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Techniques in Tissue Metabolism

4. APPARATUS FOR MAINTAINING AND RAPIDLY TRANSFERRING TISSUE SECTIONS*

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Enzymes of a given tissue can often cause the labile metabolites in that tissue to react completely in a minute or so. This speed of reaction is especially notable in excitable tissues, and in those of the central nervous system metabolites including lactic acid, phosphocreatine or acetylcholine can be doubled or halved in quantity in 2–10 sec.

Consequently, the study of such substances with preparations of isolated tissues has commonly been carried out under relatively artificial conditions in which the tissue is ground and diluted up to a 1000-fold, and quantities of substrate are added which are very great in comparison with those normally associated with a given quantity of tissue. While these conditions are often excellent for studying individual enzyme reactions, much additional information is usually required before such findings can be applied to the behaviour of the same substances in cell-containing tissues.

An alternative or complementary procedure, with which the present paper is concerned, is to examine events in cell-containing tissues over periods within the few seconds or minutes during which their native metabolites undergo reaction. The general procedure has been to establish a steady state in the tissue, to apply a given agent, and to fix the tissue for analysis at brief intervals afterwards. The agents have been added chemicals or electrical pulses. Such arrangements have in the past been employed with intact organs as muscle or nerve, but rarely with tissue slices.

* Part 3: Rodnight & McIlwain (1954).

EXPERIMENTAL

Tissues and salines

From the guinea pig or larger animals large slices, 0·35 mm. thick, can readily be obtained from the liver, kidney cortex and brain (McIlwain, 1951). Such slices, up to 2 cm. × 1·5 cm. (100 mg.), were strong enough to be picked up on wires and drained of fluid for weighing and transferring. Slices rather larger than the size mentioned were floated from the blade or cutting plate into a shallow dish of the appropriate saline (Rodnight & McIlwain, 1954; Heald, 1954), and trimmed to size with a scalpel, picked out with the rider of a torsion balance and drained on a glass surface, weighed, and returned to the saline. A transfer-holder was opened, its lower jaw put in the saline, the slice was manoeuvred over it with a spatula and lifted from the saline with the holder. The jaw of the holder was then closed on the slice, and the holder and slice were placed into the beaker (Fig. 1) containing about 5 ml. of appropriate saline.

Quick-transfer holders

For rapid transference the tissue was held, before and during transfer, in a holder (Fig. 1) from which it could be easily released. A slice must be kept extended for adequate exchange with its environment, and this was done by fixing it at nine places near each of its long edges. These places were the points of contact between the jaws of the holder (*c* or *d*, Fig. 1) and a nylon thread or metal wire wound round them. The opposing faces of the jaws were double-bevelled so as to form a continuous knife edge *f*, *f'* around each jaw. A cross-section of part of the jaws is shown in Fig. 1*D*. The knife edges closed on the wire or thread wrapped round the opposing jaw. These wires, 4 upper and 5 lower (Fig. 1*C*), formed the nine places along the front and back edges at which the tissue was gripped. The thread or wire also supported the slice, but apart from the points of contact it did so only loosely so that during oxygenation of the medium the slice could be seen to wave gently between the wires. At each point of contact the tissue was distorted as indicated in Fig. 1*D*, but much of this tissue would appear to have been displaced rather than crushed. Provision of several such points minimized tendency to tear at any one of them. In most experiments the holder with its slice fitted into a 30 ml. Pyrex beaker (Fig. 1*B*). Pieces of sponge rubber were cemented permanently to the main strips of the holder so that they formed a seal at the top of the beaker. Through this projected the top of the holder and also a fine plastic tube admitting a gas, usually O_2 or O_2+CO_2 (95:5, v/v), through the fluid in the beaker. The tube was cut at its lower end at an angle to minimize blocking and to direct the stream of bubbles (Fig. 1*A*). When these were provided in an almost continuous stream (50–250 ml. of gas/min.) from the tube in the position shown beside the holder, fluid circulated adequately under and over the slice; the gas escaped round the sponge rubber.

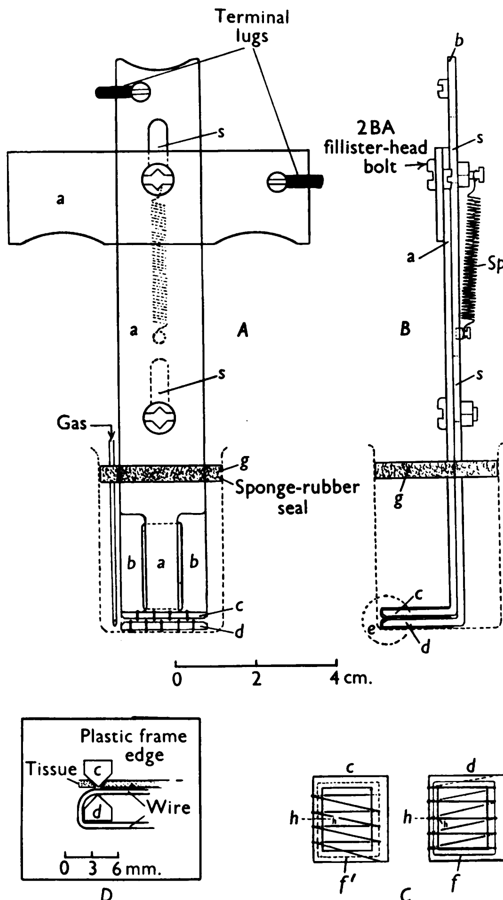

Fig. 1. For legend see bottom of next column.

Electrical characteristics. Much use has been made of a type of the holder wound with silver wire, so that electrical potential gradients can be established in tissue held by the apparatus. The type and arrangement of wire followed earlier practice with other electrode types (especially type *H* of Ayres & McIlwain, 1953). Silver wire, 0·315 mm. in diam., was insulated for us by The Temple Electric Co., Watford, Herts, with a plastic enamel. It was threaded through holes and grooves in the holder, as shown in Fig. 1, so that the centres of the wires crossing the jaws were 4 mm. apart. The upper surface of the lower jaw of the holder was crossed by five such wires, and the under surface of the upper jaw by four wires parallel to the others and spaced evenly between them. The opposing surfaces of these parallel sections of the wire were scraped clean of enamel. They thus formed a grid of electrodes, 2 mm. apart, of which alternate members were connected by the remainder of the insulated wire. An end of each coil of the wire was brought to a terminal lug at the top of the holder.

Fig. 1. Quick-release holder. (*A*) Arrangement of holder and of oxygen tube in beaker (30 ml.). (*B*) End elevation of *A* as viewed from the right-hand side. Perspex strips *a* and *b* (0·0625 in. thick) are held together with two bolts fixed to strip *a*; slots *s* in strip *b* permit the jaws *c*, *d*, to move up to 1·5 cm. Normally the jaws were kept together by the spring *Sp*, and a force of about 250–300 g. was needed to open them. The tops of the plastic strips were shaped as shown (*A*) to take the fingers and thumb of one hand. Just above the jaws, strips *a* and *b* are cut as shown in *A* so as to prevent fluid creeping up between the strips by capillary action. The nuts, bolts, terminals and spring were of 18/8 F. M. B. Staybrite Steel (Firth Vickers). The gas seal *g* was of Rubazote closed-cell sponge rubber (W. Mannering, Bermondsey) cemented to the strips with Araldite D, cold-setting (Aero Research, Duxford). (*C*) Plan of electrode jaws showing arrangement of electrode wires; these were of enamelled-silver wire which was led up the side of the strips *a*, *b*, in milled slots and soldered to the terminal lugs of tinned metal at the top of the holder. Opposing faces of the jaws are double-bevelled to form the knife edges indicated by the lines *f*, *f'*. (*D*) Enlarged view of a section of jaws showing how a slice is held between them. The portion illustrated is that of *e* of *B* at the cross-section labelled *hh* in *C*, after assembling the holder and introducing a slice. The compression of the slice occurs only at the places where the wires meet the knife edge of the opposing jaw and involves about 1 sq.mm. of the tissue; the remainder is freely bathed with saline.

Table 1. *Uniformity of metabolic characteristics of tissues in quick-transfer electrodes*

Slices of guinea-pig cerebral cortex of about 120 mg. were in the electrodes in 5 ml. of oxygenated saline at 37·5°. Pulses were alternating (100/sec.), peak potential was 10 v, and time-constant 0·4 msec. Lactic acid was determined according to Barker & Summerson (1941) after preparing the tissue and solution as described by McIlwain & Tresize (1956). Creatine phosphate was determined according to Heald (1954). Values quoted are means ± S.E. with the number of determinations in parentheses. Determinations were made after 60–80 min. incubation; values for lactate of the tissue include that of adhering fluid.

Substance measured	Duration of pulses	Amount (µmoles/g.) No pulses	Amount (µmoles/g.) With pulses
Lactic acid of fluid	60 min.	17·1 ± 0·9 (8)	31·8 ± 1·3 (4)
Lactic acid of tissue	20 sec.	2·60 ± 0·07 (6)	4·65 ± 0·08 (6)
Phosphocreatine of tissue	10 min.	1·45 ± 0·12 (9)	0·75 ± 0·06 (3)

When the quick-transfer electrodes were immersed in saline solutions and potentials applied to them, the potential gradients established were measured as described by Ayres & McIlwain (1953). When condenser impulses or sine-wave alternating currents were applied, potential varied with distance between adjacent wires as recorded in Figs. 5 and 7 of Ayres & McIlwain (1953). In glucose-phosphate saline with sine-wave current at a virtual potential of 2·25 v (6·3 v peak to peak), measurement of the characteristics of eight intervals between adjacent wires gave (± S.D.): sum of abrupt changes between wire and solution, 1·25 ± 0·17 v; gradient over the greater part of the interval, 1·42 ± 0·23 v/mm. (extending for 1·49 ± 0·26 mm.); distance with gradient of 0–1·5 v, 0·3 mm.; highest gradient in the remaining 0·2–0·3 mm., 6·9 ± 1·2 v/mm. Similar uniformity was found between a group of six electrodes, when examined also with condenser pulses of peak potential 10 v, and time-constant 0·4 msec., applied at 100/sec. The electrical characteristics of the electrodes are thus similar to those of electrodes *H* (Ayres & McIlwain, 1953), in which similar wires are arranged at the same distance apart, though in electrodes *H* the wires are shorter and fewer than in the quick-transfer electrodes.

Procedure

Six or eight tissues with holders and beakers concerned in the same experiment were fitted into a frame held in a thermostatically controlled water bath. An experiment was commenced by preparing the saline and distributing it in the beakers, and preparing slices from the chosen organ and fitting them to their holders. Incubation for the desired preliminary period was then commenced and fixing agents were prepared. The appropriate brief treatment (e.g. exposure to pulses for a few seconds) was then applied to one tissue specimen, after which it was immediately fixed by lifting its holder from the beaker, draining it if necessary and then immersing the jaws with their tissue in the fixing agent. At the same time the jaws were opened and a scooping motion was given to holder and beaker of fixing agent so that the tissue floated out from the holder. Other tissue specimens were then treated and fixed.

Metabolic behaviour of tissues in the holder

The following experiments were done to determine (1) the uniformity of behaviour of groups of tissue specimens incubated under these conditions, and (2) whether tissues so handled were comparable in metabolic behaviour to tissues under more conventional conditions. Preliminary experiments (Heald, McIlwain & Nelligan, 1954) showed especially the necessity for vigorous oxygenation and the provision of the sponge-rubber seal to maintain an atmosphere of oxygen above the tissue. Table 1 lists determinations of lactic acid in the tissues and in the medium, and of creatine phosphate in the tissue both in the presence and

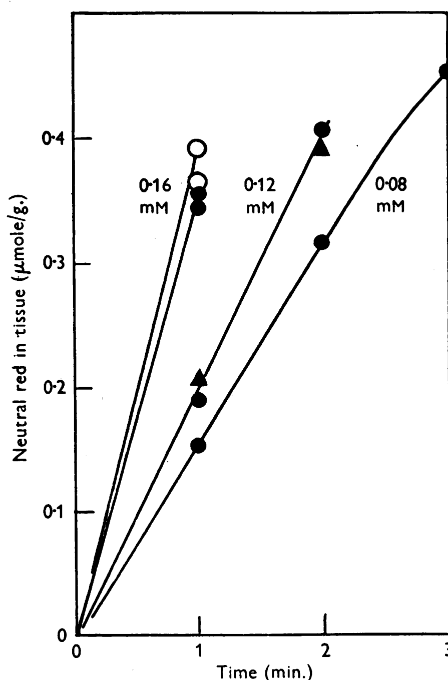

Fig. 2. Uptake of neutral red by guinea-pig cerebral cortex. Slices of cortex of about 120 mg. were held in quick-transfer electrodes in 5 ml. of phosphate saline 10 mM in glucose, except when stated otherwise. After 20 min. at 37·5° (zero time), neutral red in the same saline 0·2 ml. was added to give the concentrations stated. Slices were removed at intervals, briefly rinsed, and the dye was extracted and determined according to McIlwain & Grinyer (1950). ●, Glucose and no pulses; ▲, glucose and pulses as in Table 1; ○, no glucose and no pulses.

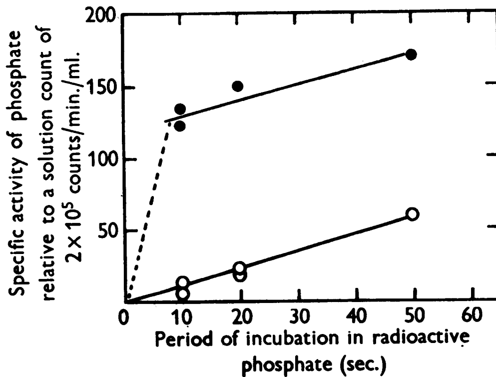

Fig. 3. The incoporation of radioactive phosphate into adenosine triphosphate (γ-P) (○) and tissue orthophosphate (●) of liver slices from fasted rats. Slices were incubated in a glucose-containing saline buffered with aminotrishydroxymethylmethane at 37·5° for 30 min. in O_2 before addition of radioactive phosphate. For procedure and analytical methods see Heald (1956*a*).

absence of electrical pulses. In the absence of pulses, creatine phosphate was maintained at levels found previously with more conventional manometric apparatus where oxygenation may be presumed to be completely adequate (McIlwain, Buchel & Cheshire, 1951; McIlwain & Gore, 1951). Levels of lactic acid in solution in absence of pulses were somwhat lower than those found previously in manometric apparatus (McIlwain, Anguiano & Cheshire, 1951). Passage of pulses through the slices doubled their lactic acid and halved their creatine phosphate content. These changes have been shown to occur in cerebral tissues in manometric apparatus (McIlwain, Anguiano & Cheshire, 1951; Heald, 1953; McIlwain & Gore, 1951). Variation between results in different holders is seen to be small. In addition, incorporation of radioative phosphate into liver slices and of neutral red into cerebral slices was studied, as shown in Figs. 2 and 3. These processes, initiated by additions to the saline surrounding the tissues, proved to proceed uniformly with time. Other investigations employing the holder have been reported by Heald (1954, 1956*b*); Greengard & McIlwain (1955); LeBaron (1955) and McIlwain & Tresize (1956).

DISCUSSION

In most biochemical work with sliced tissues, these have been floating freely in saline solutions, even when relatively brief exposure to added agents was desired. Mechanical restraint of tissues for special purposes has however been described: thus in applying electrical pulses (McIlwain, 1951) or working with non-aqueous fluids (Rodnight & McIlwain, 1954) tissues have been held mechanically without thereby interfering with their normal metabolic activities. The results of the present paper show that the mechanical arrangements and restraint for rapidly transferring the tissue are equally innocuous.

The special virtue of these arrangements lies in the facilitation of the study of changes occurring in periods one-hundredth or one-thousandth of those used in most metabolic investigations. Studies with such a time-scale, e.g. 1 sec. to 1 min., are of especial importance in excitable tissues, and are facilitated in such tissues by their being excitable: for this enables a change of metabolic state to be induced by a non-chemical agent, without problems of diffusion to the tissue, and in a time measured in msec. However, in both excitable and non-excitable tissues the present findings show that added substances do in fact reach the tissue cells from a surrounding fluid very quickly. Thus radioactive inorganic phosphate appeared in the adenosine triphosphate of liver slices 10 sec. after its addition to a fluid which bathed them.

In higher animals the body as a whole survives only for a minute or so without material exchange with its environment, and can respond to administered chemicals in a few seconds; most tissues respire at rates sufficient to exhaust their native oxygen in perhaps 10 sec. There is thus much scope for study of animal tissues within periods of this brevity.

SUMMARY

1. A tissue-holding apparatus is described by means of which thin slices of tissue can, after incubation in a suitable saline, be rapidly transferred to a fixing agent or to another saline.

2. While in the holder the tissue may be subjected to brief exposure, of from 1 sec. upwards, to other agents such as radioactive phosphate or electrical pulses, and can then be removed and fixed within 0·2–0·3 sec. of such exposure.

3. With cerebral tissues it has been shown that the normal levels of creatine phosphate and the process of glycolysis are not affected by the holder.

4. The use of the holder in following the accumulation of neutral red by cerebral slices, and in the incorporation of radioactive phosphate into liver adenosine triphosphate within 10 sec. is described.

We are greatly indebted to Mr D. Nelligan for his taking part in the construction and design of the quick-transfer holder.

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Analysis of Radioactive Phosphates in Extracts of Cerebral Tissues

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As a means of studying the metabolism of radioactive phosphates in extracts of cerebral tissue, a method of analysis was required applicable to the fractionation of small quantities (120–130 μg. of total P), and specific for adenosine triphosphate, inorganic orthophosphate and creatine phosphate; it was also desirable that it should be capable of easy extension to a survey of other compounds or groups of compounds.

The methods in general use for the analysis of phosphorylated intermediates, though of considerable value, suffer not only from the relatively large amounts of tissue necessary but also from the inherent defect of overlapping of fractions (Greenberg, 1952). This, though small, becomes important when studies of the distribution of radioactive phosphates is to be considered (Abood & Gerard, 1952; Ennor & Rosenberg, 1952; Lee & Eiler, 1953; Sacks, 1949). To overcome these difficulties it was decided to try preliminary fractionation of phosphates into groups by means of barium followed by paper chromatography.

The results of Turba & Enenkel (1951), Maurer & Schild (1951), and Schild & Bottenbruch (1953) suggested that ionophoresis on paper would achieve a rapid and complete separation of the phosphates, and studies were commenced along these lines. Recent work (Neil & Walker, 1954; Wade & Morgan, 1954, 1955), has shown the separation of phosphates by paper ionophoresis, but the application to the fractionation of tissue extracts has not been reported.

MATERIALS AND METHODS

Adenosine triphosphate (ATP). Three samples of ATP were used. Preparation 1 was prepared from the dibarium salt supplied by Boots Pure Drug Co., by means of the ion-exchange resin Deacidite FF (Permutit Co. Ltd.) with the HCl–NaCl eluants recommended by Cohn & Carter (1950). ATP was precipitated as the barium salt from the fraction removed by 0·2M-NaCl–0·01N-HCl and after conversion into the potassium salt with potassium sulphate was stored in solution at −20°. The solution showed a sharp symmetrical absorption curve with a peak at 260 mμ. The acid-labile phosphorus determined by 10 min. hydrolysis in N-H_2SO_4 was 68% of the total phosphorus. A preparation of rabbit-muscle myosin liberated 43% of the labile P in 1 hr. and 43·5% in 1·5 hr. When subjected to paper ionophoresis at pH 4·3 the solution showed only a single phosphate-containing compound. Inorganic orthophosphate was absent. The product was considered to be pure ATP.

Preparation 2 was prepared from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). The final product contained 64% acid-labile phosphorus, and it migrated mainly as a single spot on ionophoresis at pH 4·3. A faint spot corresponding to adenosine diphosphate (ADP) was present. Inorganic orthophosphate was absent.

Preparation 3 was a chromatographically pure sample of the crystalline sodium salt, and was a gift from Sigma Chemical Co., St Louis, U.S.A.

Adenosine diphosphate (ADP) was also a gift from Sigma Chemical Co. of the chromatographically pure sodium salt.

Creatine phosphate was prepared from creatine by the method of Ziele & Fawaz (1938). The product obtained as the calcium salt was stored over P_2O_5.

Glucose 1-phosphate was a gift of the pure dipotassium salt from Roche Products Ltd. The material migrated as a single spot on ionophoresis at pH 4·0 and pH 8·6, and contained 99·9% of the theoretical phosphorus content. Inorganic orthophosphate was absent.

Glucose 6-phosphate and fructose 6-phosphate were obtained as a mixed sample of the barium salts from Dr C. Long.

Myosin and potato apyrase. Myosin was prepared from rabbit muscle by the method of Bailey (1942). However, in view of the laboriousness of the preparation and the rapid loss of activity on storage the use of myosin was