'Compartmentation' of Acids in Plant Tissues

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The cells of higher plants, in contrast with those of other organisms, contain relatively large amounts of organic acids. Krebs & Lowenstein (1960) calculated that the concentrations of individual organic acids in *Escherichia coli* are about 0.01 mM, in liver or kidney cells 0.1 mM, and in yeast cells 1 mM. By using the same type of calculation it can be estimated that many plant tissues contain citrate and malate at concentrations of 10 mM, and some contain aconitate or isocitrate at this concentration. Succinate and fumarate are often present at a concentration of 1 mM, but other acids occur at lower concentrations.

Another distinguishing feature of plant cells is the large vacuole which frequently accounts for more than 90% of the volume. Stiller (1959) has given theoretical reasons for supposing that a correspondingly large proportion of the total acid is present in the vacuole. She has calculated that, if malic acid in *Bryophyllum* leaves were confined to the cytoplasm, its concentration would be 7–8 M, but, if it were in the vacuole also, its average concentration would be 0.25 M, a more credible value. Her experimental results also indicate 'compartmentation' of malate, since the malate formed from $^{14}\text{CO}_2$ remained asymmetrically labelled, but asymmetrically labelled malate provided to the tissue from outside became randomized. Bennet-Clark & Bexon (1943) have experimental evidence that individual acids may be constrained inside the vacuole so that an apparent diffusion gradient is maintained between the vacuole and the cytoplasm. The vacuole need not be considered as the only storage reservoir of metabolites, however, since a degree of 'compartmentation' within the cytoplasm itself has been invoked as a rational explanation of a variety of results in which labelling patterns and specific activities of metabolites have been measured after providing ^{14}C -labelled intermediates to various cells (Porter & May, 1955; Steward, Bidwell & Yemm, 1958; Lajtha, Berl & Waelsch, 1959; Berl, Lajtha & Waelsch, 1961; Cowie & Walton, 1956; Cowie & Bolton, 1957; Cowie & McClure, 1959).

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The experiments described below were designed to answer the question whether 'compartmentation' of the large amounts of Krebs-cycle intermediates is a general phenomenon in plant tissues and to determine the extent of 'compartmentation' where it existed. The experimental design was based on the rationale that if a labelled compound, isolated from a biological tissue, has a higher specific activity than its labelled precursor then only a fraction of the precursor pool is involved in the interconversion. The remainder of the precursor, then, is isolated from the metabolic site and mixes with the metabolic pools only when the cellular organization is disrupted by extraction procedures. The Krebs-cycle intermediates are a series of precursors to each other and to the carbon dioxide arising from the cycle. Therefore, if the carboxyl carbons of all the Krebs-cycle intermediates are labelled with ^{14}C and appropriate corrections are made for any asymmetry of labelling, and if all the acid present turns over in the cycle, then the carboxyl carbons of all intermediates and the carbon dioxide arising from the cycle should approach the same specific activity. This has been demonstrated in *Escherichia coli* (Swim & Krampitz, 1954), where equilibration time is short, endogenous metabolism is low and the pool sizes of acids are small. When $[1-^{14}\text{C}]$ acetate was used to label the Krebs-cycle intermediates in maize-root tissue (Harley & Beevers, 1963) the acetate carbon appeared to be sequentially trapped in the sizeable pools of acids of the Krebs cycle and a delay in the evolution of $^{14}\text{CO}_2$ was observed which was measurable in minutes. This indicated a mixing of the respiratory pools with part of the total acid content of the tissue, and the fact that the specific activities of the acids continued to rise during 15 min. indicated that this mixing was extensive.

The present paper describes experiments in which $[1-^{14}\text{C}]$ acetate was provided to a variety of plant tissues to label the carboxyl carbons of the Krebs-cycle intermediates. At intervals over periods of hours, the specific activities of the carbon dioxide and the carboxyl carbons of the acids were measured. As the turnover pools of acids reached equilibrium with accessible acids elsewhere in the cell, the ^{14}C in the carbon dioxide coming from the cycle reached a constant specific

activity. The amount of each individual acid involved in the Krebs cycle should be that fraction of the total amount in which the carboxyl carbons reached the same specific activity as the carbon dioxide arising from the cycle.

The results show that 'compartmentation' is a general phenomenon in plant tissues. As root tissues matured a smaller fraction of the total acid was present in turnover pools and the absolute sizes of the turnover pools showed less variation. In tissues particularly rich in acids, only small fractions were found to be in turnover pools.

MATERIALS AND METHODS

Plant material. Maize seedlings (*Zea mays*, var. Wf9 × 38-11) were germinated and prepared as described by Harley & Beevers (1963). The apical 2-3 mm. and apical 1 cm. sections were obtained from 1-day-old roots, and the third 1 cm. sections from 3-day-old seedlings. Coleoptiles were obtained from 5-day-old seedlings.

Carrots and beets were purchased locally. Cylinders of carrot-phloem tissues were removed with a cork borer (diam. 3 mm.) and sliced with a razor into disks about 0.5 mm. thick. Similar disks were cut from cylinders of beet tissue cut parallel to the long axis of the root.

Wheat plants (var. Monon) were grown in soil in the greenhouse. Leaves were removed 1 week after sowing and cut into strips 1 cm. long. *Bryophyllum calycinum* leaves were taken from greenhouse plants and sliced into 1 mm. × 1 mm. × 10 mm. pieces.

Tissues were washed in deionized water, blotted and weighed. Sample sizes were as follows: *Bryophyllum* leaves, 2.5 g.; 2-3 mm. maize root tips, 2.25 g.; maize coleoptiles, 2 g.; all other tissues, 3 g.

Labelled substrates. [^{14}C]Acetate (6.4 mc/mole) was obtained from Niche Inc.; [^{14}C]glucose (0.58 mc/mole), [^{14}C]glucose (6.54 mc/mole) and [^{14}C]glucose (6.7 mc/mole) were obtained from Volk Radiochemical Co. [^{14}C]Glucose was isolated from castor-bean endosperm that had been allowed to fix $^{14}\text{CO}_2$ for 30 min.; this glucose would contain more than 90% of its ^{14}C in C-3 and C-4 (Stillner, 1959) and it had a specific activity of 0.1 mc/mole.

Procedure. Solutions containing labelled substrate and tissue samples were placed in medium sintered-glass filter funnels. CO_2 -free air was bubbled through the base of the funnel to keep the solution above the sintered-glass disk and to aerate the suspended tissue. Respired CO_2 was carried in the air stream to a T-joint fixed in the top of the funnel. Each outlet from the T-joint led into a 50 ml. centrifuge tube containing 5 ml. of barium hydroxide, hyamine hydroxide (benzyltrimethyl[2-(*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxy]ethyl)ammonium hydroxide) or ethanolamine. A Vigreux column, in which mixing of the air stream and CO_2 absorbent occurred, provided the only outlet from each centrifuge tube. The outlets of the T-joint were alternately clamped off so that the absorbing solutions could be changed at intervals without loss of CO_2 .

To label the carboxyl groups of the organic acids, 5 μmoles of [^{14}C]acetate in 10 ml. of 0.025 M- KH_2PO_4 were provided to each of three or four replicate samples of

tissue. The specific activity of the added acetate was 1.52 $\mu\text{C}/\mu\text{mole}$ for carrot, 1.6 $\mu\text{C}/\mu\text{mole}$ for *Bryophyllum* and 0.31 $\mu\text{C}/\mu\text{mole}$ for all other tissues. CO_2 was collected continuously in barium hydroxide and its ^{14}C content determined at intervals.

In experiments in which the evolution of $^{14}\text{CO}_2$ from [^{14}C]glucose was measured, 8 μmoles of the glucose were provided in 10 ml. of 0.025 M- KH_2PO_4 . The specific activities were as follows: [^{14}C]glucose, 0.054 $\mu\text{C}/\mu\text{mole}$; [^{14}C]glucose, 0.067 $\mu\text{C}/\mu\text{mole}$; [^{14}C]glucose, 0.009 $\mu\text{C}/\mu\text{mole}$; [^{14}C]glucose, 0.065 $\mu\text{C}/\mu\text{mole}$. The $^{14}\text{CO}_2$ was collected in hyamine hydroxide-methanol (2:3, v/v) (Passman, Radin & Cooper, 1956) or in ethanolamine-ethylene glycol monomethyl ether (1:4, v/v) (Jeffay & Alvarez, 1961).

Extraction and separation of acids. At intervals, one of the replicate samples was removed, the bathing solution was drawn off, and the tissue was rinsed with deionized water and transferred to 75 ml. of boiling 80% (v/v) ethanol made acid with formic acid. The samples were then boiled for 1-2 min. The ethanol was decanted and the tissue was ground, first in a Lourdes homogenizer and then in a ground-glass hand homogenizer. The slurry was filtered and the residue, while still on the filter, was extracted successively with 80% (v/v), 50% (v/v), 20% (v/v) and 80% (v/v) ethanol. The extracts were combined and evaporated to dryness at 40° under reduced pressure. The residue was extracted successively with ethyl ether and water to yield, respectively, the lipid fraction and the water-soluble fraction. After removal of the ether, the lipid fraction was dissolved in 10 ml. of toluene. Portions (1 ml.) were removed for ^{14}C assay.

The water-soluble fraction was dissolved in 20 ml. of water and treated with Schwarz Polidase (Schwarz Laboratories, New York) to split phosphate esters. The solution was then fractionated on Dowex resins, as described by Canvin & Beevers (1961), into acidic (mainly organic acids), basic (mainly amino acids) and neutral (mainly sugars) fractions. The amino acid fraction was further separated on a column (6 cm. × 1 cm.) of Dowex 1 (X10; acetate form) resin to obtain acidic (mainly dicarboxylic amino acids) and neutral-plus-basic amino acid fractions (Hirs, Moore & Stein, 1954). After being evaporated to dryness at 40° under reduced pressure, the fractions were dissolved in 25 ml. of water and 1 ml. samples were taken for ^{14}C assay.

The organic acids were further separated by gradient elution from Dowex 1 (formate form) columns as described by Canvin & Beevers (1961). The acidic-amino acid fraction was separated by gradient elution from a column (19 cm. × 1 cm.) of Dowex 1 (acetate form). 4N-Acetic acid was used in the acid reservoir. These techniques gave peaks of pure succinic acid, malic acid, aconitic acid, glutamic acid and aspartic acid. The citric acid peak included a minor labelled component.

The quantities of organic acids were determined by titration with standardized base. Samples containing known amounts of acid were then made up to 10 ml., and 1 ml. samples were removed for ^{14}C assay.

Amino acid peaks were located by their acidity. The samples comprising the peaks were made up to 10 ml., and portions were removed for determination of radioactivity and for determination of amino N by the ninhydrin method described by Yemm & Cocking (1955).

^{14}C -labelled malate samples containing 400–3500 counts/min. were added to 100 μmoles of unlabelled malate. These samples were degraded with *Lactobacillus arabinosus* 17–5 (Nossal, 1952) to CO_2 and lactate. Lactate was purified by gradient elution from a column of Dowex 1 (formate form), and degraded to CO_2 and acetate with permanganate (Katz, Abraham & Chaikoff, 1955). The acetate was purified by steam-distillation and subjected to combustion by the persulphate method of Katz, Abraham & Baker (1954). For the degradation of ^{14}C -labelled glutamate, samples containing 700–13000 counts/min. were made 0.05 M with unlabelled glutamic acid, and 1 ml. portions were removed for decarboxylation. C-1 was removed with ninhydrin (Aronoff, 1961); C-5 was removed from a separate portion by the use of azide (Aronoff, 1961).

^{14}C -labelled citrate was decarboxylated by the oxidative-bromination method described by McArdle (1955). The resulting pentabromoacetone was extracted with petroleum solvents, and subjected to combustion for ^{14}C assay. The ^{14}C in individual carbon atoms obtained from the degradation procedures was expressed as counts/min./mg. of BaCO_3 . The percentage distribution of ^{14}C was calculated from the specific activities so obtained.

Assay of radioactivity. Except for the experiments with *Bryophyllum* all ^{14}C determinations are expressed as disintegrations/min. Corrections are made for counter efficiency and background, and for self-absorption and coincidence when necessary.

The CO_2 collected as BaCO_3 was plated on microporous porcelain planchets and counted under a thin-window continuous-gas-flow Geiger-Müller tube. Specific activity of CO_2 was determined as disintegrations/min./ $\mu\text{equiv.}$ from the weight of BaCO_3 obtained.

The CO_2 collected in ethanolamine or hyamine hydroxide was counted in a Packard Tri-Carb liquid-scintillation spectrometer. One ml. of hyamine hydroxide-methanol solution containing CO_2 was counted in 10 ml. of toluene scintillator composed of 2,5-diphenyloxazole (5 g.) and 1,4-bis-(5-phenyloxazol-2-yl)-benzene (0.25 g.) in toluene (1 l.). One ml. of ethanolamine-ethylene glycol containing CO_2 was counted in 10 ml. of toluene scintillator to which was added 1 ml. of ethanolamine and 7 ml. of ethylene glycol monomethyl ether.

One ml. of the lipid fraction was counted in 10 ml. of toluene scintillator. One ml. of the water-soluble fractions was counted in 10 ml. of dioxan scintillator consisting of 2,5-diphenyloxazole (7 g.), 1,4-bis-(5-phenyloxazol-2-yl)-benzene (0.25 g.) and naphthalene (100 g.) in dioxan (1 l.).

Benzoic acid with a known activity of 1100 disintegrations/min./mg. was used as internal standard to determine the counter efficiency.

RESULTS

General features of acetate utilization

Although there were differences in the rates of $[1-^{14}\text{C}]$ acetate utilization by the various tissues, the results in Figs. 1 and 2, obtained from carrot disks and maize-root tips, show features of acetate metabolism that were shared by the tissues examined.

The $^{14}\text{CO}_2$ output showed an initial lag followed by a linear phase and an eventual decline in rate when the $[1-^{14}\text{C}]$ acetate was exhausted.

Negligible amounts of ^{14}C were incorporated into the neutral (or sugar) fraction.

Incorporation of ^{14}C into the lipid fraction represented a small but significant drainage of acetate carbon in all tissues.

Organic and amino acids comprised the bulk of the soluble ^{14}C -labelled compounds at all times. The

Fig. 1. Total incorporation of ^{14}C into fractions isolated from 3 g. samples of maize roots (first 1 cm. section) exposed to $1.55 \mu\text{C}$ of $[1-^{14}\text{C}]$ acetate for the times indicated. $\circ$, CO_2 ; $\bullet$, organic acids; $\blacktriangledown$, amino acids (total); $\square$, acidic amino acids; $\triangle$, other amino acids; $\blacksquare$, lipid fraction; ∇ , neutral fraction.

Fig. 2. Total incorporation of ^{14}C into fractions isolated from 3 g. samples of carrot-root slices exposed to $7.6 \mu\text{C}$ of $[1-^{14}\text{C}]$ acetate for the times indicated. $\circ$, CO_2 ; $\bullet$, organic acids; $\blacktriangledown$, amino acids (total); $\square$, acidic amino acids; $\triangle$, other amino acids; $\blacksquare$, lipid fraction; ∇ , neutral fraction.

earliest determinations in Figs. 1 and 2 show that the ^{14}C content of the amino acids was already greater than that of the organic acids, although it is clear from the work of Harley & Beevers (1963) that acetate carbon is initially incorporated into non-volatile organic acids and that these are precursors of amino acids. At later times the ^{14}C content of the amino acid fraction declined though that of the organic acid fraction was maintained or increased. Apparently ^{14}C from amino acids was returned to the organic acids and this effect became evident as the $[1-^{14}\text{C}]$ acetate was depleted. Fractionation of amino acids into acidic and neutral-plus-basic fractions clearly shows that the loss in ^{14}C content occurred only from the acidic fraction: ^{14}C in the neutral-plus-basic fraction remained constant or increased throughout the period of the experiment.

Fig. 3. Changing distribution of ^{14}C from $[1-^{14}\text{C}]$ acetate between acidic amino acids and neutral and basic amino acids in: (a) carrot-root slices; (b) maize roots (first 1 cm. section). □, Acidic amino acids; ○, neutral and basic amino acids.

Fig. 3 shows the changing distribution of ^{14}C between acidic amino acids and neutral-plus-basic amino acids with time. All of the amino acid ^{14}C was in the acidic fraction at the earliest times and incorporation into the neutral-plus-basic fraction was a subsequent event. The acidic amino acids, then, are precursors of the ^{14}C -labelled neutral and basic amino acids and this conversion appears to be irreversible. The interconversions with the Krebs-cycle acids occurred without changes in the sizes of the organic acid or amino acid pools (see below) and is thus merely a cyclic transfer of carbon. It appears, then, that the dicarboxylic amino acids represent ancillary pools that can readily exchange carbon skeletons with acids of the Krebs cycle. For this reason we have treated glutamate and aspartate as Krebs-cycle acids and subjected them to the same analysis applied to the organic acids.

Distribution of ^{14}C in malate, citrate and glutamate

To determine whether $[1-^{14}\text{C}]$ acetate was labelling only the carboxyl groups of the Krebs-cycle acids, malic acid samples obtained at intervals from all tissues were degraded. The results are shown in Table 1. The ^{14}C was present only in the carboxyl groups and was distributed about equally between them, as would be expected if the malate skeleton were formed from a symmetrical succinate precursor.

Combustion of citrate samples from maize and *Bryophyllum* and combustion of the pentabromoacetone derived from C-2, C-3 and C-4 of citrate yielded the results in Table 2. C-2, C-3 and C-4 of citrate were unlabelled, showing that all of the ^{14}C in citrate was in the carboxyl groups. From the degradations of malate and citrate it was assumed that ^{14}C in all Krebs-cycle intermediates, including glutamate and aspartate, was confined to the carboxyl groups.

A comparison of the ^{14}C in the carboxyl groups of glutamate is given in Table 3. The ^{14}C was not evenly distributed between the carboxyl groups,

Table 1. Average percentage distribution of ^{14}C from $[1-^{14}\text{C}]$ acetate in the carbon skeleton of malic acid

Experimental details are given in the text.

Tissue	Time of exposure to acetate (hr.)	Distribution of ^{14}C (%)		
		C-1	C-2 + C-3	C-4
Maize root (first 2-3 mm. section)	2	46.8	0	53.2
Maize root (first 1 cm. section)	2	47.5	0	52.5
Maize root (third 1 cm. section)	3	48.2	0	51.8
Maize coleoptile	4	46.2	0	53.8
Carrot-root slices	6	47.2	0	52.8
Beet-root slices	6	48.8	0	51.2
Wheat leaf	4	50.1	0	49.9
<i>Bryophyllum</i> leaf	6	47.9	0	52.1

Table 2. *Distribution of ^{14}C in citrate produced from $[1-^{14}\text{C}]\text{acetate}$*

Radioactivity in C-1-C-6 was determined by combustion of citrate, and that in C-2, C-3 and C-4 by combustion of pentabromoacetone derived from citrate. Experimental details are given in the text.

Tissue	C-1-C-6		C-2+C-3+C-4	
	BaCO ₃ (mg.)	Sp. activity (counts/ min./mg.)	BaCO ₃ (mg.)	Sp. activity (counts/ min./mg.)
Maize root [third 1 cm. section (a)]	16.2	37	5.3	0
Maize root [third 1 cm. section (b)]	16.4	36	5.0	0
<i>Bryophyllum</i> leaf	15.6	95	5.4	0

Table 3. *Average percentage distribution of ^{14}C from $[1-^{14}\text{C}]\text{acetate}$ between C-1 and C-5 of glutamic acid*

Experimental details are given in the text.

Tissue	Time of exposure to acetate (hr.)	Distribution of ^{14}C (%)	
		C-1	C-5
Maize root (first 2-3 mm. section)	2	29.2	70.8
Maize root (first 1 cm. section)	2	31.6	68.4
Maize root (third 1 cm. section)	3	31.5	68.5
Maize coleoptile	4	32.7	67.3
Carrot-root slices	6	33.5	66.5
Beet-root slices	6	31.0	69.0
Wheat leaf	4	29.1	70.9

but approximately twice as much ^{14}C was found in C-5 as in C-1. This is the distribution expected if glutamate were derived from an asymmetrical citrate molecule in which, at equilibrium, C-5 (derived directly from C-1 of acetate) were labelled twice as heavily as C-1 or C-6 (derived directly from oxaloacetate). It is emphasized that the results in Table 3 are average values and the relative ^{14}C contents of C-1 and C-5 of glutamate varied significantly with time from 1:2, and in a way predictable from the operation of the Krebs cycle. Thus at 1.5 hr. in carrots, i.e. before the specific activity of the malate precursor had reached its peak, 72% of the ^{14}C in glutamate was in C-5, but at 6 hr. this value had fallen to 62%. Corresponding values for maize-root tips were 77% in C-1 at 30 min. and 66% at 2 hr.

It is assumed that the asymmetry observed in the glutamate labelling reflects that in the tri-carboxylic acids. Since only C-1 and C-6 of these acids (and C-1 of glutamate) are the direct precursors of respiratory carbon dioxide, the observed specific activities of these acids (an average value determined by dividing total counts/min. by total $\mu\text{equivalents}$) were corrected in order to make direct comparison with the specific activity of the respired carbon dioxide. Thus the specific activities

of citrate, aconitate and isocitrate were corrected to 75% of the observed values, and those of glutamate to 66% of the observed values.

Acid content of the various tissues

Table 4 shows the amounts of the Krebs-cycle acids found in each tissue. There was a wide range in total acid contents, and distinctive differences between the examples chosen. The succinate concentrations were relatively low in all of the tissues and not measurable in carrot, beet or *Bryophyllum*. Isocitrate was measurable only in *Bryophyllum* where it accounted for 70% of the titratable acid. Aconitate was measurable only in the maize and wheat tissues, and in the older maize-root tissues it was the major acid. Malate, citrate and glutamate concentrations were relatively high in all tissues. The amounts of each acid were not accurately determined for every tissue at each of the intervals shown in Tables 6-9, since only the major part of each peak was taken for the determination of specific activity. However, it was established that no important changes in concentration occurred. Measurements on maize-root material showed that the total acid content, including glutamic acid and aspartic acid, varied by less than $\pm 8\%$ during the experimental period, and the fluctuations in the amounts of individual acids did not exceed $\pm 11\%$.

Table 5 shows the respiration rate of each tissue in terms of carbon dioxide output/g. of tissue/hr. In the same Table, the total acid content of each tissue is shown. There is no clear correlation between acid content and respiration rate.

Specific activities of carbon dioxide and acids in experiments with $[1-^{14}\text{C}]\text{acetate}$

First 2-3 mm. of maize-root tips. The specific activity of the respired carbon dioxide reached a constant level at 1 hr. (Table 6). Succinate had the highest specific activity at 0.5 hr. and this was maintained at a higher level than that of the carbon dioxide throughout the experiment, although it declined after 1.5 hr. The specific activity

Table 4. *Acid contents of various plant tissues*

Experimental details are given in the text. N.D., Not determined; —, not measurable.

Acid	Acid content (μ moles/g. fresh wt.)							
	Maize root (first 2–3 mm. section)	Maize root (first 1 cm. section)	Maize root (third 1 cm. section)	Maize coleoptile	Carrot root	Beet root	Wheat leaf	<i>Bryophyllum</i> leaf
Citric acid	3.9	1.5	1.2	0.8	1.2	1.8	0.6	8.0
Aconitic acid	1.7	3.4	4.6	4.2	—	—	1.0	—
Isocitric acid	—	—	—	—	—	—	—	60.0
Glutamic acid	9.8	4.5	2.4	1.9	2.3	1.5	2.6	N.D.
Succinic acid	0.6	0.2	0.2	0.2	—	—	0.2	—
Malic acid	9.9	7.5	3.7	2.7	15.9	1.1	1.7	19.0
Aspartic acid	1.0	2.0	1.5	2.3	4.1	0.5	1.5	N.D.

Table 5. *Respiration rates and total acid contents of various plant tissues*

Experimental details are given in the text. Values for acid content are given: (a) for organic acids including glutamic acid and aspartic acid, and (b) for organic acids excluding glutamic acid and aspartic acid. N.D., Not determined.

Tissue	Respiration rate (μ moles of CO_2 /g. fresh wt.)	Acid content (μ moles/g. fresh wt.)	
		(a)	(b)
Maize root (first 2–3 mm. section)	57.20	26.9	16.1
Maize root (first 1 cm. section)	25.70	19.1	12.6
Maize root (third 1 cm. section)	13.57	14.6	10.7
Maize coleoptile	11.90	12.1	7.9
Carrot-root slices	4.10	23.5	17.1
Beet-root slices	5.72	4.9	2.9
Wheat leaf	8.96	7.6	3.5
<i>Bryophyllum</i> leaf	5.06	N.D.	87.0

of glutamate reached a similarly high value at 1 hr. and it too declined later. By contrast, the specific activities of malate and aconitate were relatively low at the earliest period of measurement and continued to increase for 1.5 and 2 hr. respectively.

From the carbon dioxide results we deduced that the carboxyl groups of the turnover pools of the acids of the Krebs cycle must have reached equal and maximum specific activity at 1 hr. The fact that the observed specific activities of the acids were far from equal at this time constitutes evidence for the existence of accessory pools of individual acids not in ready equilibrium with the turnover pools. If the maximum specific activities of each of the acids are compared with those of glutamate and succinate, which had high specific activities of about 11 000 disintegrations/min./ μ -equiv., the following fractional activities are obtained: citrate, 0.47; aconitate, 0.31; malate,

0.65; aspartate, 0.57. Even if all of the succinate and glutamate pools were involved in respiratory metabolism, then, at most, only these fractions of the total amounts of these acids could have been in immediate equilibrium with the turnover pools in the Krebs cycle. The progressive increase in the specific activities of individual acids indicates that there is traffic between non-turnover pools of these acids and those actively involved in the cycle, but the fact that none of the other acids reaches the same high specific activity as glutamate or succinate shows that a fraction of each of the other acids is not readily accessible to the turnover pools at the respiratory centres.

Evidence bearing on the question of what fraction of the respired carbon dioxide arises from reactions of the Krebs cycle is also provided by these results. If we assume that all of the succinate present was undergoing metabolic turnover, we arrive at a value of some 11 000 disintegrations/min./carboxyl group as the minimum specific activity of the turnover pools of the Krebs-cycle acids. Since the specific activity of the carbon dioxide never exceeded 6000 disintegrations/min. it is clear that at least 45 % of the carbon dioxide was being released in reactions outside the Krebs cycle.

First 1 cm. of maize-root tips. These segments included both meristematic and vacuolated zones. The acids in this tissue showed similar changes with time in relation to the carbon dioxide as in the younger tissues (Table 6). Citrate was the first acid to reach its highest specific activity. The specific activities of malate and aspartate showed increases to 1.5 hr. and that of aconitate was still rising at the end of the experiment. Again succinate had the highest specific activity of the acids and at 1 hr. it was almost twice that of the carbon dioxide which had reached a constant level at that time. If all of the succinate were in a turnover pool then the carbon dioxide from the Krebs cycle again must

have been diluted by an amount of unlabelled carbon dioxide equal to about 45% of the total carbon dioxide evolved.

Third 1 cm. of maize-roots. In these segments vacuolation was more complete. The specific activities of all the acids continued to rise throughout the experiment, although citrate, glutamate and succinate were nearly maximal at 1.5 hr. (Table 7). The specific activity of the carbon dioxide exceeded that of every acid at each period of measurement, but again succinate and glutamate had the highest specific activities. It is clear that the total quantity of none of the acids was involved in the turnover pools of the Krebs cycle. The generally lower specific activity of the acids in relation to that of the carbon dioxide as compared with the experiments with younger tissue indicates that large fractions of the total quantities of the acids were physically remote from the respiratory centres in the more highly vacuolated cells.

Maize coleoptile. The coleoptile tissue absorbed and metabolized acetate almost as rapidly as did the root tissues (Table 7). At 1 hr. the carbon dioxide had reached maximal specific activity and this specific activity was maintained for 2 hr. At 1 hr. the labelling in all acids except aconitate was

maximal. Aconitate began to lose activity after 3 hr. but, as in all maize tissues, the incorporation of ^{14}C into aconitate did not follow that incorporated into citrate. Glutamate was the only acid to reach the same specific activity as the carbon dioxide: the specific activities of all other acids were less by at least 25%.

Carrot. The specific activity of the carbon dioxide reached a maximal and constant level by 1.5 hr. and remained constant up to at least 3 hr. (Table 8).

Although the specific activities of citrate and glutamate approached that of the carbon dioxide, the difference between the specific activity of the carbon dioxide and that of malate was very large. At 3 hr. the carbon dioxide had a specific activity 10 times that of malate, and hence at least 90% of the malate was not in equilibrium with the respired carbon dioxide.

Beet. Beet, a storage tissue, was expected to have similar characteristics to carrot, and Table 8 shows that the specific activities of citrate and glutamate were equal to that of the carbon dioxide, as they were in carrot. In contrast with the carrot, beet tissue had relatively low concentrations of malate and its specific activity was almost 40% higher than the specific activity of the carbon dioxide. It

Table 6. *Specific activities of acids and carbon dioxide from maize-root tips supplied with $[1-^{14}\text{C}]$ acetate*

Experimental details are given in the text.

		Specific radioactivity (disintegrations/min./ $\mu\text{equiv.}$)							
		First 2-3 mm. of root tips				First 1 cm. of root tips			
Time (hr.)	...	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
Acid									
Citric acid		4446	5123	4465	4683	5033	4903	4751	4414
Aconitic acid		2381	3428	3775	4233	3131	4301	4940	6143
Glutamic acid		8413	11085	10048	7238	10778	11786	9550	6876
Succinic acid		10732	10320	10363	7664	15182	19889	14399	10872
Malic acid		2744	5544	7263	7020	2790	4678	5620	5428
Aspartic acid		2666	6295	5673	7475	4167	5319	6297	6025
Carbon dioxide		2639	5800	5900	6000	6000	10800	10800	9500

Table 7. *Specific activities of acids and carbon dioxide from maize roots and coleoptiles supplied with $[1-^{14}\text{C}]$ acetate*

Experimental details are given in the text.

		Specific activity (disintegrations/min./ $\mu\text{equiv.}$)						
		Roots (third 1 cm. section)			Coleoptiles			
Time (hr.)	...	0.75	1.5	2.25	1	2	3	4
Acid								
Citric acid		1500	2544	2957	9608	7170	4926	3640
Aconitic acid		580	1224	2041	2798	5051	6780	5374
Glutamic acid		7308	9814	11292	16582	10626	6942	5596
Succinic acid		6644	9241	10602	10140	10855	6825	5829
Malic acid		2559	4696	6887	10213	12336	9291	8784
Aspartic acid		1344	2835	3060	7388	7000	4929	4407
Carbon dioxide		7500	12000	12000	16000	16000	11000	5000

Table 8. *Specific activities of acids and carbon dioxide from carrot-root and beet-root slices provided with [1-¹⁴C]acetate*

Experimental details are given in the text. N.D., Not determined.

		Specific activity (disintegrations/min./ μ equiv.)							
		Carrot-root slices				Beet-root slices			
Time (hr.)	...	1.5	3.0	4.5	6.0	1.5	3.0	4.5	6.0
Acid									
Citric acid		74348	101348	105098	84734	1430	7427	12053	12633
Glutamic acid		74725	71127	35269	20844	7560	11388	10379	6955
Malic acid		3706	9749	15012	17730	6696	14968	17248	15177
Aspartic acid		32405	38442	17924	10101	1467	5580	3060	N.D.
Carbon dioxide		115000	105000	65000	20000	8000	12500	12500	12500

Table 9. *Specific activities of acids and carbon dioxide from wheat and Bryophyllum leaves provided with [1-¹⁴C]acetate*

Experimental details are given in the text. The values shown for the specific activity of citric acid in wheat are lower than the true values, since an additional acid was present in the citrate peak. N.D., Not determined; —, not measurable.

		Specific radioactivity (disintegrations/min./ μ equiv.)							
		Wheat leaf				Bryophyllum leaf			
Time (hr.)	...	1	2	3	4	2	4	6	
Acid									
Citric acid		1497	3530	4236	5720	629	968	1252	
Isocitric acid		—	—	—	—	43	125	173	
Aconitic acid		200	392	607	707	—	—	—	
Glutamic acid		4770	7130	12898	18037	N.D.	N.D.	N.D.	
Succinic acid		7649	13422	17086	29032	—	—	—	
Malic acid		1415	3698	6273	8956	1637	2957	3130	
Aspartic acid		3130	6247	7798	10075	N.D.	N.D.	N.D.	
Carbon dioxide		3600	8900	14100	19400	5400	5400	5400	

is evident, then, that a large part of the acid content of beet is in equilibrium with the respiratory pools.

Wheat leaves. The specific activity of the carbon dioxide failed to reach a constant level during the period of the experiment, and the specific activities of the individual acids also increased (Table 9). Succinate, the acid present in smallest amount, had the highest specific activity and, as in the younger maize-root tissues, this was considerably greater than that of the carbon dioxide. The specific activity of aconitate was always considerably lower than that of the other acids and rose continuously during the experiment.

Bryophyllum leaves. The distinctive feature of this tissue was the high concentration of isocitrate. Although the carbon dioxide rapidly reached a high and constant specific activity, indicating that the carboxyl groups of the turnover pools had reached constant specific activities, the measured specific activities of the acids were much lower than this value, indicating partial equilibration. Attention is drawn to the extremely low specific activity of isocitrate (Table 9).

Correction of specific activity of respired carbon dioxide

From the above results it is evident that the specific activities of the carboxyl groups of the individual extracted acids were usually much smaller than that of the respired carbon dioxide. It is emphasized that the specific activity of the respired carbon dioxide was not the specific activity of the carbon dioxide from the Krebs cycle. No allowance was made for the fact that only a portion of the carbon dioxide collected had arisen from reactions subsequent to acetyl formation. Since the acetate added was not a large amount and hexoses were still the major respiratory substrate, one-third of the carbon dioxide (released in the conversion of pyruvate into acetyl) probably arose from reactions before the Krebs cycle, and this value would be increased if hexose were diverted through the pentose phosphate pathway. Moreover, irreversible drainage of carbon from acetate to lipid and of Krebs-cycle intermediates to amino acids (other than glutamate and aspartate) and protein would still further decrease the fraction of

the respired carbon dioxide provided by reactions subsequent to acetyl formation.

Although it is not possible to determine precisely what correction factor should be applied to the specific activity of the carbon dioxide from each tissue in order to make a meaningful comparison with the acids, some information on this point is shown in Table 10 where the contributions of the different carbon atoms of supplied glucose to the respired carbon dioxide are shown. These results show that the contributions of C-3 and C-4 to the carbon dioxide are in fact higher than the total of one-third expected if complete conversion of glucose into carbon dioxide had occurred. More than one-half of the respired carbon dioxide was produced from these carbon atoms in each tissue, except wheat leaf and maize coleoptile where the values were 44%. The selective utilization of C-1, C-2, C-5 and C-6 (which give rise directly to acetate) in the reactions mentioned above and in the production of acids other than those of the Krebs cycle (Harley & Beevers, 1963) would bring about this result. When the disproportionate contri-

bution of C-1 of glucose to carbon dioxide in carrot, wheat, beet and *Bryophyllum* is also considered it seems that not more than one-half of the carbon dioxide actually observed was being produced in reactions of the Krebs cycle. The results for maize-root-tip tissues (Table 6), from which it was calculated that at least 45% of the carbon dioxide came from reactions outside the cycle, also lend support to this estimation. Thus the values for specific activity of the carbon dioxide may be conservatively corrected by a factor of 2.

This has been done in Table 11 where the maximum specific activities of the individual acids in each tissue were divided by the corrected specific activity of the carbon dioxide. These values are estimates of the maximum fraction of the total amount of individual acids which readily equilibrate with the turnover pools in the Krebs cycle.

From the values presented in Tables 4 and 11 we estimated the absolute amount of individual acids which equilibrate with the turnover pools in the Krebs cycle. These calculations are presented in Table 12.

Table 10. *Percentage contribution of different carbon atoms of glucose to respiratory carbon dioxide*

Replicate samples (2 g.) of tissue were provided with 8 μ moles of [1- 14 C]-, [2- 14 C]-, [3,4- 14 C₂]- or [6- 14 C]-glucose in 10 ml. of 0.025M-KH₂PO₄. Experimental details are given in the text. The measurements of the evolution of 14 CO₂ were converted into μ moles of CO₂ and the percentage contribution of each carbon of glucose was calculated from these values. C-3 and C-4 were assumed to contribute equally and the contribution of C-5 was taken to be equal to that of C-2.

Tissue	Distribution of 14 C (%)					
	C-1	C-2	C-3	C-4	C-5	C-6
Maize root (first 2-3 mm. section)	11	10	27	27	10	15
Maize root (first 1 cm. section)	11	8	32	32	8	10
Maize root (third 1 cm. section)	16	8	25	25	8	18
Maize coleoptile	14	14	22	22	14	14
Carrot-root slices	22	10	26	26	10	6
Beet-root slices	22	9	23	23	9	14
Wheat leaf	18	12	22	22	12	14
<i>Bryophyllum</i> leaf	14	11	27	27	11	10

Table 11. *Percentage of acid contents estimated to be in turnover pools*

The values were determined by comparing the specific activities of the carboxyl groups of the acids with the specific activity of CO₂ respired from the Krebs cycle, by assuming that 50% of total CO₂ arises from the Krebs cycle. N.D., Not determined; —, not measurable.

Acid	Maize root (first 2-3 mm. section)	Maize root (first 1 cm. section)	Maize root (third 1 cm. section)	Maize coleoptile	Carrot-root slices	Beet-root slices	Wheat leaf	<i>Bryophyllum</i> leaf
Citric acid	44	23	13	30	50	48	15	12
Aconitic acid	32	23	9	16	—	—	2	—
Isocitric acid	—	—	—	—	—	—	—	2
Glutamic acid	96	55	47	52	34	46	47	N.D.
Succinic acid	89	92	44	34	—	—	75	—
Malic acid	62	26	36	39	5	69	23	29
Aspartic acid	55	29	13	23	18	23	26	N.D.

Table 12. *Amounts of individual acids estimated to be in turnover pools*

The results were calculated from the values given in Tables 4 and 11. N.D., Not determined; —, not measurable.

Acid	Acid in turnover pools (μ moles/g. fresh wt.)							
	Maize root (first 2-3 mm. section)	Maize root (first 1 cm. section)	Maize root (third 1 cm. section)	Maize cole-optile	Carrot-root slices	Beet-root slices	Wheat leaf	<i>Bryophyllum</i> leaf
Citric acid	1.72	0.35	0.16	0.24	0.60	0.86	0.09	0.96
Aconitic acid	0.54	0.78	0.41	0.67	—	—	0.02	—
Isocitric acid	—	—	—	—	—	—	—	1.20
Glutamic acid	9.41	2.48	1.13	0.99	0.78	0.69	1.22	N.D.
Succinic acid	0.54	0.18	0.09	0.07	—	—	0.15	—
Malic acid	6.10	1.95	1.33	1.05	0.80	0.76	0.39	5.50
Aspartic acid	0.55	0.58	0.20	0.53	0.74	0.12	0.39	N.D.

DISCUSSION

There is now no doubt that the major pathway of acetate dissimilation in higher-plant cells is the Krebs cycle. To the previously available evidence is now added the results obtained from degradations of acids from each of the tissues. These findings agree with the predicted asymmetry of the C_3 unit and the symmetry of the C_4 units in the cycle. If acetate were condensed to form citrate and if each molecule, as it formed, were completely oxidized, all of the ^{14}C from each entering $[1-^{14}C]$ acetate unit would be eliminated after two turns of the cycle. Under these conditions it would be expected that the ^{14}C content of the organic acids would be restricted to small pools of transient intermediates, the cumulative $^{14}CO_2$ emission would greatly exceed the cumulative ^{14}C in the organic acids, and the specific activity of the carbon dioxide would become constant shortly after acetate oxidation began. Figs. 1 and 2 show that these expectations were not realized.

The organic acids were heavily labelled at all times. The cumulative label in the $^{14}CO_2$ did not exceed that in the organic acids until late in the experiments (after $[1-^{14}C]$ acetate was depleted) and never by more than twofold. In most tissues the ^{14}C contents of the organic acids were very large before linear $^{14}CO_2$ emission (indicative of a constant specific activity) occurred. Moreover, the curve of $^{14}CO_2$ emission declined in many tissues while the ^{14}C contents of the organic acids were still high. These findings show that turnover of the organic acids was not restricted to catalytic amounts at the respiratory centres; apparently mixing with acids elsewhere in the tissues was taking place. However, the results are clearly not those expected if the total quantity of each acid was present at the site of the reactions of the Krebs cycle or in ready equilibrium with the acids at those sites. This qualitative conclusion may be drawn from the

large and sustained differences in specific activity of the acids when $[1-^{14}C]$ acetate was being metabolized. These differences, more than tenfold in the acids of carrot and *Bryophyllum*, take on added significance when compared with the specific activity of the respired carbon dioxide when this had reached its maximum level (Tables 6-9).

Even before the specific activity of the carbon dioxide was corrected to take account of the non-labelled carbon dioxide not arising from acetate utilization, it was clear that this was greatly in excess of the specific activity of some of the acids that were its precursors. The fact that in some of the maize tissues the specific activities of succinate and glutamate actually exceeded those of the carbon dioxide (before correction) shows that a greater proportion, and perhaps all of these particular acids, rapidly attained equilibrium with the succinate and glutamate undergoing reactions at the site of Krebs cycle.

The relative specific activities of the succinate and carbon dioxide from maize-root tips provide one basis for a minimum estimate of the fraction of the respiratory carbon dioxide arising from the Krebs cycle, and findings on the contribution of the different carbon atoms of glucose to the carbon dioxide for all tissues lead to a similar conclusion, that the reactions of the Krebs cycle produce not more than 50 % of the respired carbon dioxide. By applying this correction to the observed specific activity of the carbon dioxide it was possible to make quantitative estimates of the amounts of individual acids which came into equilibrium with the turnover pools (Table 11).

From this Table it is clear that the youngest maize-root tissue, which contains largely meristematic cells, has the greatest fraction of its total acid engaged in turnover. In progressively older regions of the same organ (with correspondingly greater degrees of vacuolation) larger amounts of the acids are out of equilibrium. In all tissues in

which it was present, aconitic acid appeared to be largely out of contact with the turnover pools. In *Bryophyllum*, which has a particularly high acid content, not more than 30% of any of the acids, and less than 2% of the isocitrate, was in equilibrium with the carbon dioxide. Of the two storage tissues, carrot is distinguished by its relatively high malate content, and the great bulk of this does not equilibrate with that in the Krebs cycle. It thus appears that, in particular tissues, individual acids are separated from the turnover pools to very different degrees. A noteworthy feature is that, in all tissues, a sizeable fraction of the glutamate, even when it is present in relatively large amounts, rapidly reaches the same specific activity as the carbon dioxide.

The values in Table 11 should be considered in relation to the total amount of each acid present (Table 4). This allows a calculation of the amounts of each acid which reaches isotopic equilibrium with themselves and the carbon dioxide (Table 12). In some tissues, such as carrot and beet, these amounts are about equal, but in most these turnover pools vary greatly in size.

No positive information is available from these plant materials on the possible intracellular location of the various functional pools. However, the magnitude of some of the turnover pools argues against their being confined to the mitochondria. The relatively slow approach to maximum labelling shown by some of the acids (Tables 6–9) would result if the pools actually at the respiratory centres were equal and small, and the remainder of the turnover pools remote but in ready equilibrium with these. Specific transfer of individual acids to the vacuole seems to offer the most logical explanation of the existence of extensive non-turnover pools, though further degrees of cytoplasmic 'compartmentation' are not to be ruled out.

SUMMARY

1. The amounts of various Krebs-cycle acids and of glutamate and aspartate present in maize-root tissue of three different ages, maize coleoptiles, beet- and carrot-root tissue, and *Bryophyllum* and wheat leaves were determined.

2. Features of [^{14}C]acetate utilization by these tissues were examined.

3. ^{14}C from [^{14}C]acetate was found equally and only in the carboxyl carbons of malate, it was confined to the carboxyl groups of citrate, and distributed in a 1:2 ratio between C-1 and C-5 of glutamate. These results confirm that acetate was being utilized by the Krebs cycle.

4. The progress of incorporation of ^{14}C from [^{14}C]acetate into acids, amino acids and carbon dioxide with time was determined, and changes in

the specific activities of these components were measured.

5. When the specific activity of the carbon dioxide reached a constant value, indicating that equilibrium had been reached in the turnover pools of the acids of the Krebs cycle, there were great differences in the observed specific activities of the acids.

6. These differences are ascribed to the existence of pools of acids and amino acids not in ready equilibrium with the turnover pools, but which mixed with them during extraction.

7. By correcting the specific activity of the carbon dioxide to allow for the production of carbon dioxide in reactions other than those of the Krebs cycle, and comparing this value with the specific activities of the carboxyl groups of the acids, the fraction of each acid which was in equilibrium with the turnover pools was estimated.

8. The results show that large amounts of individual acids are physically remote from the respiratory centres, and that the amounts of acids in turnover pools in the various tissues are different.

9. The evidence from the maize-root tissues of different ages shows that, as vacuolation occurs, the relative amount of acids not in turnover pools (i.e. out of equilibrium with the carbon dioxide) increases markedly.

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Depolymerization of Ribonucleic Acid by Enzymes in Basic Proteins from Liver Nuclei and Ribosomes

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Recent studies on the proteins associated with nucleic acids have revealed the composite nature of histones and the basic proteins of ribosomes. Nuclear histones from a variety of tissues have been separated into three main fractions by chromatography on resins (Crampton, Moore & Stein, 1955) and on carboxymethylcellulose (Davison, 1957; Phillips & Johns, 1959; Davis & Busch, 1959; Johns, Phillips, Simson & Butler, 1960). Multiple bands can be produced from these histones and from histone fractions when they are subjected to starch-gel electrophoresis (Neelin & Connell, 1959; Neelin & Neelin, 1960; Setterfield, Neelin, Neelin & Bayley, 1960; Johns & Butler, 1962). The amino acid composition of ribosomal proteins from bacterial, plant and animal cells is reported to be similar in certain respects to the composition of histone protein (Ts'O, Bonner & Dintzis, 1958; Crampton & Petermann, 1959; Butler, Cohn & Simson, 1960).

Pirie (1950) reported the presence of latent ribonucleases in ribonucleoprotein of tobacco leaf, and recent work has suggested that this enzyme is an integral part of the ribonucleoprotein particles of animal, plant and bacteria cells (Elson, 1958; Tashiro, 1958; Ts'O, Bonner & Vinograd, 1958; Siekewitz & Palade, 1958; Dickman & Trupin, 1958; Roth, 1960; Spahr & Hollingworth, 1961; Zillig, Schachtschabel, McCorquodale, Krone & Horne, 1960). The latent nucleases of rat-liver ribosomes have been extracted by dilute acids (Tashiro, 1958; Roth, 1960), and it is possible that they are components of those ribosomal proteins that are similar in chemical composition to histone proteins (Butler *et al.* 1960). The possibility that latent nucleases act as gene regulators in metazoan cells has been proposed by Leslie (1961).

Basic proteins of guinea-pig-liver nuclei and ribosomes have been isolated and examined for RNA-depolymerase activity. Depolymerase activity has been found and the conditions for optimum activity of the preparations are described. The distribution of depolymerases within the complex of basic proteins has been investigated by electrophoresis on starch gel.

METHODS

Isolation of nuclei. Fresh guinea-pig livers (60 g.) were perfused in 0.9% NaCl and the cells broken in a solution containing sucrose (0.25M), CaCl₂ (3 mM) and tris (0.035M), at pH 7.1, by homogenization for 20 sec. in a M.S.E. blade homogenizer, at a 1:1 (w/v) concentration. All operations were carried out at 0–4°. The nuclei were collected by centrifuging at 1000 rev./min. for 5 min. after filtering through nylon gauze and diluting the homogenate to a 1:10 (w/v) concentration. The nuclear pellets were combined and washed by resuspension in 100 ml. of a solution of sucrose (0.25M), citric acid (0.01M) in a Potter-Elvehjem-type homogenizer and were then centrifuged. The total time of the washing procedure was kept to a minimum. Nuclei were stored at –20°.

Nuclei were isolated in the following manner from two strains of human cells. HEP 1 cells were derived from an epitheloid cervical carcinoma (Fjelde, 1955), and HLM cells from the liver of a 20-week human foetus (Leslie, Fulton & Sinclair, 1956). Approx. 5 g. of cells was suspended in 20 ml. of sucrose–CaCl₂–tris solution and 5 ml. volumes were treated initially with a M.S.E. Mullard ultrasonic oscillator (output 60 w, and tuned to 20 kcyc./sec.) by using a stainless-steel probe of 1.9 cm. diam. and glass tubes of 2.3 cm. internal diam., immersed in ice-cold water. This treatment was limited to periods of 15 sec. to reduce to the minimum the destruction of liberated nuclei, and the extent of cell disruption was checked by examination with an interference microscope. When necessary, a second 15 sec. treatment was applied with the oscillator. The