lipids. Collins (1960) has proposed that the complex phospholipids may be phosphate triesters and that this structure is consistent with the above-mentioned facts.

In the present study of the phospholipids of rat-liver mitochondria and microsomes it is apparent that the difference in the complex phospholipids in these two fractions is due to the presence of a component in microsomes which is absent from mitochondria. The fact that one component of the complex phospholipids seems to be associated with the endoplasmic reticulum raises speculation about the role of triester phospholipids in the functioning of the cell.

SUMMARY

1. The phospholipids from rat-liver mitochondria and microsomes have been examined by the countercurrent distribution of the dinitrophenylated and methylated derivatives.

2. The mitochondrial lipids contain a higher proportion of cardiolipin than do the microsomes.

3. The mitochondria lipids lack a component of the complex amino-phospholipids which is present in microsomes. No lysolecithin could be detected.

We wish to thank Miss H. Doery of the Commonwealth Serum Laboratory, Australia, for a gift of lysolecithin, Dr P. Plackett of the Animal Health Division, Commonwealth Scientific and Industrial Research Organization, for a gift of cardiolipin, the Standard Vacuum Refining Co. Aust. Pty. Ltd. for a gift of *n*-heptane and the National Health and Medical Research Council for financial support. One of us (V. L. S.) held a Commonwealth Post-graduate Award and a grant from the University of Melbourne. We wish to express our appreciation for the technical assistance of Miss T. B. G. Kaak.

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Mammalian Glycosidases

3. THE INTRACELLULAR LOCALIZATION OF β-GLUCURONIDASE IN DIFFERENT MAMMALIAN TISSUES*

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(*Received* 27 *September* 1960)

Kerr & Levvy (1951) and Walker & Levvy (1951) showed that a large part of the β-glucuronidase activity in water homogenates of mouse liver was associated with the cytoplasmic granules, and was apparently spread over particles of all sizes. The enzyme was fully active in water homogenates, and the fraction associated with the granules could be brought into a soluble state by incubation in acetate buffer, mechanical disintegration, and freezing and thawing, as well as by treatment with acetone or with the non-ionic, surface-active agent Triton X-100 (Rohm and Haas Co.). Walker (1952) extended this work to mouse liver homogenized in sucrose solution (0·25 M), and found that nearly all the enzyme was then associated with the cytoplasmic granules. The enzyme within the granules now displayed only a fraction of its total activity, owing apparently to slow diffusion of the substrate into the particles, since the activity rose when the substrate concentration was increased. The enzyme could be released by the measures already described, and, in addition, by dilution of the medium with water. These methods made the enzyme fully active, the most convenient being the use of Triton X-100. It was concluded that semi-permeable membranes must play a considerable part in regulating the activity of the enzyme *in vivo*.

* Part 2: Conchie & Hay (1959).

As a prelude to similar work on other mammalian glycosidases, a comparative study has now been made of the localization of β-glucuronidase in white-mouse liver, kidney, spleen and cancer tissue. Two tissues from C3H mice, together with pig liver, were included to observe whether the low β-glucuronidase activity in these organs (Morrow, Carroll & Greenspan, 1951; Conchie, Findlay & Levvy, 1959*a*) resulted in a different distribution of the enzyme within the cell.

EXPERIMENTAL

Materials

Phenolphthalein β-D-glucuronide was prepared biosynthetically as described by Levvy & Marsh (1959).

The adult white mice were from our Institute colony (Tyler's Original: Mill Hill Colony). The C3H strain was obtained from the Institute of Physiology, Glasgow University. Only male-mouse kidney was used because of the sex difference in the β-glucuronidase activity of this organ (Conchie, Findlay & Levvy, 1959*b*).

Mouse tumours, Ehrlich and T2146, were kindly supplied by Dr A. J. Carr, Department of Pathology, University of Aberdeen, and the S37 tumour by Professor P. C. Koller, Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3. All tumours were maintained in our own colony of white mice by subcutaneous transplantation. Suspensions for transplanting were made by grinding the tissue by hand in a glass homogenizer with a diameter clearance of 0·6 mm.

Pig liver was obtained immediately on slaughter from Robert Lawson and Sons (Dyce) Ltd. On removal, the liver was stored under 0·25M-sucrose solution at 0° and used as soon as possible.

Preparation of homogenates

Tissues were homogenized in a cooled all-glass homogenizer of the Potter–Elvehjem type with a diameter clearance of 0·23–0·43 mm. between the pestle and the tube, driven by an electric motor, average speed 1500 rev./min. For the preparation of sucrose homogenates, tissues were washed with ice-cold 0·25M-sucrose, pressed between pieces of hardened filter paper, weighed and homogenized in 0·25M-sucrose. The homogenates were used as soon as possible. Periods of homogenizing were varied, as stated in the text.

Water homogenates were prepared by homogenizing for ¼ min. or 1 min. as stated below. The preparations were made 0·1M with respect to acetic acid–NaOH buffer, pH 5·2, and kept for 30 min. before use. This delay permitted full activity to be reached, and avoided the small variable activation of water homogenates by Triton X-100 sometimes observed by Walker & Levvy (1951, 1953).

Enzyme assay

Assays were carried out as described by Levvy (1952), with 0·05M-acetic acid–NaOH buffer, pH 5·2. In experiments involving the measurement of the activity of untreated homogenates in 0·25M-sucrose solution, the incubation buffer for all assays contained 0·25M-sucrose, final concentration. For measurement of the total activity of sucrose homogenates, Triton X-100 (0·1%, w/v) was also included (Walker, 1952). The final supernatant from centrifugal fractionation (see below) did not require Triton X-100 to display full activity. The addition of the non-ionic surface-active agent Triton X-100 (0·1%, w/v) to sucrose homogenates of all the tissues resulted in the display of activities equal to those obtained by homogenizing the corresponding tissues in water, after due allowance had been made for the small inhibitory effect of 0·25M-sucrose on β-glucuronidase activity.

Because of their low activity, pig-liver preparations were incubated with the substrate for 4 hr. (Tables 2 and 3) or 17 hr. (Table 1), instead of 1 hr. as with the other tissues, in order to get accurate readings. The activity did not appear to alter on prolonged incubation.

RESULTS

Variation in homogenizer clearance

The distribution of β-glucuronidase activity in the fractions obtained from various tissues (see below) depended upon the clearance between the pestle and tube of the homogenizer. Potter (1955) found that the optimum clearance for each tissue was different, and the critical fit of the homogenizer could be determined only by experience, depending upon the purpose of the experiments. The tissues studied here, with the possible exceptions of the mouse tumours and pig liver, were all of types which have been found by Potter to require a similar homogenizer clearance. As a result of preliminary experiments, homogenates were prepared in homogenizers with a diameter clearance of 0·23–0·43 mm. between the pestle and the tube. When the clearance became greater than 0·43 mm., the homogenizer was discarded in order to keep the time relationships constant. Within the above clearances, reproducible results were obtained. Homogenizers with a smaller clearance tended to destroy cytoplasmic particles as soon as the cell was disrupted, since there was an immediate appearance of an abnormally large activity in the soluble enzyme fraction.

Fractionation procedure

This was a modification of that described by Hogeboom (1955) for liver-cell components, all separations being done at 0°. The 0·25M-sucrose homogenate (10 ml.; 5% moist tissue) was layered over 0·34M-sucrose (10 ml.) and centrifuged at 700*g* for 10 min. in a MSE Minor centrifuge. After removal of the supernatant with a capillary pipette, the residue was suspended in 0·25M-sucrose (2–3 ml.) and again centrifuged at 700*g* for 10 min. The washed residue, consisting mainly of unbroken cells and nuclei, was called fraction I.

Supernatant and washings were centrifuged at 5000*g* for 10 min. in a MSE Superspeed 25 centrifuge, the entire supernatant, including the

'fluffy layer', was removed with a capillary pipette, and the residual pellet washed by resuspension in 0·25 M-sucrose (5–6 ml.) by using the homogenizer for a brief period at low speed. After recentrifuging, washing was repeated, the final washed residue, which contained the larger cytoplasmic particles, being called fraction II.

The combined washings and supernatant were made 0·1 M with respect to acetate, with acetic acid–sodium hydroxide buffer, pH 5·2, containing 0·25 M-sucrose, and centrifuged at 1500*g* for 10 min. The supernatant was removed, and the residue, after washing with 0·25 M-sucrose (10 ml.) containing 0·1 M-acetic acid–sodium hydroxide buffer, pH 5·2, again centrifuged, giving fraction III (Walker & Levvy, 1951; Walker, 1952), the small cytoplasmic granules.

The final supernatant and washings, which were particle-free when examined microscopically, were called fraction IV. A number of assays were also carried out on homogenates fractionated without the aid of acetic acid–sodium hydroxide buffer, fraction III being obtained by centrifuging the supernatant from fraction II at 60 000*g* for 60 min. Identical results were obtained with the two procedures and the acetate precipitation method was adopted as a general fractionation technique. By either procedure, fraction IV is defined as 'soluble' enzyme.

For assay, all fractions were suspended in appropriate volumes of 0·25 M-sucrose, and assayed in the presence of Triton X-100 (0·1 %), the resulting activity being expressed as a percentage of the activity obtained when the unfractionated homogenate in 0·25 M-sucrose was assayed in the presence of Triton X-100.

Table 1. *Distribution of enzyme activities in sucrose homogenates after fractionation*

For details of homogenization and fractionation procedures see text. Results are expressed as a percentage of the activity of a sucrose homogenate: all assays were done in the presence of Triton X-100 (0·1 %).

		Period of homogenization				
	Fraction	'Hand ground'	¼ min.	1 min.	3 min.	4 × ¼ min.
Mouse liver	I	49	11	8	7	8
	II	24	34	30	19	32
	III	19	37	31	27	36
	IV	6	9	18	32	14
	Recovery	98	91	87	85	90
Mouse kidney	I	40	32	21	19	19
	II	38	30	19	16	26
	III	13	17	14	13	26
	IV	9	16	39	43	24
	Recovery	100	95	93	91	95
Mouse spleen	I	65	21	14	15	19
	II	10	5	3	2	5
	III	12	12	9	5	12
	IV	18	62	75	79	60
	Recovery	105	100	101	101	106
S 37-mouse tumour	I	27	23	19	7	10
	II	17	16	15	11	16
	III	10	10	10	12	14
	IV	45	51	52	64	55
	Recovery	99	100	96	94	95
C 3 H-mouse liver	I	62	30	24	16	25
	II	11	21	16	12	20
	III	20	28	24	28	37
	IV	2	14	35	50	15
	Recovery	95	93	99	106	97
C 3 H-mouse kidney	I	30	14	9	3	1
	II	13	10	6	1	10
	III	44	41	28	9	51
	IV	13	28	50	81	29
	Recovery	100	93	93	94	91
Pig liver	I	36	29	16	2	2
	II	28	42	24	8	35
	III	21	9	21	23	36
	IV	13	17	37	72	27
	Recovery	98	97	98	105	100

Effect on fractionation of varying the period of homogenizing

To ascertain the conditions which gave maximum breakdown of whole cells with minimum disruption of cytoplasmic particles, each tissue studied was subjected to homogenization for various periods of time, the fractionation procedure described above being carried out in each case. The results for a series of such determinations are shown in Table 1.

'Hand ground' homogenates were obtained by placing the tissue and sucrose solution in the homogenizer tube and depressing and raising the plunger by hand till an even suspension was obtained. Usually 10 strokes of the plunger were sufficient. An additional series of fractionations ($4 \times \frac{1}{4}$ min.) was also done, in which, after a $\frac{1}{4}$ min. period of homogenization, fraction I was separated and subjected to homogenizing for three further $\frac{1}{4}$ min. periods, the supernatant (fractions II, III and IV) being removed at the end of each period and pooled for further fractionation. The object of this procedure was to obtain maximum disintegration of the cells while subjecting the granules to the minimum risk of damage. No differences were observed when 1 mM-disodium ethylenediaminetetra-acetate was added to the sucrose solutions used for homogenization.

The 'hand ground' results show that, compared with other tissues, a very large percentage of the total β-glucuronidase activity in the tumour was found in fraction IV, the cytoplasm. Similar results were obtained with two other types of mouse tumour, Ehrlich and T2146. Mouse spleen also displayed a pattern of distribution different from the other tissues. After being homogenized for $\frac{1}{4}$ min., fraction I retained about the same percentage enzyme activity as in the case of the other tissues, but by far the greater part of th activity released from fraction I appeared in the cytoplasm.

The tissues studied varied to some extent in their rate of breakdown in 0·25 M-sucrose. Whereas with mouse liver homogenizing for $\frac{1}{4}$ min. was sufficient to break down the cells almost completely, other tissues were not always so rapidly disrupted. Prolonged homogenizing of the entire tissue did bring about a decrease in the activity of fraction I, but there was some loss of enzyme activity from fractions II and III, indicating breakdown of the granules, and a progressive increase in fraction IV. When fraction I alone was subjected to repeated homogenization, further breakdown of this fraction was obtained with minimum loss of activity in fractions II and III, and this presumably gave a truer comparison of the distribution of enzyme activity in tissues which were variable in their resistance to homogenization.

Enzymic activities in sucrose homogenates

Walker (1952) showed that homogenates of mouse liver in 0·25 M-sucrose solution only displayed about 60% of their total β-glucuronidase activity on direct assay. Similar phenomena were now found with sucrose homogenates of other tissues, though the percentage of total activity displayed varied with the tissue.

Table 2 shows the activities of the various tissues in sucrose solution, expressed as a percentage of the activity measured in the presence of Triton X-100, and the percentage of the total tissue activity that was found in the soluble fraction in sucrose and water homogenates. Particularly high percentage activities in the whole sucrose homogenates were observed with white-mouse spleen, C3H-mouse liver and pig liver.

Table 2. *Enzyme activities of tissue homogenates in 0·25 M-sucrose, and of the soluble fractions from sucrose and water homogenates*

Whole tissues were homogenized for the single periods shown. The whole sucrose homogenate was assayed in the absence of Triton X-100, and the results were expressed as a percentage of the total activity determined in the presence of Triton X-100. The soluble fractions were prepared by removing all particulate material with 0·1 M-acetic acid–NaOH buffer, pH 5·2, and the results are expressed as a percentage of the total activity in the homogenate. Total activities are expressed as μg. of phenolphthalein liberated/g. wet wt. of tissue in 1 hr.

	Period of homogenizing (min.)	Sucrose homogenate		Water homogenate.	
		Whole homogenate	Soluble fraction	Soluble fraction	Total activity
White-mouse liver	$\frac{1}{4}$	51	12	52	2440
kidney	$\frac{1}{4}$	59	24	67	2090
spleen	$\frac{1}{4}$	86	50	92	4050
S37 tumour	1	57	47	75	2600
T2146 tumour	1	—	52	82	2870
Ehrlich tumour	1	52	47	84	2120
C3H-mouse liver	$\frac{1}{4}$	85	15	87	228
kidney	$\frac{1}{4}$	69	21	56	870
Pig liver	1	85	25	83	138

Whereas water homogenates of all the tissues were fully active without Triton X-100 only a proportion of this activity appeared in the soluble fraction, and this fraction varied from tissue to tissue. A high figure under this heading appeared to depend upon either (*a*) ready penetration of the granules, as betrayed by a high activity for the sucrose homogenate in absence of Triton X-100, or (*b*) a high figure for the cytoplasmic fraction obtained from the sucrose homogenate. The mouse tumours were an example of the latter, the soluble fraction containing a much greater percentage of the total enzyme activity in the water homogenate than that of white-mouse liver or kidney. This factor must have led to exaggeratedly high figures for cancer tissue in early assays of β-glucuronidase carried out with the soluble fraction from water homogenates (see review, Levvy & Marsh, 1959).

Sucrose homogenates were diluted with 6 vol. of water. The soluble fraction then reached the figure for the same tissue homogenized in water, whereas the total activity (assayed in absence of Triton X-100) was within 10% of full potential activity of the tissue (Triton X-100 present).

Effect of incubation in acetate buffer on the release of enzyme

Table 3 gives details of the results obtained when sucrose homogenates were incubated for various periods of time in the presence of 0·1 M-acetic acid–sodium hydroxide buffer, pH 5·2, the tonicity of the medium being kept constant. At the end of each incubation period both the activity of the whole homogenate and that of the soluble enzyme (obtained after centrifuging the homogenate at 1500*g* for 10 min.) were assayed and expressed as a percentage of the activity obtained on assaying the homogenate in presence of Triton X-100. No inactivation was observed even after incubation for 22 hr.

In all cases there was a considerable release of enzyme, tending to a maximum at the figure for soluble enzyme in a water homogenate (cf. Table 2). Moreover, though in some cases the enzyme became more accessible to substrate, as was shown by higher percentage total activity figures, in no case did the activity level reach that of the fully activated enzyme. In both respects, the time relationships varied from tissue to tissue. The results suggest that there was no great change in the location of the enzyme or in its accessibility to substrate under our assay conditions.

DISCUSSION

In all the tissues examined, particulate β-glucuronidase was distributed between the large and small granules. This is in accord with observations on rat liver (Gianetto & de Duve, 1955; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), where part of the enzyme was found in the microsomes, in which it displayed the same 'latency' as the 'lysosomal' fraction. In the microsomes there is no reason to believe that β-

Table 3. *Effect of incubation at* 37° *and* pH 5·2 *on the activity of a tissue homogenate in sucrose solution and of the particle-free supernatant prepared from it*

Homogenates prepared in 0·25 M-sucrose were incubated for various periods of time at 37° in 0·1 M-acetic acid–NaOH buffer. Assays were carried out in a buffer containing 0·25 M-sucrose. Results are expressed as a percentage of the activity of the corresponding whole homogenate assayed in the presence of Triton X-100. The whole tissues were homogenized for the single periods shown.

	Period of homogenizing (min.)		Period of incubation (hr.)					
			0	½	1	2	4	22
Mouse liver	¼	Whole homogenate	45	46	47	53	74	89
		Soluble fraction	7	8	8	22	56	67
Mouse kidney	¼	Whole homogenate	52	53	46	70	74	91
		Soluble fraction	15	20	35	41	46	66
Mouse spleen	¼	Whole homogenate	87	88	87	86	87	88
		Soluble fraction	57	71	77	81	85	83
Mouse S 37 tumour	1	Whole homogenate	43	52	55	61	—	65
		Soluble fraction	30	28	34	40	—	68
Mouse Ehrlich tumour	1	Whole homogenate	52	49	48	53	—	59
		Soluble fraction	45	40	47	51	—	69
C 3 H-mouse liver	¼	Whole homogenate	85	81	84	85	89	91
		Soluble fraction	31	45	61	65	74	83
C 3 H-mouse kidney	¼	Whole homogenate	64	73	72	84	83	89
		Soluble fraction	21	48	49	53	55	63
Pig liver	1	Whole homogenate	91	89	88	82	88	86
		Soluble fraction	28	38	44	54	70	77

glucuronidase should be any less active *in vivo* than, for example, the microsomal enzyme glucuronyl transferase (Dutton & Storey, 1954; Strominger, Kalckar, Axelrod & Maxwell, 1954) which synthesizes β-glucuronides. In the same way, there appear to be no grounds as regards physiological activity for distinguishing between β-glucuronidase and mitochondrial dehydrogenases that are known to exhibit latency, such as succinic dehydrogenase (Singer & Lusty, 1960), and glutamic, malic and D-β-hydroxybutyric dehydrogenases (Bendall & de Duve, 1960). Even though in short-term experiments *in vitro* the activity of an enzyme is decreased when the integrity of the granules is preserved, there is no reason to believe that substrate equilibrium across the granular membrane could not be established eventually *in vivo*. Moreover, the possibility of a steady secretion *in vivo* of enzyme from the granules into the cytoplasm cannot be excluded. 'Latency' might thus be regarded as representing a device for regulating the catabolic activity of the healthy cell, that breaks down on the death of the cell (de Duve, 1959; Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959; Sellinger, Beaufay, Jacques, Doyen & de Duve, 1960).

Whereas mouse kidney resembled mouse liver in that the bulk of the enzyme was situated within granules, and thereby markedly decreased in activity in 0·25M-sucrose solution, a large part of the enzyme in spleen and cancer tissue was free in the cytoplasm. It would therefore appear that the β-glucuronidase activity of the latter tissues must be markedly enhanced *in vivo* in comparison with tissues like kidney and liver. This finding opens up interesting possibilities with regard to the use of saccharo-1 → 4-lactone (Levvy, 1952) as an inhibitor of the enzyme in pathological conditions (Boyland & Williams, 1956). It has an obvious bearing on the suggestion that cancer drugs might be usefully administered as their β-glucuronides (Danielli, 1950) with a view to selective hydrolysis at the desired site of action. Carcinogen β-glucuronides in the circulation could also be subject to selective hydrolysis at different sites.

No matter how elaborate the centrifugal analysis of the distribution of an enzyme in homogenates may be, it must be recognized that the homogenizing technique may itself introduce artifacts. Liver is very nearly an ideal tissue for studying the intracellular localization of an enzyme by centrifugal methods, in that the cells are almost completely fragmented before breakdown of the granules occurs, provided the homogenizer clearance is not too small (see fraction IV, Table 1). Kidney, on the other hand, behaved in this respect as though the cells were tougher than those of liver, whereas the granules were more fragile. In the tumours, both the cells and the granules appeared to be tougher than those of liver.

The low β-glucuronidase activity of the C3H-mouse tissues and of pig liver compared with most other strains of mice and other mammalian species (for review, see Levvy & Marsh, 1959) was not compensated for (as might have been anticipated) by an increase in the fraction of the enzyme free in the cytoplasm, but C3H-mouse liver and pig liver did show a greater accessibility of the granular enzyme to substrate, as well as greater release of the enzyme from the granules on incubation of the homogenate in 0·25M-sucrose solution or on homogenizing the tissue in water A few experiments with female C3H kidney, done since this work was completed, suggest that the lower overall enzyme activity (about 30% of the male) is compensated for by greater penetration of the granules by substrate in sucrose homogenates. The fact that homogenizing a tissue in water releases only a fraction of the β-glucuronidase activity from the granules has been noted (Kerr & Levvy, 1951; Walker & Levvy, 1951), and may be explained in two ways, either by assuming that part of the enzyme within the granule is bound more firmly than the rest, or that there are within the cell two types of particle, differing in their fragility.

SUMMARY

1. A study has been made of the intracellular localization by differential centrifuging of β-glucuronidase in 0·25M-sucrose homogenates of adult white-mouse liver, kidney, spleen and cancer tissue, adult C3H-mouse liver and kidney, and pig liver.

2. In all tissues except mouse spleen and cancer tissue most of the enzyme was associated with the cytoplasmic granules, within which it displayed only a fraction of its total activity. In mouse spleen and cancer tissue a large part of the enzyme was free in the cytoplasm, and most of the remainder was associated with the cytoplasmic granules.

3. The remarkably low β-glucuronidase activity of pig liver and C3H-mouse liver and kidney as compared with white mice was not compensated for by any increase in the amount of the enzyme free in the cytoplasm, but the enzyme within the granules was more freely accessible to substrate.

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Electrical Pulses and the Potassium and Other Ions of Isolated Cerebral Tissues

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(*Received* 27 *September* 1960)

The differential distribution of sodium and potassium salts which is found in most animal tissues is of special significance in neural systems, for here the substances take part in the rapid ion movements associated with the nerve impulse. Loss of K^+ ions and gain in Na^+ ions on electrical stimulation of peripheral nerve is well documented (Keynes, 1951; Hodgkin, 1958), and it is a general impression that a major part of energy utilization by cerebral tissues is connected with the work performed in maintaining their characteristic ion distribution. When cerebral tissues are removed from an animal, promptly sliced and placed in media similar in content of Na^+ and K^+ ions to the extracellular fluids of the body they lose K^+ ions and gain Na^+ ions and water. Respiration in glucose is, however, associated with restoration of a large part of the K^+ ions and with extrusion of Na^+ ions (Terner, Eggleston & Krebs, 1950). Studies with isotopically-labelled potassium (^{42}K) salts showed that this was accompanied by a considerable turnover of the K^+ ions of the slices, of approximately 3·5–4% of the tissue content/min. (Krebs, Eggleston & Terner, 1951).

The metabolism of isolated cerebral tissues in media similar to those employed in studying potassium movement, is affected by applied electrical pulses; energy-rich phosphates of the tissue are diminished and energy-yielding reactions accelerated (McIlwain, 1951, 1959). The content of Na^+ and K^+ ions and the exchange of K^+ ions have now been examined in electrically stimulated tissues. The tissues' respiratory and glycolytic responses to pulses have already been shown to be dependent on the addition of Na^+ ions to the medium in which they are incubated (Gore & McIlwain, 1952), and a similar dependence on K^+ ions is reported below.

EXPERIMENTAL

Ion movements

Tissues and electrodes. The procedures described by McIlwain (1960, 1961) were followed. Guinea pigs were used throughout the studies; they were stunned by a blow on the neck, killed by bleeding and the brain was removed in less than 3 min. The cerebral cortex was sliced to an average thickness of 0·35 mm. with a blade and recessed glass guide, weighed after draining on glass and placed in vessels which were incubated at 37·5° within 30 min. of the animal's death. Electrical pulses were supplied from the generator of Ayres & McIlwain (1953) to silver-grid electrodes at 100/sec., and were condenser pulses of peak potential 10v and time constant 0·4 msec.; for choice of these conditions see McIlwain (1954).