homogenates, but is remarkably stable in the spleen. The chromatogram showed no evidence of the formation of the corresponding radioactive γ-glutamyl peptides from incubations with radioactive glycine, valine or phenylalanine in the presence or absence of unlabelled γ-glutamylglycine.

4. The formation of acidic metabolites, including γ-glutamyl peptides, from neutral substances was studied using chromatography with Amberlite IR-4B resin. An upper level of 5 % was obtained as the possible extent of formation of γ-glutamyl peptides.

5. Attempts to confirm the formation of γ-glutamylvaline and γ-glutamylphenylalanine, employing radioactive amino acids, by the sheep kidney system of Hanes *et al.* (1950, 1952) and modifications of this system with rat kidney were unsuccessful.

6. A micro synthesis of γ-glutamyl-[1:2-$^{14}$C]-glycine is described.

[1:2-$^{14}$C]Glycine and DL-[2-$^{14}$C]valine were synthesized under the direction of Dr E. M. Gal of this Department, the DL-[4:4′-$^{14}$C]valine was prepared by the Bio-organic Group of the Radiation Laboratory of the University by R. Ostwald, P. T. Adams and B. Tolbert (unpublished method). We are indebted to Mr G. Ellis of this Department for construction of the vacuum-driven stirring motor and the scanning windowless gas-flow counter. The paper represents work condensed from a thesis submitted by R. W. H. to the Graduate division of the University of California for the degree of Ph.D., June 1952, and was aided by research grants from the cancer research funds of the University of California and the Hobson Fund of the School of Medicine.

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# Techniques in Tissue Metabolism

## 3. STUDY OF TISSUE FRAGMENTS WITH LITTLE OR NO ADDED AQUEOUS PHASE, AND IN OILS

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In conducting metabolic experiments with fragments of tissues, a common procedure during the past 30 years has been to suspend the tissue in some 30–200 times its volume of aqueous fluid. The artificial nature of this situation can be appreciated by noting that the volume of blood in an animal tissue at any one time is usually between one-thirtieth and one-third of its total volume. Even consideration of the rate of blood flow *in vivo* does not always justify the high fluid/tissue ratios which have been employed *in vitro*. Thus blood flows through the human brain at about 50 ml./100 g./min.; in an experiment with cerebral tissue in 100 vol. of fluid, in which initial substrate level and utilization *in vitro* equalled those *in vivo*, over 3 hr. would be required *in vitro* to decrease the added substrate concentration to its level in venous blood *in vivo*. In metabolic studies with separated tissues the volume of added fluid is rarely considered in relation to the duration of metabolism or to the metabolic rate of a tissue. When substrate may become limiting, the expedient of increasing its

concentration *in vitro* rather than increasing fluid volume is not often employed.

Accumulating products of *in vitro* metabolism may give more serious reason for addition of much fluid; few homeostatic devices are applied *in vitro* in contrast to the many *in vivo*. On the other hand, certain accumulation of products is normal and high fluid/tissue ratios greatly increase the time needed to establish *in vitro*, levels of products normal *in vivo*. Thus blood enters the brain 1·1 mM in lactate and leaves it at 1·28 mM (Gibbs, Lennox, Nims & Gibbs, 1942). Cerebral tissues placed in 100 vol. of glucose–saline have their lactate (about 15 mM) greatly reduced and require about 3·5 hr. to establish 1·28 mM lactate in the saline (see, for example, McIlwain & Grinyer, 1950). Constituents other than those formed from added substrates are also lost from tissue to fluid. Increasing fluid/tissue ratios probably increases such loss or the osmotic work necessary to oppose such dilution. Loss from cerebral tissues respiring in 50 vol. of glucose–saline was shown by a 30% fall in respiratory rate when the saline was replaced by a fresh specimen of the same initial composition (McIlwain, 1953).

In the present work, tissues have been studied with little or no added aqueous fluid. This has raised problems of provision of substrates and support for the tissue, for which answers have been sought by use of high substrate concentrations, non-aqueous solvents, and additional mechanical arrangements. We were encouraged in this by previous observations (McIlwain, 1951; McIlwain & Ochs, 1952) that the metabolism of cerebral tissues can remain normal while they are fixed in frames and after they have been bathed with liquid paraffin.

## EXPERIMENTAL

### *General*

*Salines.* The saline used in all experiments except those involving phosphate determinations contained: 134 mM-NaCl, 5·2 mM-KCl, 2·8 mM-$CaCl_2$, 1·3 mM-$KH_2PO_4$, 1·3 mM-$MgSO_4$, and 10·4 mM-$Na_2HPO_4$. Substrate when desired was added from a concentrated stock solution without diluting the saline by more than 3%. For preparation the following stock solutions were stored in the refrigerator: *A*, 0·725 M-NaCl, 0·029 M-KCl, 7·25 mM-$KH_2PO_4$ and 7·25 mM-$MgSO_4$; *B*, 0·11 M-$CaCl_2$; *C*, 0·1 M phosphate buffer (17·8 g. $Na_2HPO_4$ brought to pH 7·4 with N-HCl and diluted to 1 l.). To prepare 120 ml. of medium without precipitating Ca salts, 21·2 ml. of *A* was diluted to 106 ml. with glass-distilled water, 3·0 ml. of *B* added and the solution gassed with $O_2$ for 7 min.; 12·0 ml. of *C* were added slowly whilst gassing, after which gassing was continued for a further 10 min. When phosphates were to be determined the medium was buffered with aminotrishydroxymethylmethane (final concentration 0·025 M) in place of phosphate.

*Experimental animals.* For most experiments adult guinea pigs of 300–400 g. were used; they were stunned by a blow on the back of the neck and bled by decapitation before removal of the tissues. To obtain cerebral cortex slices of 100–120 mg. moist wt., the cerebral hemispheres were prepared as described by McIlwain (1951). Tissues from rats were obtained similarly; rabbits were killed by injection of air into the marginal ear vein.

*Fresh tissue slices.* Slices were cut from the freshly excised organ without the use of saline on blade or tissue. A narrow blade 10 cm. long and 2–3 mm. wide, held taut in a frame of silver-steel wire, was used and guided by a recessed glass slide to obtain slices of uniform thickness (approx. 0·35 mm.) After being cut, the slices adhered to the block of tissue and from this they were picked up by the wire rider of a torsion balance. In some cases they were transferred immediately with a spatula to a tared support (see below), for insertion into manometric vessels. In other experiments (see below) slices were cut with the minimum quantity of saline required to lubricate a blade of 15 mm. width and in these cases the slices adhered to the glass slide used as a guide. Any surplus saline was removed from the tissue immediately after cutting by placing the torn edge of a hardened filter paper at the side of the tissue as it lay on the slide.

*Moist tissue slices.* These were cut in the more usual fashion with blade and guide wet with the saline employed in the remainder of the experiment. After cutting they were floated from blade or guide to the experimental saline, and then drained from saline to a glass surface for weighing or further handling; in this condition they are referred to throughout as 'moist tissue'. Slices handled moist were mounted on their holders in a shallow dish of saline. They were floated as an open sheet above the holder which was held by forceps, and the holder lifted out of the saline so that it carried the slice with it. Excess saline was drained by placing on a pad of filter paper for 3–5 sec.

*Supports for slices.* A problem common to measurement of respiratory rates both with no added fluid and in non-aqueous media was that of providing adequate support for the tissue. Slices suspended in oil adhered to the vessel or to themselves, curling or forming a blob which could not be opened without damage to the tissue. The grid tissue-holders type *D* (McIlwain, 1951), devised previously as electrodes, were found very suitable for supporting the tissue in the present experiments. For moist tissue to which electrical pulses were not to be applied, a piece of metal gauze was used. The gauze should not be closely woven; silver wire gauze of 20-mesh was found suitable and gauze of stainless steel gave the same results.

*Manometric vessels.* These were vessels *A* (McIlwain, 1951) with a wide mouth (C24 standard socket) to admit the tissue-holders, and a displaced alkali-well to allow the holders to rest on the base of the vessel. They were conical, of 18–20 ml. volume, with a side arm with a valve-stopper.

### *Determinations*

*Lactic acid* was determined according to Barker & Summerson (1941).

*Inorganic and creatine phosphates.* Methods described previously (McIlwain, Buchel & Cheshire, 1951) were modified as follows. The trichloroacetic acid extract (1 ml.) of the slice, centrifuged at 0°, was neutralized at 0° with N-NaOH (0·5 ml.; phenolphthalein) in a tapered centrifuge tube of 3–5 ml. Of a 10% (w/v) solution of $CaCl_2$ saturated with $Ca(OH)_2$, 0·3 ml. was added and precipitation of the inorganic (and other) phosphates was allowed to proceed for 10 min. at room temp.; the tube was then centrifuged strongly.

For inorganic phosphate the precipitate, in its tube drained and wiped free from solution, was dissolved in 0·5 ml. 0·1 N-HCl and Berenblum & Chain's (1938) method applied on a small scale as described by Long (1943). Creatine phosphate was similarly determined in the supernatant or a measured portion, after adding 0·3 ml. of 10 N-$H_2SO_4$.

*Measurements with fresh or moist tissue*

A tissue holder carrying a slice was placed in a vessel already containing 0·15 ml. 5 N-NaOH in its well. A filter-paper wick was added to this and moist $O_2$ which had bubbled through 0·9% NaCl passed through the vessel for 7 min., after which it was placed in the thermostat and respiration followed in the usual manner.

*Measurements with non-aqueous media*

*Fluids used.* Light liquid paraffin, sp.gr. 0·835–0·850, was obtained from British Drug Houses Ltd. Silicone fluid D.C.200 was kindly supplied as samples of viscosities 0·65 centistokes (cS.), 1 and 5 cS. by Midland Silicones Ltd. Olive oil of B.P. (1948) specification was freshly obtained. In use, the liquids were equilibrated with the experimental saline and gas phases in the apparatus described below.

*Measurement of solubility of $O_2$ in the fluids.* The solubility is known to be high. Data relating specifically to the present experimental conditions were required for calculating manometric constants, and were obtained as described in the Appendix to this paper.

*Preliminary experiments.* Initial difficulties, apparently due to the high solubility of atmospheric gases in the non-aqueous fluids used, are illustrated by the following experiment. Light liquid paraffin (5 ml.), freshly gassed with $O_2$, was pipetted into a manometric vessel which was immediately placed on a manometer through which $O_2$ was passing. After 7 min. with $O_2$ flowing at room temperature the manometer and flask were put to the thermostat at 37° and shaken. After 5 min. the manometer tap was closed. Equilibrium was not reached until 40 min. later, and during this time 100 $\mu$l. of gas were gradually evolved. Only slight improvement was effected by passing the $O_2$ at 37° rather than at room temperature. The solubility of $N_2$ in the oil is such that when in equilibrium with air the volume used could contain about 400 $\mu$l. $N_2$, and it appeared that much $N_2$ had redissolved during pipetting.

*Apparatus* (Fig. 1). This was designed to equilibrate the non-aqueous fluid with the saline and gas employed, and to enable the pipetting to take place without exposure to air. The reservoirs *A* and *C* were permanently clamped in the thermostat used for manometric measurements. The manometric vessel *D* was temporarily held by a screw clamp in the position shown. The manometer *F* (described below) during initial procedures was fixed on the shaking arm of the thermostat by an additional clip on its back-board, which held the ground glass joint of the manometer about 2 cm. above the water level. Connexions from the gas cylinders to the reservoirs were of thick-walled rubber tubing; those from reservoir *C* to the vessel and manometer, of thin-walled surgical drainage tubing (approx. 2–3 mm. int. diam.). Oxygen from a cylinder was continually led through a two-holed rubber bung into the reservoir *A*, which contained the oil to be used in the experiment, in contact with a few ml. of saline. The pipette *B*, used for transferring the oil to the vessel, was a loose sliding fit in the other hole of the bung and when not in use was left in the position shown. Oxygen from the same cylinder also passed through the reservoir *C*, which contained oil and saline to saturate the gas with vapour; from here it was led either through the side-arm tap of the manometric vessel *D*, or through the three-way tap of the manometer *F*, depending on the position of the heavy 'bulldog' artery clip *E*.

*Procedure.* Before starting an experiment, $O_2$ was passed through both reservoirs for at least 30 min. to saturate the oil. Meanwhile, 0·15 ml. 5 N-NaOH was pipetted into the wells of all manometric vessels and tissue slices were cut and mounted. Vessels were then dealt with successively, the experimental ones first and an oil thermobarometer last. This contained only 5 ml. of oil from *A*. The holder (*G*) with its slice was fitted into an experimental vessel (*D*) which was immediately clamped in the thermostat and $O_2$ passed through the side-arm tap as shown in Fig. 1. Oil (5 ml.) from the reservoir *A* was rapidly pipetted into the vessel, which was then loosened from its clamp and attached to the appropriate manometer, already in position on the shaking arm, with its tap in position '*a*' and its right-hand limb filled with manometer fluid up to the tap. Concurrently with this last operation the clip *E* was moved from position 'i' to position 'ii' and the connexion to the vessel side-arm tap broken. Oxygen now passed into the flask via the manometer, which was lowered to its usual position, thus immersing the vessel. Gassing was now continued for a further 1·5 min., during which time the manometer and vessel were shaken at 40 oscillations/min. Finally the side-arm tap was closed and the manometer tap turned first to position '*b*' and then to position '*c*', and the vessel rubbed on to the joint. With two workers setting up an experiment consisting of four experimental vessels and one oil thermo-

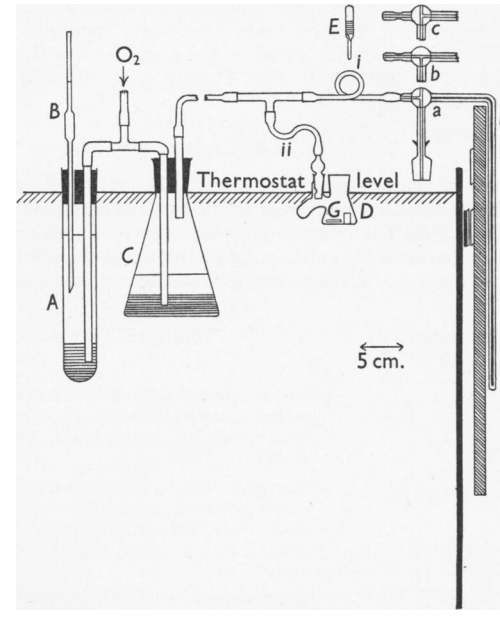


Fig. 1. Equilibration apparatus for manometric experiments in oils. Semi-diagrammatic. See text for description.

barometer, readings could normally commence 30–35 min. after killing the animal. The time taken to deal with one vessel was 3–4 min. and the moist tissue was never exposed to atmospheric air at room temperature for more than 15 sec. To complete the procedure in this time careful allocation of tasks was essential and involved overlapping in the preparation of successive slices and vessels.

In addition to the oil thermobarometer referred to above, a thermobarometer containing ungassed oil in atmospheric air was always included, as a check on the stability of the thermobarometer containing oil and oxygen. This should be placed at 37° at least 1 hr. before starting the experiment, in order to allow time for the oil vapour-pressure to become established. The rate at which $O_2$ was passed through the vessel and manometer was controlled by a flow meter (type FMO/V, Quickfit and Quartz Ltd.). A gas flow of 300–400 ml./min. was found adequate (and may have been excessive).

*Design of manometers.* The manometers used for most of these experiments were of an unconventional pattern illustrated in Fig. 1; they show some resemblance to the McGilvery-Machlett manometer described by McGilvery (1949). They were designed to overcome a difficulty encountered in using conventional manometers for experiments with the silicone fluids of viscosity 0·65 and 1 cS. These oils are appreciably volatile at 37°, with the result that when $O_2$ saturated with silicone vapour was passed through the manometer, some silicone condensed and formed a layer above the manometer fluid. With the new manometers gassing could be accomplished, as explained above, with the manometer fluid isolated from the gas. These manometers are more robust and flexible than conventional patterns and have been found very satisfactory for conventional procedures. They also possess certain advantages: for example the gas in the vessel can be shut off from that in the U-tube of the manometer by turning the tap to an intermediate position between those illustrated; further it is possible to evacuate the vessel on the manometer without disturbing the manometer fluid. The tap must be carefully greased and well ground.

## RESULTS

### *Respiration of tissue without fluid*

Tissues have been examined 'fresh' (cut and mounted without saline), 'moist' (cut with saline and mounted after floating in a dish of saline, but drained free from excess saline after mounting) and 'in saline' (the conventional procedure). In each case rates have been determined with and without glucose as substrate.

*Initial rates of 'fresh' tissues.* These rates proved surprisingly high; values with cerebral cortex are quoted in Table 1.

Rates with tissues which in their preparation had been left in saline were found to be lower because of two factors: (1) An apparently lower rate due to absorption of water before weighing; Table 1 shows this can amount to 20 % or more of the tissue weight, and such increase is adequate to explain the difference of respiratory rates (*c*) and (*d*) in Table 1. (2) An inherently lower rate of the tissue which had been exposed to saline. This is necessary to explain the difference between rates (*b*) and (*c*). It could be due to loss of material from the tissue to the saline or to dilution of tissue reactants by the absorbed saline.

*Initial rates of 'moist' tissues.* Such rates are compared in Table 2 with values for tissues immersed in about 50 vol. saline. All tissues had been handled in saline and their rates are thus lower than those of Table 1 (*a*)–(*c*) and of some previously recorded values (e.g. Wollenberger, 1947; see Cutting & McCance, 1946, and Kratzing, 1951).

Table 1. *Initial respiratory rates of 'fresh' cerebral cortex without added substrate*

Slices of guinea pig cerebral cortex 0·35 mm. thick were cut with a blade and guide without saline. Their fresh weight was determined and they were then treated as described in col. 2. Respiratory rates with specimens (*a*) were calculated on the basis of the fresh weight of tissue, and with (*b*), (*c*) and (*d*) on the basis of the last weighing described in col. 2. Conditions (*b*) imitated the cutting of slices with a blade moist with saline; conditions (*d*) imitated a commonly used procedure, followed in Table 2. Rates are followed by S.E. and number of observations in parentheses.

| Specimen | Additional treatment | Respiratory rate ($\mu$moles $O_2$/g./hr.) | Increase in weight (%) |
|---|---|---|---|
| (*a*) | None | 104±5·4 (7) | — |
| (*b*) | Slice moistened with saline on cutting blade; saline removed immediately afterwards and slice reweighed; then placed in manometric vessel | 101±3·0 (17) | 1, 3 |
| (*c*) | Slice moistened with saline on cutting blade; saline removed immediately afterwards and slice reweighed; subsequently slice left in saline 7 min. and then placed in manometric vessel | 89±1·7 (8) | 1, 3 |
| (*d*) | Slice moistened with saline on cutting blade, but removed immediately afterwards and slice weighed; left in saline 5 min., reweighed; returned to saline 2 min., then placed in manometric vessel | 68±5·9 (8) | 20, 24 |

Table 2. *Initial respiratory rates of tissue slices with and without added saline*

Slices of guinea pig tissues were cut in the presence of saline and floated on to dishes of saline for mounting. Tissues weighed approx. 100 mg. and under conditions (*A*) were with 5 ml. saline while under (*B*) the adhering saline was about 0·09 ml. (see text). Values quoted are mean rates, followed by their S.E. and number of observations, for the period 15–45 min. after placing at 37·5°. Glucose when present was initially 10 or 33 mM.

| Tissue | Glucose in saline | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) (*A*) Tissue immersed | (*B*) Tissue moist |
|---|---|---|---|
| Cerebral cortex | – | 40±1·2 (7) | 44±1·7 (10) |
| Cerebral cortex | + | 63±1·4 (9) | 68±1·9 (10) |
| Kidney cortex | – | 86±2·0 (14) | 127±3·2 (12) |
| Kidney cortex | + | 98±2·2 (14) | 147±4·4 (6) |
| Liver | – | 51±3·0 (7) | 61±5·0 (6) |

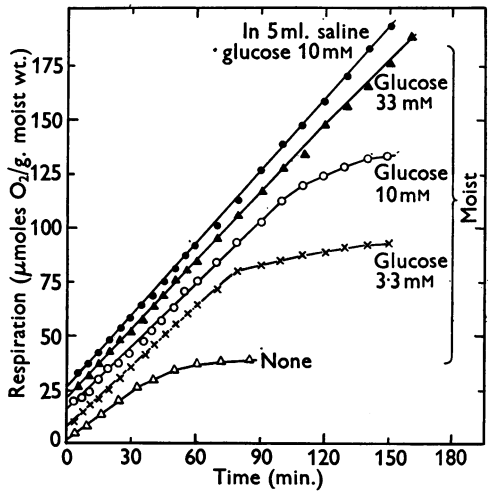


Fig. 2. Respiration of moist guinea pig cerebral cortex. Slices were cut in and floated into saline of glucose concentrations quoted. They were supported in manometric vessels in grid holders. The ordinates for slices respiring with glucose have been displaced upwards for clarity. The greater respiratory rate of the moist tissue, seen in the average values of Table 2, is not evident in the particular experiment illustrated here.

The average respiratory rates of tissues respiring moist were in every case greater than those of tissues immersed in saline. The difference was especially notable with kidney cortex, when rates with glucose were half as great again when the volume of saline was decreased to 0·002 of the normal amount. The presence of substrate afforded higher rates with the cerebral and renal tissues (it was not examined with liver), in spite of the small quantity of nutrient saline involved.

In some initial experiments with moist cerebral cortex and liver, 0·35 mm. slices were laid on filter paper moist with glucose salines on the base of conical vessels. The tissues were found to respire at rates some 10–15% lower than those of Table 2, presumably due to poor oxygenation.

*Maintenance of respiration.* Rates such as those of the fresh tissue in Table 1 decreased after about 30 min. This occurred also with moist tissues prepared in absence of substrate, but with substrate respiration could remain high for some 2 hr. (Figs. 2 and 4). With cerebral cortex in saline the effect of added glucose in increasing respiratory rate could be seen in the first few minutes of metabolism. This was evident also with the moist tissue which had received glucose only from the saline in which it has been prepared. The glucose so provided is limited, and higher concentrations of glucose were found necessary to maintain respiration after the first hour, than was the case when the relatively large volume of saline was provided in normal metabolic practice (Fig. 2). However, by preparing the tissue in saline with 33 mM glucose in place of the usual 10 mM, respiration remained high for at least 2·5 hr.

*Respiration of slices bathed with non-aqueous fluids*

Certain of the functions of aqueous suspending media in metabolic experiments may possibly with advantage be carried out by non-aqueous media. To examine this, respiratory rates of tissues in saline have been compared with those of moist tissues supported and immersed in liquid paraffin, olive oil or silicone fluid which were saturated with oxygen and saline as described in the experimental section.

*Initial rates of respiration.* Guinea pig cerebral cortex was examined in all three media. In liquid paraffin and olive oil respiratory rates were not significantly different from those found by conventional procedures in saline. With silicone fluid as suspending agent a rate was obtained 10 or 12% higher than that in saline ($P<0·05$) or in liquid paraffin ($P<0·01$). Other tissues from other animal species gave results summarized in Table 3.

*Maintenance of respiration and provision of substrate.* Unless additional substrate was provided, respiratory rates of moist tissue in oil fell after about 30 min. at 37° (Fig. 3). However, it was again easy to provide substrate by preparing the tissue in saline containing 33 mM glucose.

Table 3. *Initial respiratory rates of moist tissue slices bathed with non-aqueous media*

Slices were prepared in glucose salines except those marked * which were without substrate. All were mounted in tissue-holders type *D* (McIlwain, 1951). When sufficient values are available the mean rate is followed by its S.E. and the number of observations. The initial rate refers to the period 20–40 min. after placing at 37·5°. The respiratory rates in saline were obtained in the same experiment, or in experiments conducted at about the same time as the rates recorded in non-aqueous fluids.

| | | Moist tissue in non-aqueous fluid | | Respiratory rate of tissue in saline ($\mu$moles $O_2$/g. moist wt./hr.) |
|---|---|---|---|---|
| Animal | Tissue | Fluid | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) | |
| Guinea pig | Cerebral cortex | Silicone | 68·6±2·01 (12) | 62·9±1·37 (9) |
| | Cerebral cortex | Paraffin | 59·8±1·35 (6) | |
| | Cerebral cortex | Olive oil | 60, 62 | — |
| Rabbit | Cerebral cortex | Paraffin | 40, 40 | 48, 52 |
| Guinea pig | Kidney cortex | Paraffin | 108 | 110 |
| | Liver | Paraffin | 67 | 66 |
| | Spleen | Paraffin | 50 | 47 |
| Rat | Liver | Paraffin | 63 | 73 |
| | Spleen | Paraffin | 56 | 66 |
| | Diaphragm | Paraffin | 51 | 59 |
| Guinea pig | Kidney cortex* | Paraffin | 112 | 98 |
| | Liver* | Paraffin | 62 | 40 |

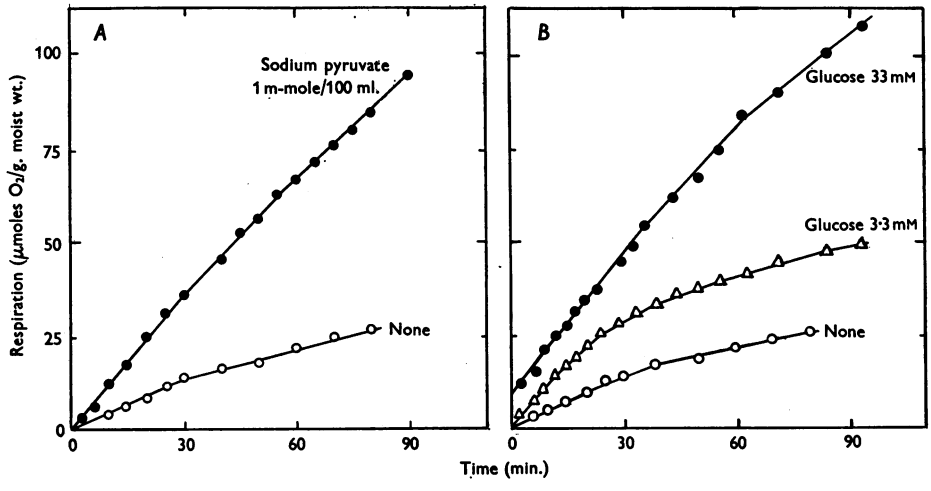


Fig. 3. Respiration of moist guinea pig cerebral cortex immersed in silicone fluid. Slices cut and floated into salines and supported in manometric vessels in grid holders. *A*: substrate (sodium pyruvate) supplied by 'homogenization' in oil in quantity quoted (preparative saline contained no substrate). *B*: substrate supplied by incorporation in preparative saline at concentrations quoted (the ordinates for the slices respiring in glucose have been displaced upwards for clarity).

An estimate of the amount of substrate available to slices of guinea pig cerebral cortex from the small quantity of preparative saline remaining associated with the slice was obtained in one series of experiments by weighing. From the weight of the tissue-holder plus moist slice, the original moist weight of the slice and the weight of the holder, the weight of saline transferred to the manometric vessels was calculated. The average for six slices, whose weights ranged from 100 to 150 mg. was 90 mg., range 73–117 mg. However, a proportion of this saline was obviously adherent to the tissue-holder and some of this was observed to be washed off subsequently by the oil in the vessel. A determination of amount of saline which remained associated with the slices themselves at the end of a 2 hr. experiment in liquid paraffin was therefore made. This was done by removing the slices from their holders and weighing them together with adherent oil and saline. The aqueous phase was then removed by drying in a vacuum desiccator and the slices were reweighed. From the difference in these two weights and the dry weight of the tissue (found to be 19 % of its original wet weight) the weight of the remaining

saline was found to average, for 6 slices, 15–20 mg. In these experiments the substrate in the saline was 33 mM glucose. Taking from the above determinations a figure of 0·05 ml. as representing the average volume of saline in contact with the slices during the experiments, it follows that 1·7 μmoles of glucose are available to the tissue from this source. The oxidation of this glucose would require 10·2 μmoles oxygen. The mean oxygen consumption by the tissues in the 2 hr. before their oxygen uptakes began to fall was in fact 10 μmoles. This agreement doubtlessly is partly coincidental, for no allowance has been made for endogenous substrate nor the possibility of preferential assimilation of glucose from saline before the slice was transferred to the vessel. The values, however, show that appreciable quantities of substrate can be made available for metabolism in a very much smaller bulk of saline than is normally employed.

The non-aqueous fluid has also been used as a vehicle for providing substrate. Sodium pyruvate when ground in oils in an all-glass homogenizer afforded a suspension from which it was only slowly washed by shaking with water. Such a suspension in silicone was used as substrate in the experiment of Fig. 3 and is seen to be actively oxidized.

### *Exchange, removal, or addition of fluid to respiring slices*

These procedures enable the effects of different conditions to be examined on the same specimen of tissue. As shown in Table 4 and Fig. 4, the greater part of the saline may be removed from slices of kidney and cerebral cortex respiring under conventional conditions and the tissue continues to respire with little change in rate. When succinate was provided in the first period as substrate for cerebral tissues, the rate was lower in the second period but this is the case whether or not the medium has been removed.

On the other hand, when saline was added to moist tissues, their respiratory rates invariably fell. This is shown to be the case with cerebral cortex in Table 4; the fall was greater with kidney cortex (Fig. 4; Table 4) and took place whether or not glucose had initially been provided in the saline used for preparing the tissue.

### *Application of electrical pulses to tissues*

One of the aims of the present studies was to obtain conditions under which cerebral tissues might respond to applied electrical pulses (see McIlwain, 1951) in the absence of much saline. When electrical pulses adequate for metabolic response in saline were applied to moist tissue or tissue in oil, increase in respiration was usually found, but the increase was small. Larger and more regular response was obtained when the slices were first incubated under conventional conditions. The aqueous medium was then removed and the slice left moist or with different oils. Electrical pulses of characteristics adequate for response in salines (McIlwain, 1951, 1954) were then also effective when the tissue was moist or when it was surrounded by liquid paraffin (Table 5 and Fig. 5). No response was given in silicone or olive oil.

### *Addition of inhibitory and stimulating agents to moist tissue and tissue in liquid paraffin*

Inhibitory substances could be added satisfactorily in liquid paraffin. It was especially interesting to examine substances which in saline showed inhibition of respiration in presence of applied electrical pulses though not in their absence. Table 6 shows that such effects were observed with oil containing atropine at 1 mM but not with atropine at 0·1 mM. When atropine was incorporated

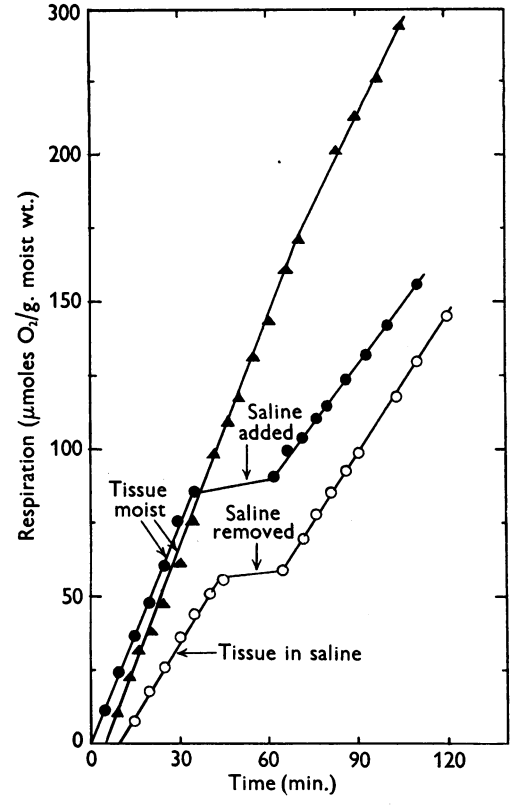


Fig. 4. Respiration of moist guinea pig kidney cortex. Slices cut and floated into saline containing 10 mM glucose, and supported in vessels in grid holders. Saline (5 ml.) was added and removed from vessels where indicated. (The abscissae for two slices have been displaced to the right for clarity.)

Table 4. *Respiratory rates of tissue slices before and after exchange, removal or addition of fluid*

Guinea pig tissues were prepared as in Table 2 and were mounted in tissue-holders. Fluid volumes were 5 ml. except in the first part of Expt. *B*, when the fluid adhering to the tissues was about 0·09 ml. Replacement or removal of fluid occupied 2–6 min., during which time the tissue was at room temp.

Expt. *A*. Removal of saline or its replacement by non-aqueous media

| | First 30 min. | | Second 30 min. | |
|---|---|---|---|---|
| Tissue | Substrate in saline (mM) | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) | Non-aqueous medium or state of tissue | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) |
| Cerebral cortex | Glucose, 13·3 | 64, 62 | Silicone | 86, 66 |
| | Glucose, 33 | 72, 75, 68 | Silicone | 70, 78, 79 |
| | Glucose, 33 | 70, 76, 74 | Olive oil | 65, 63, 59 |
| | Glucose, 33 | 68, 74 | Paraffin | 65, 76 |
| | Glucose, 33 | 65, 79, 77 | Saline removed and moist tissue left supported in vessel | 69, 73, 69 |
| | Na glutamate, 50 | 90, 82 | | 83, 82 |
| | Na pyruvate, 50 | 79, 73 | | 81, 82 |
| | Na succinate, 50 | 100 | | 64 |
| Kidney cortex | Glucose, 10 | 102, 94 | As above | 96, 94 |
| | None | 98, 98 | As above | 90, 90 |

Expt. *B*. Addition of saline after a period of respiration with no fluid

| | First 30 min. | | Second 30 min. with added saline | |
|---|---|---|---|---|
| | Substrate in preparative saline (mM) | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) | Substrate in added saline (mM) | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) |
| Cerebral cortex | Glucose, 33 | 60, 66 | Glucose, 33 | 44, 48 |
| | Glucose, 10 | 148 | Glucose, 10 | 78 |
| Kidney cortex | None | 125, 115 | None | 84, 84 |
| | None | 128 | None | 78 |

Table 5. *Application of electrical pulses to tissue respiring moist or in non-aqueous media*

Guinea pig cerebral cortex slices were prepared in saline with 33 mM glucose and allowed to respire for 30 min. in this medium before its removal or replacement by non-aqueous media. Respiratory rates are not quoted for this preliminary period. Alternating condenser pulses were applied at 100/sec. and were of other characteristics described in the table. When sufficient values are available the mean rate is followed by its S.E. and the number of observations, and the increase in respiration is calculated as the difference between each group.

| | Electrical pulses applied | | Respiration during second period ($\mu$moles $O_2$/g. moist wt./hr.) | | Increase with electrical pulses (%) |
|---|---|---|---|---|---|
| Medium during second period | Peak potential (v) | Time constant (msec.) | Unstimulated | Stimulated | |
| Silicone | 18 | 1·0 | 77±2·3 (5) | 81±1·9 (5) | 5 |
| Olive oil | 18 | 1·0 | 60, 62 | 66, 63 | 5 |
| Paraffin | 18 | 1·0 | 66±1·7 (8) | 88±3·0 (8) | 34 |
| | 12 | 1·0 | 75 | 95 | 27 |
| | 12 | 0·25 | 65 | 82 | 26 |
| | 24 | 1·0 | 75 | 105 | 40 |
| | 24 | 0·25 | 65 | 88 | 35 |
| None; tissue supported moist in vessel | 18 | 1·0 | 67±2·0 (14) | 98±2·1 (14) | 48 |

in saline at these concentrations, and the saline removed leaving the moist tissue, comparable inhibition was not found. The oil was thus performing a valuable function in maintaining a supply of the inhibitor.

Cocaine or butobarbitone added to saline in which slices were respiring did however affect their subsequent behaviour after the saline had been removed leaving the moist tissue. Applied electrical pulses normally increasing respiration then had very little effect.

The use of a non-aqueous medium enabled oil-soluble agents to be added from side arms during metabolism. 2:4-Dinitrophenol, added to form a 50 $\mu$M solution in the oil, greatly increased the respiration of moist cerebral tissue and electrical pulses were then ineffective (see McIlwain & Gore, 1951). Phenobarbitone and butobarbitone added during electrical stimulation inhibited the respiratory response (Fig. 5).

### *Lactic acid formation*

In early experiments, measurements were made of the pH of ground suspensions of cerebral cortex slices after periods of respiration moist or in non-aqueous media, using bromothymol blue. With slices prepared in saline containing glucose at 10 mM or less, the pH of the suspension after $O_2$ uptake had fallen off (e.g. Fig. 2) was invariably about 7·0. With 33 mM glucose in the preparative saline the pH was about 6·0 after 2 hr. of respiration. This suggested some accumulation of acid metabolites, amongst which lactic acid might be expected to preponderate.

Lactic acid appeared in the saline or slice in absence of added substrate when guinea pig tissues were examined (Table 7; see also McIlwain, 1953). The amount from the moist tissue was smaller in total amount but higher in concentration than with the tissue in saline. This was true also when glucose was added as substrate. Lactic acid in the moist tissue with or without liquid paraffin appeared to reach a maximum level of about 7–9 $\mu$moles/g. when 10 mM glucose was provided and metabolism progressed for less than 1 hr. Metabolism from 33 mM glucose or for 100 min. did not greatly increase this quantity. Previous experiments have shown that 6·7 mM or higher concentrations of lactic acid in saline increase respiration of cerebral tissues while 2 mM does not (McIlwain, 1953). Probably such further oxidation limits the level of lactic acid attained in the present experiments. This also makes understandable the greater quantity of lactic acid which accumulates in the saline under conventional manometric conditions. The last column of Table 7 gives an estimate of the percentage of the glucose metabolized, which has been converted into lactic acid. The calculation depends on a previous finding (McIlwain, Anguiano & Cheshire, 1951) that the glucose not so converted is oxidized completely. The proportion oxidized is greater with minimal fluid.

### *Phosphocreatine and inorganic phosphate in cerebral cortex with minimal saline*

The phosphocreatine and inorganic phosphate levels of cerebral tissues *in vivo* are grossly disturbed during excision and preparation of the brain for

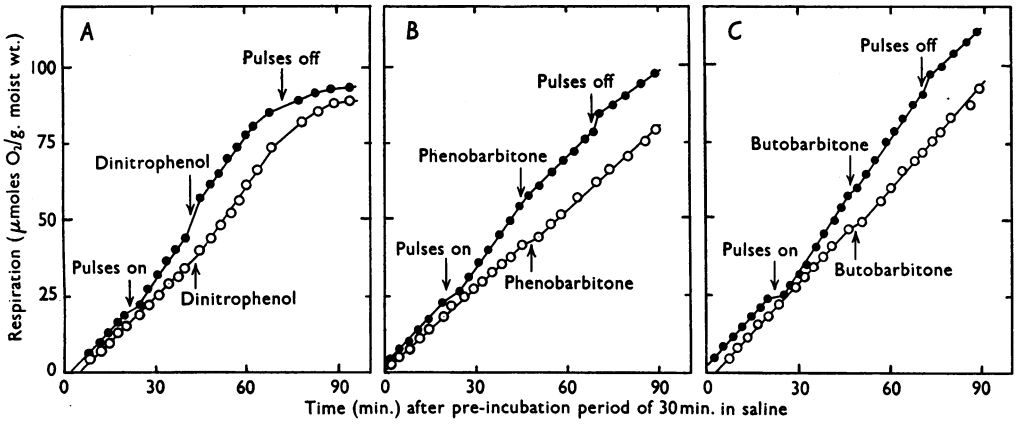


Fig. 5. Stimulation and inhibition of respiration of moist guinea pig cerebral cortex tissue immersed in liquid paraffin. Slices were cut and floated into saline containing 33 mM glucose, mounted in grid holders and allowed to respire in this saline for 30 min., before its removal and replacement by oil. Alternating condenser pulses, 100/sec., 18 v peak potential and time-constant 1 msec. were applied where indicated. *A*: effect of 50 $\mu$M 2:4-dinitrophenol on stimulated and unstimulated respiration; *B*: 0·1 mM phenobarbitone; *C*: 0·1 mM butobarbitone. Added substances were initially in side arms in paraffin at 10 times the concentrations quoted, and tipped as indicated.

Table 6. *Action of inhibitory agents on stimulated respiration of moist tissue and tissue respiring in liquid paraffin*

Guinea pig cerebral cortex slices were prepared in salines with 33 mM glucose. Electrical condenser pulses were alternating, of frequency 100/sec., peak potential 18 v and time-constant 1·0 msec.

| First period | | Second period | | Third period (medium as in second period) | | Increase in respiratory rate in third period (% of that in second period) |
|---|---|---|---|---|---|---|
| Substance added to glucose saline (mM) | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) | Medium | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) | Agents (mM) | Respiratory rate ($\mu$moles $O_2$/g. moist wt./hr.) | |
| None | 68, 66, 61 | None | 68, 66, 60 | Pulses | 104, 100, 104 | 53, 51, 73 |
| Atropine, 0·3 | 64, 65 | None | 57, 54 | Pulses | 80, 93 | 36, 67 |
| Atropine, 1·0 | 70, 72, 72, 62, 64 | None | 67, 64, 65, 57, 62 | Pulses | 93, 95, 82, 81, 83 | 38, 48, 26, 42, 34 |
| Butobarbitone, 1·0 | 64, 53 | None | 48, 51 | Pulses | 51, 54 | 6, 6 |
| Cocaine, 1·0 | 60 | None | 58 | Pulses | 62 | 7 |
| None | 65 | Paraffin | 69 | Pulses | 100 | 45 |
| None | 70, 74 | Paraffin with 0·1 mM atropine | 60, 67 | Pulses | 92, 83 | 53, 24 |
| None | 64, 68 | Paraffin with 1 mM atropine | 63, 56 | Pulses | 64, 61 | 1, 9 |
| None | 62 | Paraffin | 59 | 2:4-Dinitrophenol, 0·025 | 89 | 50 |
| None | 69 | Paraffin | 68 | Pulses | 94 | 38 |
| | | | | Pulses + phenobarbitone, 0·1 | 75 | 10 |
| None | 63 | Paraffin | 67 | Pulses | 90 | 34 |
| | | | | Pulses + butobarbitone, 0·1 | 79 | 18 |

Table 7. *Lactic acid formation by cerebral cortex slices respiring moist, in liquid paraffin and in saline*

Guinea pig cerebral cortex slices (except * which were from rabbit) in grid holders were incubated at 37·5° for 90–100 min. At the end of this period the tissue was ground in 16 ml. 4% (w/v) $CuSO_4$ to which washings or samples from the vessel and holder were also added, and lactic acid was determined in the suspension after adding $Ca(OH)_2$ and centrifuging, according to Barker & Summerson (1941).

| Tissue (mg. moist wt.) | Medium | Saline in vessel: Volume (ml.) | Saline in vessel: Glucose (mM) | Oxygen uptake (μmoles) | Lactic acid formed (μmoles) | $\frac{\text{Lactate} \times 100}{\text{Lactate} + \frac{1}{3}O_2}$ (see text) |
|---|---|---|---|---|---|---|
| 80 | Saline | 5 | 0 | 3·1 | 0·39 | — |
| 70 | Saline | 5 | 10 | 7·7 | 3·9 | 60 |
| 103 | Saline | 5 | 33 | 10·2 | 5·4 | 62 |
| 105 | None | Approx. 0·1 ml./ 100 mg. adhering to tissue | 0 | 6·0 | 0·18 | — |
| 106 | None | | 3·3 | 10·1 | 0·17 | 5 |
| 83 | None | | 10 | 8·3 | 0·51 | 16 |
| 80 | None | | 33 | 10·4 | 0·56 | 14 |
| 83 | Paraffin | Approx. 0·05 ml./ 100 mg. adhering to tissue | 33 | 4·9 | 0·63 | 27 |
| 72 | Paraffin | | 33 | 6·6 | 0·56 | 20 |
| 86* | Paraffin | | 33 | 5·7 | 0·69 | 27 |
| 72* | Paraffin | | 33 | 4·6 | 0·67 | 30 |

Table 8. *Inorganic and creatine phosphates in guinea pig cerebral tissues*

Tissues under conditions *A* were on silver gauze and in *B* and *C* were in grid holders. After incubation, tissues *A* were washed from the gauze with trichloroacetic acid to homogenizers and *B* and *C* were transferred rapidly to trichloroacetic acid in homogenizers. Values given are each the mean of 2 or 3 results which were within ±8% of the mean.

| Condition of tissue when incubated | Time of incubation (min.) | Phosphocreatine (μmoles/g.) | Inorganic phosphate (μmoles/g.) |
|---|---|---|---|
| *A*. Fresh; no previous contact with saline | 0 | 0·49 | 17·1 |
| | 15 | 0·51 | 15·9 |
| | 30 | 0·20 | 17·7 |
| *B*. Moist; previously floated in saline with 0·01 M glucose | 0 | 0·39 | 5·9 |
| | 30 | 0·81 | 5·7 |
| | 60 | 0·50 | 8·2 |
| *B*. Moist; previously floated in saline with 0·033 M glucose | 0 | 0·66 | 6·1 |
| | 30 | 0·98 | 6·1 |
| | 60 | 1·09 | 6·2 |
| *C*. In saline with 0·01 M glucose | 60 | 1·71 | 3·1 |
| *C*. In saline with 0·033 M glucose | 60 | 1·63 | 2·9 |

metabolic experiments, but are largely restored by incubation in glucose salines (McIlwain, Buchel & Cheshire, 1951).

In fresh tissue incubated without contact with salines or substrates, phosphocreatine remained low and inorganic phosphate high (Table 8). Thus endogenous substrates here, as in experiments with saline, support respiration but not maintenance of the phosphate levels. Slices prepared in glucose-containing salines were found to have more normal levels of phosphocreatine and inorganic phosphate, but values tended to be erratic. However, after restoring the creatine phosphate by incubation in glucose-containing salines, it was well maintained in the moist tissue which retained only some 0·1 ml. fluid/0·1 g. tissue. With 30 mM glucose during pre-incubation, phosphocreatine levels fell by no more than 15% in 15 min., though with 10 mM glucose the fall was of 38% in 30 min. (Fig. 6).

## DISCUSSION

Present observations bridge a gap between two methods of handling animal tissues. For observing mechanical or electrical response, intact muscle and nerve have often been studied in moist gas but with little or no added aqueous phase. With these tissues, such conditions may suffice for respiratory measurements (Meyerhof & Schulz, 1927; Gerard, 1932). They have also been employed with the cornea (Langham, 1952). Yet with tissue fragments whose cut surfaces increase likelihood of loss, a tradition of suspension in much fluid was early established. Warburg (1923, 1924) and Minami (1923) used 30–800 vol. of saline with liver and carcinoma specimens, and Dixon (1951) suggests 60–400 vol. Warburg (1915, 1924) measured respiration at different fluid/tissue ratios with spermatozoa and with tissue slices, but considered only two or three-

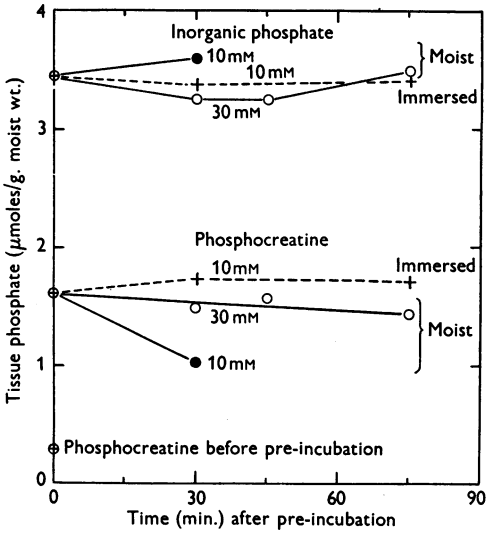


Fig. 6. Maintenance of creatine phosphate and inorganic phosphate in slices of guinea pig cerebral cortex cut in salines of the glucose concentrations quoted in the figure. All slices were initially incubated in 5 ml. of saline for 30 min., after which the saline was removed from the vessels indicated and their incubation continued for 30, 45 or 75 min.

fold variation in ratios 100–400 in value. Dixon (1951) pointed out discrepancies which could arise especially in muscular tissues when suspensions probably already in much fluid were diluted threefold, but did not raise the question of the general use of high fluid/tissue ratios.

The studies of the present paper indicate that many metabolic observations may be made with sliced tissues from brain, kidney, spleen and liver with volumes of saline no greater than those of the tissues themselves. This is 0·002–0·01 of the conventional quantity, but was adequate to supply substrates and maintain respiration at 37° for 1–2·5 hr. Change in composition of this saline brought about changes in behaviour of the tissue. Moreover, in the case of cerebral cortex, creatine phosphate could be adequately maintained in the moist tissue. The level of creatine phosphate in the brain *in situ* is very susceptible to any failure in supply of materials from the bloodstream and in the separated tissue in saline is promptly lowered if oxygen and glucose are not supplied or if inorganic constituents of the medium are much altered from normal (McIlwain, 1952). Hence its maintenance in the moist tissue gives further indication that such conditions are metabolically satisfactory. Also contributing to this impression and possibly interconnected with the maintenance of creatine and inorganic phosphates are the observations that respiration of the tissue can be increased by electrical pulses and by 2:4-dinitrophenol. This stimulation implies that the restraint or control imposed on the level of respiratory (and other) metabolism in the cell-containing tissue in saline is also reproduced in the moist tissue.

Apart from these similarities which indicate conditions with little saline to be not inferior to those with excess, special interest attaches to circumstances when little saline may be advantageous. Thus respiratory rates were in some cases higher with minimal saline. Oil-soluble substances were supplied to the tissue. Substrate utilization may more closely resemble its *in vivo* course. An example of this is seen in the formation of lactic acid from glucose by cerebral tissues. *In vivo* about 15% of the glucose metabolized by the brain is found by arterial-venous difference to be converted to lactic acid, and almost all the remainder oxidized completely (Himwich & Himwich, 1946). Under conventional conditions *in vitro* some 60% or more of the glucose appears as lactic acid (McIlwain & Grinyer, 1950; McIlwain, Anguiano & Cheshire, 1951), and many agents increase the proportion so appearing. With moist tissue alone or in oil, assuming lactic acid and $CO_2$ to be the only products, the fraction appearing as lactic acid was 15–30% (Table 7). Reasons were given above for considering the artificially high formation of lactic acid under conventional conditions to be due to its dilution in the large quantity of aqueous fluid supplied. Possibly one reason for the frequent use of large fluid volumes lies in their acting in this way as trapping agents for intermediary metabolites, a situation which offers analytical advantages although limiting quantitative comparison with the behaviour of the tissue *in vivo*.

## SUMMARY

1. Respiration of sliced cerebral cortex, kidney cortex, liver and spleen from guinea pig, rat, or rabbit has been measured in the presence of minimal aqueous fluid. The tissue rested on a grid in apparatus designed for these procedures.

2. Tissues examined fresh or moist (prepared in saline but drained from it before use) respired at rates initially similar to or higher than those under conventional conditions which employed 100–1000 times as much aqueous phase. Rates subsequently fell unless substrate was provided.

3. Comparably high respiratory rates were observed with the tissues immersed in a silicone fluid, light liquid paraffin, or olive oil.

4. Substrate was provided either during preliminary preparation of the tissue, or in a non-aqueous phase during metabolism.

5. Inhibitory and stimulating substances were also effective when so added. Respiration of

moist cerebral cortex was increased by electrical pulses and the increase opposed by certain added agents.

6. Formation of lactic acid by cerebral tissues in minimal aqueous fluid was closer to its normal *in vivo* value than was its formation with the conventional excess of saline.

7. A considerable level of phosphocreatine could be maintained in moist cerebral tissues for at least 75 min., while they respired at 37° in oxygen.

We are greatly indebted to the Board of Governors of the Bethlem Royal Hospital and Maudsley Hospital for support from the Hospital Research Fund for these studies.

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## APPENDIX

# Manometric Determination of the Solubility of Oxygen in Liquid Paraffin, Olive Oil and Silicone Fluids

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For the calculation of manometric vessel-constants for experiments conducted in non-aqueous fluids, the solubility of oxygen in the medium at the temperature of the thermostat was required.

The manometric apparatus of Van Slyke & Neill (1924) is not convenient for measurements at temperatures above that of the laboratory; the present method employs a conventional thermostat and shaking apparatus and involves measuring the entry of oxygen into the de-gassed oil; by this technique information can also be obtained about the rate at which the gas dissolves in the oil. A method somewhat similar in principle but for larger volumes especially of solid fats which have lower vapour pressures and in which gases are less soluble than the present oils, has recently been described by Davidson, Eggleton & Foggie (1952).

## EXPERIMENTAL

The essential part of the apparatus is illustrated in Fig. 1. The oil reservoir *A* contained, besides the oil, anhydrous $Na_2SO_4$ and was mounted in the thermostat. Before a determination, $O_2$ (dried by passing over $P_2O_5$) was bubbled through the reservoir and out into the atmosphere via the three-way stop-cock *B*. Semi-pressure tubing (ext. diam. 8 mm.; int. diam. 3 mm.) connected the manometer *C* (Rodnight & McIlwain, 1954) via the T-piece *D* to a high-vacuum pump and also to *B*. The special manometer vessel *E*, of approx. 30 ml. total capacity, was fitted with a stop-cock (5 mm. tap bore and greased with high-vacuum rubber grease) leading to a side chamber; the latter, together with the tap bore, had a capacity of 1·5 ml. 2 ml. of oil were pipetted into the vessel, which was placed on the manometer cone (greased with silicone high-vacuum grease) and immersed in the thermostat. With the manometer stop-cock in the position illustrated in Fig. 1, the pump was switched on