A CONVENIENT ENZYMATIC-CHEMICAL METHOD FOR DEGRADATION OF MICROAMOUNTS OF GLUCOSE.

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SUMMARY

A convenient method for degradation of 1 micromole of glucose is described. The hexose is split enzymatically to triose phosphate. In one half of this solution glyceraldehyde-3-phosphate is converted enzymatically to alkali stable 3-phosphoglycerate, in the other half, dehydroxyacetone phosphate to alkali stable glycerol-3-phosphate. In each sample the enzymatically unconverted triose phosphate is hydrolized by alkali to lactic acid which is oxidized by cerium^{IV} to acetaldehyde and carbon dioxide. Acetaldehyde is degraded further to iodoform and formic acid. The latter is oxidized to carbon dioxide.

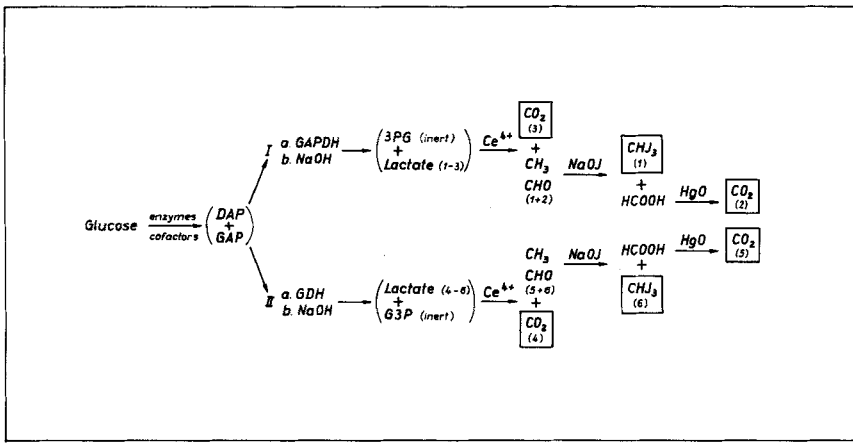
The omitting of laborious chromatographic separation procedures as well as the isolation of degradation products by two step microdiffusion in a single vessel and their trapping by reagents directly applicable for photometric quantification and measurement of radioactivity makes the procedure time saving and easily performable. These advantages compensate for the loss of 50% of glucose carbon.

MATERIALS AND METHODS

In the course of our studies on the incorporation of ^{14}C-labelled precursors into metabolites of rat brain in vivo it was necessary to localize ^{14}C within the carbon skeleton of cerebral glucose. Its presence in minimal amounts excluded the application of common

chemical and microbiological degradation methods. Already existing enzymatic-chemical microtechniques do not discriminate between individual carbon atoms [1, 2] or are laborious due to chromatographic separation of intermediary products ([2] method b, [3]). Independently from these methods we have worked out a procedure for complete degradation of microamounts of glucose. The initial steps were used recently for orientation of the ^{14}C-distribution within the glucose molecule [4].

The procedure consists of the following steps (fig. 1):



Legend figure 1.

Scheme of degradation procedure.

In parenthesis number of carbon atom of origional glucose.

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1. the quantitative enzymatic splitting of glucose to DAP-$^{x)}$ and GAP-hydrazones, their decomposition to free triose-P;

2. in sample I of the divided triose-P solution, GAP is converted enzymatically to alkali resistent 3PG, in sample II DAP to

alkali resistent G3P; in each sample the unchanged triose-P is hydrolyzed to lactic acid;

3. the oxidation of lactic acid to acetaldehyde and carbon dioxide, their separation by microdiffusion;

4. the oxidation of acetaldehyde to iodoform and formic acid, isolation of iodoform by extraction and

5. final oxidation of formic acid to carbon dioxide.

Procedure for 1 /umole glucose in 1 ml of fluid.

1. Quantitative enzymatic splitting of glucose to triose-P [5].

The glucose was praeincubated for 5 minutes at 37°C with the reagents listed in table 1 and the enzymes hexokinase and phosphoglucose isomerase. Fructose-6-P kinase and aldolase were added and the reaction was stopped after 40 minutes by acidification with perchloric acid to 0,3 N and perchlorate was removed as the insoluble potassium salt. In the neutral supernatant the triose-P-hydrazones were decomposed by shaking with benzaldehyde (3 x 1 ml). Benzaldehyde and benzaldehyde hydrazone were extracted with diethylether (4 x 1 ml) and residual ether was removed by aeration. The enzymatically controlled yield of triose-P was > 95 %.

2. Selective conversion of DAP in sample I and GAP in sample II to lactic acid (enzymatic steps according to [6] and [7]).

The solution of triose-P was divided in 2 parts. A mixture of 0,5 ml pyrophosphate buffer pH 8.4 containing 5 /umoles sodium pyrophosphate, 8 international units GAPDH, 5 /umoles sodium arsenate, 2 /umoles NAD and 3 /umoles cysteine was preincubated for 7 minutes at room temperature. Then sample I was added and the oxidation of GAP to 3PG proceeded over 15 minutes. Successive alkalinization to 1 N with sodium hydroxide hydrolyzed DAP in 20

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x) The following abbreviations are used:
DAP = dihydroxyacetone phosphate; GAP = glyceraldehyde-3-phosphate; triose-P = triose phosphate; GAPDH = D-glyceraldehyde-3-phosphate+dehydrogenase; 3PG = 3-phosphoglycerate; GDH = L-*glycerol*-3-phosphate+dehydrogenase; G3P = glycerol-3-phosphate.

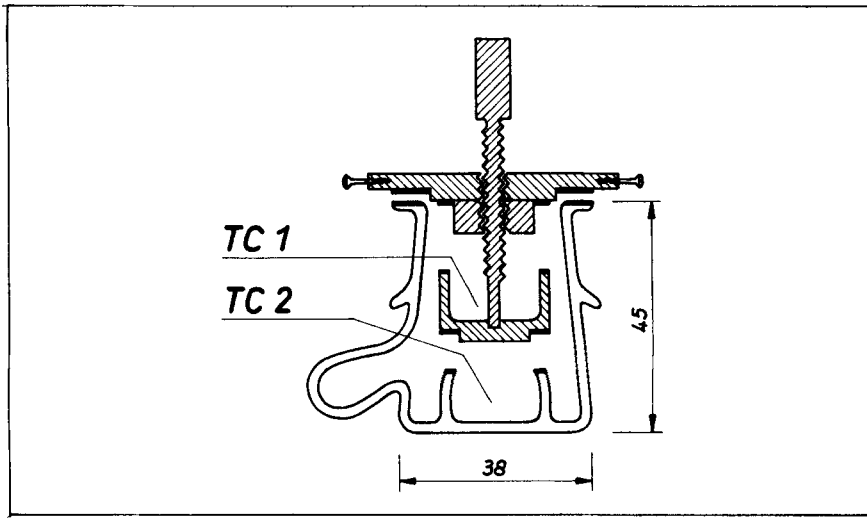
minutes to lactic acid [8]. Sample II of the triose-P was added to 0.5 ml pyrophosphate pH 8.4 containing 5 µmoles pyrophosphate, 2 µmoles NADH and 4 international units of GDH. DAP was reduced to G3P at room temperature within 5 minutes. Then GAP was hydrolyzed to lactate as described above.
Sample I and II were acidified with sulfuric acid to 0,2 N and the precipitated enzyme proteins were centrifuged down. To remove carbon dioxide, nitrogen was streamed through the sample for a few minutes. The amount of lactate was completed to 1 µmole by addition of carrier lactate.
All subsequent steps were performed in an atmosphere and with reagents free of carbon dioxide.

3. Oxidation of lactic acid [9] and microdiffusion of acetaldehyde and carbon dioxide in special diffusion vessel.

The vessel was prepared as outlined in legend of figure 2. Trapping chamber 2 is closed, the cerium IV solution was tipped in and mixed. Cerium phosphates precipitated out so that the final cerium IV concentration was in the range of 0.015 molar. Oxidation and microdiffusion of acetaldehyde at 37°C was complete within 3.5 hours. Successively trapping chamber 2 was opened and 1 was closed and carbon dioxide was absorbed from the color reagent at room temperature quantitatively over 2.5 hours.
An aliquot of the semicarbazide solution was diluted 1:10 with distilled water and the concentration of acetaldehyde semicarbazone was determined by measuring $A_{224\ nm}$. The amount of trapped carbon dioxide was determined by measuring the drop in $A_{555\ nm}$ of the color reagent.
Aliquots were transferred in the vial for liquid scintillation counting (for carbon dioxide alkaline scintillator is recommended).

4. Oxidation of acetaldehyde to iodoform and formic acid [11].

The acetaldehyde semicarbazone was made alkaline by addition of 0,2 ml 1 N sodium hydroxide (dissociation of the carbazone). The sample was chilled in an ice bath and with agitation was added 0,1 N iodine in potassium iodide until a faint yellow color persisted. After 30 minutes the iodoform was extracted with heptane



Legend figure 2.

Diffusion vessel (dimensions in mm).
Main compartment (glass, capacity 28 ml) with trapping chamber 2 (TC, 3 ml) and side-bulb (1,2 ml).
Cover (polymethacrylate) with spindle and TC 1 (2,5 ml). Polished contact planes sealed with mixutre of paraffin oil and wax (1:1).

Trapping reagent for acetaldehyde ([9], modified): semicarbazide hydroohloride, 15 molar in destilled water, pH adjusted to 3.
Color trapping reagent for carbon dioxide [10]: 3 ml 0,1 molar sodium carbonate and 6 ml 0,1 molar sodium bicarbonate mixed and diluted to 100 ml with freshly doubly destilled water. To this buffer solution 1 % phenolphthalein in methanol is added until the extinotion at 555 nm is about 0.9 (addition of approximately 150 µl indicator). The pH is adjusted to 9.8 .
One ml of semicarbazide reagent is brought in TC 1, 1 ml of color reagent in TC 2. The side bulb contains 0.45 ml cerium IV soluti-on (6 N sulfuric acid saturated with cerium^{IV} sulfate at room temperature).

(2 x 1 ml). To remove residual iodine, the combined organic phases were washed with 0.1 N sodium hydroxide (2 x 1 ml) and doubly distilled water (3 x 2 ml). The amount of iodoform in the heptane was determined by measuring $A_{349\ nm}$ (molar extinction coefficient 2080). For measurement of radioactivity, an aliquot was pipetted into the scintillation liquid.

5. Oxidation of formic acid to carbon dioxide [12].

The water phase was adjusted to approximately pH 3 with phosphoric acid and residual iodine was extracted into heptane (5 x 2 ml). Traces of carbon dioxide were removed by bubbling through nitrogen for a few minutes.

The sample was then placed in the main compartment of a Warburg standard vessel whose center well contained 1 ml of 0,1 N sodium hydroxide. To the formic acid solution were added 2 ml of a suspension of mercuric oxide (1,25 g in 10 ml phosphate buffer pH 3) and the closed vessel was placed in a water bath at 85°C. The evolution and absorption of carbon dioxide was complete within 45 minutes. Successively, the sodium hydroxide from the center well was transferred in the main compartment of a Conway dish (unit No. 2) and acidified with 0,4 ml 4 N sulfuric acid. Diffusion and trapping of carbon dioxide in 1 ml color reagent proceeded as described (step 3).

Discussion

The degradation procedure described compensates for a loss of 50 % of glucose carbon in favour of:

1. avoidance of laborious separation techniques for intermediate products. This enables one to keep the volumes of reaction mixtures in all steps below 2.5 ml;
2. simple enzymatic control of yields in steps 1, 2, 3 and
3. termination of degradation at choice when C-1+C-2, C-3, C-4 and C-5+C-6 are directly applicable for determination of quantity and radioactivity.

The data in table 2 inform on the precision of discrimination between the carbon atoms of glucose and lactic acid. The fixation

of the aldolase products DAP and GAP as hydrazones provides for quantitative splitting of the glucose present and protects against triose-P isomerase as a possible contaminant in the enzyme solution of step 1. The equilibria of the enzymes involved in step 2 favour the conversion of DAP and GAP to the alkali resistent compounds which is practically quantitative. When mixed with the cerium IV reagent, no other substances present in the sample beside lactate - cofactors, coenzymes, adenine nucleotides and the alkali stable C_3-intermediates - evolve carbon dioxide or volatile carbonyl compounds which react with semicarbazide. The trapping of substantial amounts acetaldehyde by the alkaline color reagent - in standard experiments 4 ± 1.8 % ^{14}C-acetaldehyde (N = 10) - requires the initial diffusion of acetaldehyde when trapping chamber 2 is closed. Traces of carbon dioxide which diffuse in the semicarbazide reagent - in standard experiments 0.5 ± 0.8 % (N = 8) - are eliminated by bubbling through nitrogen for a few seconds. With this colorimetric method, the recovery of carbon dioxide standards was 101 ± 4.5 % in agreement with a quantitative trapping of ^{14}C-carbon dioxide in amounts up to 2 µmoles.
The oxidation of acetaldehyde by hypoiodite gives maximal yields of iodoform (94 ± 5 %, N = 10) only when the reagents were added strictly in the manner described [11]. The yield of 88 ± 7 % carbon dioxide (N = 10) from the oxidation of formic acid by mercuric oxide could not be increased further.

Table 1

Reagents and enzymes for conversion of 1 µmole glucose to triose-P hydrazones.

Reagents (final volume 1 ml)	µmoles per ml
Phosphate buffer pH 6.5[x)]	15
Magnesium chloride	10
Hydrazine sulfate	40
ATP-Na_2	5
AMP-Na_2[xx)]	5

Enzymes[xxx]	international units
Hexokinase	14
Phosphoglucose isomerase	20
Fructose-6-phosphate kinase (yeast)	6
Aldolase	6

Table 2

Validity of the ^{14}C-discrimination of specifically labelled glucose and lactic acid. Average values (n = 6).

Substrate	relative per cent ^{14}C in position					
	1	2	3	4	5	6
[1-^{14}C]-glucose	100	0.5	1.8	1.5	0	2.8
[6-^{14}C]-glucose	3.2	0	1.1	2.2	0.9	100
[1-^{14}C]-lactate	100	0.5	0.5	-	-	-
[2-^{14}C]-lactate	0.8	100	1.1	-	-	-
[3-^{14}C]-lactate	0.8	0.4	100	-	-	-

x) for fructose-6-phosphate kinase from muscle pH 7.5

xx) counteracting the inhibition of fructose-6-phosphate kinase by ATP

xxx) enzymes from C.F. Boehringer and Soehne, Mannheim (Federal Republic of Germany).

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