycolytic pathway and the tricarboxylic acid cycle, whereas C-1 would be oxidized preferentially by the pentose phosphate pathway.

This approach has been widely used, the experimental technique typically involving collection of total respiratory $^{14}\text{CO}_2$ for a period of 2–3 h from tissue preparations incubated with labelled glucose at a concentration of 10 mM. It was early realized, however (e.g. Wood, 1955), that quantitative evaluation of the results would be complicated by factors such as the recycling of labelled metabolites, and a number of schemes were proposed to correct for these, requiring the use of further specifically labelled substrates and the measurement of the incorporation and distribution of radioactivity in various metabolites. These have been reviewed by Cheldelin *et al.* (1962) and since then there have been further refinements necessitating a wide range of measurements (e.g. Katz & Wood, 1963; Flatt & Ball, 1964; Landau *et al.*, 1964; Landau & Bartsch, 1966; Katz & Rognstad, 1967).

It is clear that some of the difficulties of interpretation result from the use of a system in which the whole complex of metabolic processes is allowed to proceed for several hours.

A kinetic approach, involving the measurement of radioactive CO_2 at intervals during the incubation, was introduced by Wang (e.g. Wang *et al.*, 1956, 1958), who used the term radiorespirometry for this technique. However, these were prolonged experiments in which micro-organisms were incubated with

comparatively large amounts of labelled substrates for periods of up to 15 h with CO_2 -sampling intervals of 0.5–1 h, and the same criticisms of interpretation are valid.

It seemed probable that a simpler picture would be obtained if it were possible to follow the appearance as CO_2 of various carbon atoms in the chosen substrate on a much shorter time-scale. The present paper describes an experimental method based on this concept and some illustrative results are presented that have been obtained with various types of cell.

Materials and Methods

General considerations

The aim of the method is to allow a small amount of labelled substrate to be metabolized as rapidly as possible, and to follow closely the appearance of any resulting $^{14}\text{CO}_2$. The effect would be a pulse of labelled material passing along the metabolic pathways, rather than the continued flow from a large exogenous supply that occurs with the usual technique. This aim implies a number of requirements: (1) penetration of labelled substrate into the tissue should be as rapid as possible; (2) the substrate should be in a low enough concentration, and the amount of tissue sufficient, for substrate utilization to be substantially complete in the course of a short incubation; (3) any $^{14}\text{CO}_2$ formed should be released rapidly from the medium; (4) it should be possible to follow the changing radioactivity in the effluent gas stream accurately and rapidly.

These requirements are fulfilled, as far as possible, in the following ways. (1) Free cell preparations are used so as to minimize the limitation of accessibility of substrates to cells that occurs with tissue slices and fragments. (2) High cell concentrations are used, with labelled substrates at the highest specific radioactivities available. (3) and (4) A

small quantity of carbonic anhydrase in the incubation medium decomposes the [^{14}C]bicarbonate, in which form the metabolic $^{14}\text{CO}_2$ is bound, and the resulting $^{14}\text{CO}_2$ is swept from the medium by a stream of air. This bubbles through a CO_2 -absorbing solution contained in rapidly interchangeable containers. The rationale for this technique is described below (see 'Apparatus').

Requirement (2) may not always be realizable in practice. Thus the substrate concentration is dictated by the amount of radioactivity required to obtain measurable radioactivity in the $^{14}\text{CO}_2$ samples. This may vary considerably according to the quantity of cells available and their metabolic activity. Examples are given in the Results section. Further, when comparing the oxidation of different carbon atoms in one substrate it is essential that the substrate concentration should be the same in all experiments done on the same cell preparation. This again may necessitate using different amounts of radioactivity in the various runs, or the addition of non-radioactive substrate, or both.

Apparatus

The requirement that the radioactivity of the respired CO_2 should be capable of being measured at short time-intervals precluded the use of a flow device such as an ionization chamber or scintillation flow cell. Effects of gas mixing in such detectors, together with the inherent defects of radioactivity measurement by rate-meter, cause the observed response to be a distortion of the changing pattern of radioactivity emanating from the metabolic system, and the flowing sample also causes a low effective counting efficiency.

A method has therefore been devised in which the total CO_2 produced in a short time-interval is collected quantitatively and counted for radioactivity by liquid-scintillation counting. The changing radioactivity can thus be followed quite accurately and counted with high efficiency.

The design finally adopted is shown in Fig. 1. The incubation flask A is made from a Quickfit screw-thread joint (catalogue no. SQ 18) on which a small bulb is blown, with a short side arm B. Over the latter fits a serum cap C (Suba Seal, size 21; William Freeman and Co. Ltd., Barnsley, Yorks., U.K.). A side arm with a B10 cone, D, is attached near the top of the flask, on to which fits a B10 socket and a two-way capillary bore stopcock, E. Into the two outlets of this are cemented (by using thermosetting resin) lengths of stainless-steel tubing (1.5mm outer diam.), F, which dip into CO_2 -absorption medium (see below) contained in standard liquid-scintillation-counting vials, G. The

Fig. 1. Apparatus for incubation of cells and absorption of respiratory CO_2

Details are described in the text.

two-way stopcock and somewhat flexible tubing allow rapid changeover and replacement of the vials.

The lower end of A is immersed in a thermostatically controlled water bath and appropriate gas, usually CO_2 -free air saturated with water vapour at the temperature of the water bath, is bubbled through the system at 200ml/min via the centre tube H. The lower part of the latter has a trace of Silicone MS Antifoam A (Hopkin and Williams, Romford, Essex, U.K.) applied to the outside.

Carbon dioxide absorption

The vials contain 8ml of a 30% (v/v) solution of 2-aminoethanol in 2-methoxyethanol, having an absorption capacity of about 20mmol of CO_2 .

Liquid-scintillation counting

After CO_2 absorption, 12ml of the following scintillator solution is added to each vial: 1% (w/v) butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (Ciba-Geigy, Duxford, Cambridge, U.K.) in toluene-methanol (2:1, v/v). Normal scintillation counting is then carried out, an efficiency of 55–65% being obtained with a Packard 2450 Tri-Carb counter. For many comparative purposes the

observed radioactivity counting rates (corrected for background and as described below) are sufficiently accurate, since quenching is almost identical in all samples, but if desired the absolute radioactivities may be obtained by a suitable standardization method. In either case the CO_2 sample-counting rates are further corrected (a) for different substrate specific radioactivities and (b) for different CO_2 -collection periods (see under 'General procedure').

The radioactivity of aqueous solutions of substrates is measured by counting portions of accurate dilutions in the following scintillator solution: 0.4% PPO (2,5-diphenyloxazole)+0.08% dimethyl-POPOP (1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene) in xylene-Triton X-100 (2:1, v/v), followed by standardization.

Labelled substrates

[3,4- ^{14}C]Glucose was obtained from NEN Chemical G.m.b.H., Frankfurt, Germany. All others were from The Radiochemical Centre, Amersham, Bucks., U.K. Specific radioactivities ranged from 13.5 to 60mCi/mmol.

Substrates were made up to a concentration of 10 or 1 μCi (nominal)/ml, dispensed out in 100 μl quantities in 2ml screw-top polypropylene ampoules (Sterilin Ltd., Richmond, Surrey, U.K.) and stored in a liquid- N_2 refrigerator. The complete contents of an ampoule were used immediately after thawing. In no case was unused material refrozen for subsequent use. The weight of substrate per ampoule was calculated from the specific radioactivity quoted by the suppliers and the radioactivity content of representative ampoules determined as described above. The radiochemical purity was checked by using paper or thin-layer chromatographic systems described in the suppliers' technical data, followed by quantitative radioactive scanning.

Enzymes

Carbonic anhydrase (EC 4.2.1.1) (from bovine erythrocytes) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Pronase (from *Streptomyces griseus*) was obtained from Koch-Light, Colnbrook, Bucks, U.K.

Cell preparations

Escherichia coli. Wellcome Research Laboratories culture no. 2236, glucose-fermenting, grown aerobically, was used. A 16h culture in nutrient broth (200ml) was centrifuged, washed three times with 100ml of 0.85% NaCl and suspended in 2ml of Krebs-Ringer phosphate buffer, pH7.4 (Krebs & Henseleit, 1932).

Human lymphoblastoid cells (Wellcome Research Laboratories strain 7). About 6×10^8 cells from a cultured cell line were treated as above.

Yeast (commercial dried bakers' yeast). About 4g wet wt. of cells from an aerobic culture actively fermenting glucose were treated as above.

Baby-hamster kidney (BHK) cells. About 7×10^8 cells from tissue culture were treated as above.

EL4 leukaemia cells. About 4×10^9 cells were treated as above.

Chlorella pyrenoidosa. This was from the Culture Centre of Algae and Protozoa, Cambridge, U.K. (no. 211/8h). Cells from an agar culture were grown aerobically in a liquid medium. Cells (2.5g wet wt.) were treated as above. Washing and incubation of cells was carried out in very dim tungsten lighting and in addition the glass incubation apparatus was painted black to minimize photosynthesis.

Rat brain cells. Brain tissue from two 130g rats was passed through 110-mesh nylon cloth as described by Rose (1967), and collected in 20ml of ice-cold Krebs-Ringer phosphate medium. After low-speed centrifugation the sedimented cells were taken up in 2ml of the buffer and used for the incubation experiments.

Rat liver cells. The liver from a fed male 130g rat was minced into 20ml of Krebs-Ringer phosphate medium containing 50mg of Pronase (Gwatkin & Thomson, 1964). This was stirred gently at room temperature for 2h, strained through fine stainless-steel gauze and centrifuged for 20min at 4°C and approx. 50g. The supernatant was discarded and the residue resuspended in the buffer and again centrifuged. The final residue was suspended in 2ml of the buffer.

Incubations with all of the cell types described above were carried out in Krebs-Ringer phosphate buffer containing carbonic anhydrase and the appropriate labelled substrate. No other additions were made to the incubation medium.

General procedure

The apparatus is set up with the B10 joint greased, the water bath running at the appropriate temperature and the vials charged with the CO_2 -absorbing fluid.

With a 1ml disposable plastic hypodermic syringe and needle 0.5ml of Krebs-Ringer phosphate buffer is taken up, transferred to a small specimen vial containing 2mg of carbonic anhydrase and, when the latter is dissolved, transferred to the ampoule containing the desired amount of labelled substrate. The ampoules are hydrophobic and the total solution can readily be drawn into the syringe. The contents are now ejected into the incubation vessel via the lower side arm (with serum cap removed) and washed in with a further 0.3ml of buffer. The serum cap is attached, the air flow turned on at 200ml/min and timing started. For this initial period before the

cells are injected (to check that there is no significant non-enzymic release of $^{14}\text{CO}_2$ from the substrate) CO_2 collections are usually made at 1 min intervals for 2–3 min. At the end of each interval the two-way stopcock is turned and the used vial removed and replaced by another already charged with absorber. Scintillator solution from a dispenser is added to used vials immediately, if time permits, or at the end of the run.

After the desired blank collection period, 0.5 ml of cell suspension in buffer is injected through the serum cap, the stopcock is simultaneously turned and sampling continued. It is possible to sample as frequently as every 10 s, but for most purposes it is sufficient to sample at $\frac{1}{2}$ min intervals for the first 4 or 5 min and thereafter at 1 min intervals for a total of 15–20 min. After scintillation counting, the observed radioactivity is corrected as described above. Values for 1-min collection periods are halved so as to make them consistent with the $\frac{1}{2}$ -min collections for plotting purposes. For calculation and plotting of cumulative radioactivity this correction is omitted.

It has been found convenient to have the apparatus set up in duplicate with at least four incubation flasks available. Two operators can then use two differently labelled substrates and immediately continue with two more labels in clean flasks, the tube of stock cell suspension meanwhile having been kept in ice-water. In this way it is possible, for instance, to investigate the oxidation of 1-, 2-, 3,4- and 6- ^{14}C -labelled glucose in a total time of less than 1 h.

The incubated cell suspension may be further investigated at the end of the CO_2 -collection period according to requirements. Thus it may be washed out and a portion taken for determination of residual radioactivity or a protein-free filtrate could be chromatographed and scanned to check on radioactivity in a particular chemical form.

The general procedure was modified in preliminary experiments on the characteristics of the release and absorption of $^{14}\text{CO}_2$. Here the substrate was [^{14}C]bicarbonate in CO_2 -free water, and the $^{14}\text{CO}_2$ was released by the injection either of carbonic anhydrase or of acid or by gassing with $\text{O}_2 + \text{CO}_2$ (95:5).

Results

Efficiency of $^{14}\text{CO}_2$ absorption

For this experiment a train of three absorption vials was made up, connected by stainless-steel tubing sealed through airtight screw caps so that the effluent gas bubbled through each absorber in turn. Lactic acid (0.2 ml of 10%, v/v) was used to release the CO_2 from 0.033 μmol of [^{14}C]bicarbonate

(0.01 μCi , nominal) in 1 ml of water and absorption continued for 2.5 min at a gas-flow rate of 400 ml/min. The three vials contained 28838, 38 and 9 d.p.m. of ^{14}C radioactivity respectively. Thus even at this comparatively high gas-flow rate some 99.8% of the $^{14}\text{CO}_2$ was absorbed in the first vial. It was concluded that a single absorption vial would be adequate, and this was used in all subsequent work.

Time-course of $^{14}\text{CO}_2$ release and absorption

With [^{14}C]bicarbonate (0.066 μmol ; 0.02 μCi nominal) in the flask CO_2 collections were made at 10 s intervals for 1 min. Lactic acid was then injected as above and sampling continued at 10 s intervals for another 3 min. Fig. 2(a) shows that with a gas-flow rate of 200 ml/min a considerable amount of radioactivity is collected within 10 s after acidification. Plotting of the cumulative radioactivity (not shown here) reveals that 33% appears within 10 s and 90% within 1 min. The pattern was substantially similar for gas flows of 200–400 ml/min, whereas at 100 ml/min the tailing was slightly prolonged. A flow rate of 200 ml/min was used subsequently.

Release of $^{14}\text{CO}_2$ from [^{14}C]bicarbonate by carbonic anhydrase

When 2 mg of carbonic anhydrase in 0.5 ml of water was used instead of lactic acid a similar pattern of appearance of $^{14}\text{CO}_2$ was found (Fig. 2b), though with a slight increase in the time to reach maximum radioactivity. In three similar experiments the radioactivity remaining in the medium was less than 0.3% of the total collected. Similar results were obtained with quantities of carbonic anhydrase ranging from 1 to 10 mg.

Exchange of $^{14}\text{CO}_2$ between dissolved [^{14}C]bicarbonate and gaseous CO_2

A small amount of dissolved [^{14}C]bicarbonate would be expected to lose its radioactivity rapidly if vigorously gassed with CO_2 . This was confirmed with labelled bicarbonate in water gassed with $\text{O}_2 + \text{CO}_2$ (95:5), the response being similar to that shown in Fig. 2(b). This might therefore form the basis for a convenient method of releasing metabolically produced $^{14}\text{CO}_2$. For biological work, however, an iso-osmotic buffer must be used, and this must not contain more than a very small amount of non-radioactive bicarbonate, otherwise metabolically produced $^{14}\text{CO}_2$ will become trapped in a comparatively large pool of bicarbonate and its release to the gas phase will be slow. The use of Krebs–Ringer bicarbonate buffer is thus precluded, and the pH stability of Krebs–Ringer phosphate buffer gassed

Fig. 2. Release of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$ bicarbonate by (a) lactic acid and (b) carbonic anhydrase

Procedures were as described in the text. The arrows indicate addition of lactic acid or carbonic anhydrase.

with $\text{O}_2 + \text{CO}_2$ was unacceptable for biochemical purposes. Acceptable buffering was obtained with Krebs-Ringer buffer (without extra phosphate) containing 100 mM-Tes [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid] when saturated at pH 7.4 with $\text{O}_2 + \text{CO}_2$, but the bicarbonate content was still not sufficiently low, and the release of radioactivity from $[^{14}\text{C}]$ bicarbonate in such a solution when gassed with the $\text{O}_2 + \text{CO}_2$ mixture was prolonged.

It was concluded from all the experiments described so far that 'flushing out' metabolic $^{14}\text{CO}_2$ with inactive CO_2 in the gas phase is not satisfactory for biochemical purposes, and that the use of carbonic anhydrase is preferable. With 2 mg of the enzyme and a gas-flow rate of 200 ml/min any metabolically produced CO_2 is released from the medium and absorbed in the collecting vial almost quantitatively within about 2 min of formation. These conditions were therefore used for subsequent biochemical experiments.

Oxidation of glucose carbon atoms by E. coli

The appearance in CO_2 of the radioactivity of C-1, C-2, C-3+C-4 and C-6 of ^{14}C -labelled glucose is shown in Fig. 3(a). Samples were taken each 0.5 min for 5 min after addition of the cells in each case, and thereafter at intervals of 1 min.

The most noteworthy feature is the rapid onset and high degree of oxidation of C-1 compared with the other carbon atoms. In other experiments using even shorter sampling times significant amounts of C-1 were detected in CO_2 within 10 s of addition of the cells, giving a picture very similar to that of Fig. 2(b). Since the shape of the latter is determined by the physical characteristics of the CO_2 release and absorption it is evident that there is a very rapid overall process involving penetration of labelled glucose into the cells, passage through the metabolic pathways oxidizing C-1 and release of CO_2 from the cells.

The possibility that this C-1 peak is an artifact related to non-enzymic release of the labelled C-1 atom is excluded by the observation that negligible amounts of radioactivity are produced until the cells are added. This has been found with all biological systems tested.

After the appearance of C-1 as CO_2 the other carbon atoms follow in the order C-3 + C-4, C-2, C-6, this pattern being consistently found in experiments with this organism.

The interpretation of this pattern requires an examination of the patterns to be expected from various pathways of glucose utilization. If the Embden-Meyerhof-Parnas glycolytic sequence only is operating then C-3 and C-4 of glucose become the carboxyl carbon atom of pyruvate and would, if pyruvate is decarboxylated, be the first glucose carbon atoms to appear as CO_2 . If the tricarboxylic acid cycle is also functional then C-2 + C-5 would next appear as CO_2 (via pyruvate C-2 and acetate C-1) and lastly C-1 + C-6 (via pyruvate C-3 and acetate C-2).

If the pentose phosphate pathway only is operating then C-1 appears first, followed by C-2 and later by C-3, C-6, C-5 and C-4 (Axelrod, 1967). The precise order of appearance at these later stages cannot be as well defined as with the glycolytic pathway, owing to the complexities of recycling and of recombination of triose phosphates to hexose phosphate.

In the Entner-Doudoroff pathway one molecule of glucose ultimately forms one molecule of pyruvate (in which C-1, C-2 and C-3 are derived from C-1, C-2 and C-3 of glucose respectively) and one molecule of glyceraldehyde 3-phosphate, in which C-1, C-2 and C-3 are derived from C-4, C-5 and C-6 of glucose (Cheldelin *et al.*, 1962). The first glucose carbon atom to appear as CO_2 would then be C-1, by decarboxylation of pyruvate, followed by C-2 and then C-3 via the tricarboxylic acid cycle. C-4, C-5

Fig. 3. Oxidation of glucose carbon atoms by *E. coli*

Procedures were as described in the text. (a) Time-course of oxidation of various carbon atoms: —, C-1; - - - -, C-2; - - - -, C-3 + C-4; ·····, C-6. The incubation temperature was 37°C . The glucose concentration was $6\mu\text{M}$, and $0.1\mu\text{Ci}$ (nominal) was used for each experiment. The arrow indicates addition of the cells. (b) Ratio of C-1/C-6 glucose carbon atoms oxidized, calculated from the results of (a). Each point represents the ratio of the cumulative values for C-1 and C-6 oxidized to that time. (c) Effect of substrate concentration. $[1\text{-}^{14}\text{C}]\text{Glucose}$ ($0.1\mu\text{Ci}$, nominal) was used for each experiment at a concentration of 0.011mM (—) or 1.1mM (- - - -). The incubation temperature was 37°C , and cells were added at the arrow.

and C-6 might be oxidized via glycolysis or via the pentose phosphate cycle after recombination.

The glycuronic acid pathway, in which C-6 of glucose is the first carbon atom to appear as CO_2 (Burns, 1967), may also be operative, and further complications may be caused by such reactions as the direct entry of pyruvate carbon into the tricarboxylic acid cycle after CO_2 fixation.

The appearance of glucose carbon atoms as CO_2 through the various pathways is conveniently summarized in chart form by Wang (1967, 1972).

It is apparent that the observed pattern of appearance of glucose carbon atoms in the present experiment cannot result from the operation of any single oxidative pathway. The rapid appearance first of C-1 can be explained only by the operation of the pentose phosphate and/or Entner–Doudoroff pathways, and the relative order of appearance of C-2 and C-6 is consistent with these. The observed position of C-3 + C-4, however, does not fit in with either of these schemes and almost certainly results from the operation of the glycolytic pathway, followed by decarboxylation of pyruvate. The positions of C-2 and C-6 are consistent also with this pathway if the tricarboxylic acid cycle is operative.

The fact that C-6 radioactivity, though appearing later than that of C-2, rises to a peak higher than that of C-2 is apparently significant, since it has been found repeatedly in similar experiments. A calculation of the cumulative radioactivity in CO_2 from the various carbon atoms shows that the total of C-6 oxidized is some 30% greater than that of C-2. Some C-6 is thus being oxidized in excess of that via the glycolytic pathway, since by this pathway C-6 radioactivity would tend to equal that of C-2 after a time but could not exceed it. Results from oxidation experiments are not sufficient alone to identify the route of formation of this extra CO_2 .

The comparatively small total oxidation of C-3 and C-4, the mean being lower than that of any of the other carbon atoms, also shows that oxidative routes other than glycolysis and the tricarboxylic acid cycle are important, though it should be noted that the mean radioactivity for C-3 and C-4 might conceal a considerably greater degree of oxidation of one of these than of the other, and this cannot be resolved by using $[3,4\text{-}^{14}\text{C}]\text{glucose}$.

A plot of the cumulative C-1/C-6 ratio in CO_2 (Fig. 3b) shows one of the advantages of the technique of frequent sampling. Collection of total CO_2 over a period as short as 20 min would have indicated a C-1/C-6 ratio of about 1.15, suggesting a comparatively small contribution from a pathway preferentially oxidizing C-1. In fact the ratio is more than 170 during the first $\frac{1}{2}$ min of incubation.

The possibility that some of the radioactivity in the effluent gas from the incubation flask might be

due to volatile metabolites other than CO_2 was investigated. In this experiment the effluent gas was first passed through a glass spiral trap cooled to -78°C and then through the normal CO_2 -absorbing solution. Air was passed through a suspension of *E. coli* cells metabolizing $[\text{U-}^{14}\text{C}]\text{glucose}$ for 21 min. The total CO_2 radioactivity collected was 1.46×10^6 d.p.m., and the total radioactivity in material from the chilled trap was about 150 d.p.m. It is unlikely that there is any metabolite of glucose other than CO_2 that would pass through a trap at -78°C and it may be concluded that when labelled glucose is used with *E. coli* the technique is specific for the collection of CO_2 .

Effect of substrate concentration

Fig. 3(c) shows the oxidation by *E. coli* of $[1\text{-}^{14}\text{C}]\text{glucose}$ at two different concentrations. At the lower concentration, 0.011 mM, there is the sharp initial peak as already reported, falling fairly rapidly to a low value. At 1.1 mM the time to reach maximum radioactivity is delayed and later there is a continued steady evolution of $^{14}\text{CO}_2$. It should be noted also that the maximum rates of oxidation are not proportional to the substrate concentrations. These observations suggest that the cell concentration is rate-limiting for glucose oxidation at the higher substrate concentration and that under such conditions one of the requirements considered earlier (see the Materials and Methods section) is not fulfilled.

Oxidation of glucose carbon by yeast

As with *E. coli* there is a large and rapid initial output of CO_2 from C-1 of glucose (Fig. 4a), but here the peak for C-2, though considerably smaller, follows closely in time that for C-1. Oxidation of C-6 is negligible and that of C-3 + C-4 very small and later than that of C-2. These findings would be consistent with only a small proportion of glucose being oxidized via glycolysis and the tricarboxylic acid cycle, the chief contribution being from the pentose phosphate and/or Entner–Doudoroff pathways.

Oxidation of glucose carbon by rat brain cells

An entirely different pattern is observed with brain tissue (Fig. 4b). The early peak of C-3 + C-4 followed by C-2 is consistent with a major contribution to oxidation via glycolysis and the tricarboxylic acid cycle (cf. Bloom, 1955). The equal radioactivities of C-1 and C-6 in the latter part of the experiment also support this, the earlier broad peak in C-1 indicating a small contribution from the pentose phosphate or Entner–Doudoroff pathways.

Fig. 4. Oxidation of glucose carbon atoms by various organisms

Procedures were as described in the text. Arrows indicate addition of cells. —, C-1; ---, C-2; ----, C-3 + C-4; ·····, C-6. (a) Yeast cells, (b) rat brain cells, (c) EL4 leukaemia cells, (d) rat liver cells, (e) *Chlorella pyrenoidosa*. The incubation temperature was 30°C for (a) and (e), and 37°C for (b), (c) and (d). The glucose concentration was 60 μ M for (d), and 6 μ M for (a), (b), (c) and (e). The radioactivity used in each experiment was 1 μ Ci for (d), and 0.1 μ Ci for (a), (b), (c) and (e).

Oxidation of glucose carbon by other organisms and cells

In a number of other systems tested the initial peak of C-1 radioactivity is again the predominant feature. In leukaemia cells (Fig. 4c) the pattern is similar to that for yeast, though the radioactivities of C-3 + C-4 and C-6 suggest a somewhat greater contribution from glycolysis. The metabolic activity of rat liver cells (Fig. 4d) is evidently very low, as shown by the low CO_2 radioactivity and by the finding, from chromatography and radioautography, that much of the radioactivity in the media after 20 min was still in the form of glucose. However, the initial rapid peak for C-1 again indicates appreciable relative activity of the pentose phosphate and/or

Entner–Doudoroff pathways. Very low radioactivity in CO_2 was also found in a similar preparation from the liver of a rat starved for 18 h. The low radioactivities are thus not due to dilution of the tracer glucose with glucose derived from endogenous glycogen. It is likely that some damage is caused to the cells by the method of preparation.

In *Chlorella pyrenoidosa* (Fig. 4e) the C-1 peak is again apparent though at a noticeably later time than in other cells, possibly owing to slower penetration of glucose through the cell walls.

Other cells that have been investigated include a human lymphoblastoid cell line and baby-hamster kidney cells, in both of which the initial C-1/C-6 ratio is very large.

Fig. 5. Oxidation of succinate carbon atoms by *E. coli*

The procedure was as described in the text. The succinate concentration was $88 \mu\text{M}$, and $1 \mu\text{Ci}$ (nominal) was used for each experiment. The incubation temperature was 37°C , and cells were added as indicated by the arrow. —, C-1 + C-4; ----, C-2 + C-3.

Oxidation of succinate

Fig. 5 shows the oxidation of $[1,4-^{14}\text{C}]$ succinate and $[2,3-^{14}\text{C}]$ succinate by *E. coli*. The pattern is consistent with the expected appearance first of CO_2 from the carboxyl groups of succinate via the tricarboxylic acid cycle and then with the oxidation of the methylene carbon atoms after further turns of the cycle.

A search for volatile radioactive metabolites other than CO_2 was carried out as described above. *E. coli* metabolizing $[1,4-^{14}\text{C}]$ succinate for 20 min produced 2.023×10^6 d.p.m. in the CO_2 and 4370 d.p.m. in the condensate in the -78°C trap. The small amount of radioactivity in the trap, about 0.2% of the total radioactivity collected, indicated that there is no significant amount of labelled metabolite likely to interfere with measurements of true CO_2 radioactivity.

Discussion

The biochemical results presented are intended only to indicate, with a variety of cell types, some potentialities of the method which, in principle, can be used to investigate any process in which a carbon atom appears as CO_2 . It is not suggested that it alone will necessarily provide sufficient data for the quantitative evaluation of metabolic pathways. Indeed the use of complementary information such as the distribution of radioactivity in metabolites will usually be required, but even without this it is possible to obtain valuable qualitative results.

Other possible applications that have not been

investigated include the use of other labelled substrates, the effect of pure oxygen for gassing, anaerobic production of CO_2 , the use of subcellular fractions and the use of inhibitors and stimulators. In any new application it would be necessary to confirm, by using suitable traps, that no radioactive product other than CO_2 is being collected in the CO_2 absorber.

It must be emphasized that the reactions being measured include the whole series of steps from substrate to CO_2 . Negative results must therefore be interpreted with caution, and may only indicate that the last step involving CO_2 release is not operative. Thus lack of radioactive CO_2 from $[3,4-^{14}\text{C}]$ glucose would not necessarily mean that the glycolytic sequence as far as pyruvate is not active.

The present results demonstrate some of the advantages of the method over those used hitherto. The very large glucose C-1/C-6 ratio observed at an early stage with many tissues is much more convincing as evidence for a pathway preferentially oxidizing glucose C-1 than the low cumulative ratio obtained after long incubation times. The order of appearance of the various carbon atoms may be significant and this can only be seen in detail over a short time-span, and the rate of appearance of the first detectable radioactivity may provide information on substrate transport.

The use of very low substrate concentrations means that the pattern of CO_2 evolution in later stages of the experiment, when recycling may be occurring, does not have superimposed on it the continued primary oxidation of substrate that would be found with higher concentrations. This does, however, require a tissue preparation of sufficiently high metabolic activity, which was probably not the case, for instance, with the rat liver-cell experiment reported here.

Other advantages of the short-term experiment include less deterioration of the cells, less possibility of the growth of contaminating micro-organisms and less accumulation of possibly inhibitory end-products.

A major limitation is that the tissue preparation must provide ready access for substrate and oxygen to the cells. The final entry of substrate will of course be dependent on the mechanism of transport for the substrate and cell concerned, but the access of substrate to the cell will depend on the size of tissue pieces being used. In the experiments described free cells have been used, so that access is not a limiting factor. Thick tissue slices would obviously diminish the resolution of response obtained with free cells, since the cells within the slices would not be supplied with substrate immediately. It is possible that thin slices might give useful results; this has not been examined.

Thanks are due to Mr. K. D. Townsend for technical assistance, and to the following colleagues for supplying some of the biological material: Dr. D. C. Edwards, Mr. J. F. Spilsbury, Mr. T. P. Varley and Dr. Barbara Woodward.

References

- Axelrod, B. (1967) in *Metabolic Pathways* (Greenberg, D. M., ed.), vol. 1, chapter 5, Academic Press, New York and London
- Bloom, B. (1955) *Proc. Soc. Exp. Biol. Med.* **88**, 317–318
- Bloom, B. & Stetten, DeW., Jr. (1953) *J. Amer. Chem. Soc.* **75**, 5446
- Burns, J. J. (1967) in *Metabolic Pathways* (Greenberg, D. M., ed.), vol. 1, chapter 7, Academic Press, New York and London
- Cheldelin, V. H., Wang, C. H. & King, T. E. (1962) in *Comparative Biochemistry* (Florkin, M. & Mason, H. S., eds.), vol. 3, chapter 9, Academic Press, New York and London
- Flatt, J. P. & Ball, E. G. (1964) *J. Biol. Chem.* **239**, 675–685
- Gwatkin, R. B. L. & Thomson, J. L. (1964) *Nature (London)* **201**, 1242–1243
- Katz, J. & Rognstad, R. (1967) *Biochemistry* **6**, 2227–2247
- Katz, J. & Wood, H. G. (1963) *J. Biol. Chem.* **238**, 517–523
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Landau, B. R. & Bartsch, G. E. (1966) *J. Biol. Chem.* **241**, 741–749
- Landau, B. R., Bartsch, G. E., Katz, J. & Wood, H. G. (1964) *J. Biol. Chem.* **239**, 686–696
- Rose, S. P. R. (1967) *Biochem. J.* **102**, 33–43
- Wang, C. H. (1967) in *Methods of Biochemical Analysis* (Glick, D., ed.), vol. 15, pp. 311–368, Interscience Publishers, New York
- Wang, C. H. (1972) in *Methods in Microbiology* (Norris, J. R. & Ribbons, D. W., eds.), vol. 6B, chapter 7, Academic Press, London and New York
- Wang, C. H., Gregg, C. T., Forbusch, I. A., Christensen, B. E. & Cheldelin, V. H. (1956) *J. Amer. Chem. Soc.* **78**, 1869–1872
- Wang, C. H., Stern, I., Gilmour, C. M., Klungsoyr, S., Reed, D. J., Bialy, J. J., Christensen, B. E. & Cheldelin, V. H. (1958) *J. Bacteriol.* **76**, 207–216
- Wood, H. G. (1955) *Physiol. Rev.* **35**, 841–859