reaction is not given by the limiting slopes but could be obtained by the use of an electronic computer.

ACKNOWLEDGEMENTS

I much appreciate the advice and encouragement which I received from Drs. E. M. CROOK, H. KESTELMAN AND R. A. BUCKINGHAM of University College, London (England). I also gratefully acknowledge a grant from the Medical Research Council.

SUMMARY

The differential equation describing the Michaelis-Menten scheme is modified by a change of time and concentration units so as to contain only one rate parameter. In this form it is solved numerically. Solutions are obtained by the "steady state" and "pre-steady state" approximations and are compared with the numerical solutions. Methods are suggested for the derivation of individual rate constants and the molar concentration of enzyme from kinetic experiments on the overall reaction.

REFERENCES

1 L. MICHAELIS AND M. L. MENTEN, *Biochem. Z.*, 49 (1913) 333.
2 B. CHANCE, in E. S. G. BARRON, *Trends in Physiology and Biochemistry*, Academic Press Inc., New York, 1952, p. 25.
3 B. CHANCE, D. S. GREENSTEIN, J. HIGGINS AND C. C. YANG, *Arch. Biochem. Biophys.*, 37 (1952) 322.
4 P. A. T. SWOBODA, *Discussions Faraday Soc.*, 20 (1955) 275.
5 F. J. W. ROUGHTON, *Discussions Faraday Soc.*, 17 (1954) 119.
6 H. GUTFREUND, *Discussions Faraday Soc.*, 20 (1955) 167.
7 K. J. LAIDLER, *Can. J. Chem.*, 33 (1955) 1614.
8 M. F. MORALES AND D. E. GOLDMAN, *J. Am. Chem. Soc.*, 77 (1955) 6069.
9 O. H. STRAUS AND A. GOLDSTEIN, *J. Gen. Physiol.*, 26 (1943) 559.
10 C. C. YANG, *Arch. Biochem. Biophys.*, 51 (1954) 419.
11 W. E. MILNE, *Numerical Solution of Differential Equations*, Chapman and Hall, Ltd., London, 1953, p. 64.
12 G. E. BRIGGS AND J. B. S. HALDANE, *Biochem. J.*, 19 (1925) 338.
13 S. G. WALEY, *Biochim. Biophys. Acta*, 10 (1953) 27.
14 K. J. LAIDLER AND I. M. SOCQUET, *J. Phys. Chem.*, 54 (1950) 519.

Received April 6th, 1956

THE OUTPUT OF ^{45}Ca FROM FROG MUSCLE

E. J. HARRIS

Biophysics Department, University College, London (England)

It is well known that calcium exists in biological materials in both ionic and bound states. It was thought that the application of the calcium isotope ^{45}Ca might reveal to what extent the element is bound in frog muscle and what factors affect its subsequent liberation. Earlier work (TAUBMANN[1]; BERWICK[2]) has shown that little muscle Ca is removed by perfusion with Ca-free solution, or even by grinding in KCl solution. It therefore seemed likely that only a small proportion of the total Ca would be exchangeable, as proves to be the case. Recently GILBERT AND FENN[3] using ^{45}Ca have reached similar conclusions.

References p. 87.

METHODS

A mixture was prepared containing a proportion of ^{45}Ca as chloride and of the following composition: Na 120, K 2, Ca 0.5, Cl 93, HCO_3 30, in mmoles/l. Sartorius muscles of *Rana temporaria* were immersed in the mixture to introduce tracer Ca. The immersion was usually for 16 hours at 4°, but occasionally shorter times at room temperature were used. No difference in subsequent behaviour was found. Excitability was retained throughout experiments in which muscles were treated only with physiological saline mixtures; it was lost after a period of spontaneous activity when ethylenediaminetetraacetic acid was added to the solution.

The radioactivity of the Ca measured about 2000 impulses/min/0.005 μmole, measured under a thin window (type G.M.4) counter. When samples of low radioactivity were encountered in the experiments use was made of a helium flow counter (HARRIS[4]) into which the sample could be inserted.

Tracer output experiments were made by passing the previously loaded muscles along a series of sample tubes each containing 2 ml of test solution. The composition of the solution was usually Na 120, K 2, Ca 1, Mg 1, Cl 96, HCO_3 30 mM. To facilitate handling and to permit stirring by introduction of O_2/CO_2 mixture the muscle was tied so that it hung along the tube carrying the gas mixture into the fluid. At intervals the muscles were moved from one sample tube to the next, the times of transfer were chosen to keep the radioactivity collected in each portion at a convenient level. Portions of 1 ml were taken from the sample tubes after the muscle had been moved on; these were dried down on nickel planchettes for radioactivity measurement. At the end of the experiment the muscle was dried, and ashed at 550° on a platinum planchette.

Two radioactivity standards were used, both had the ^{45}Ca from 0.01 ml of the loading solution but to one was added 1 ml saline solution, this was evaporated off. The standard with the added salts from the saline solution was used for comparison with the activities measured in the dried portions of test solution. The standard without added salts was used for comparison with the activity of the muscle ash. The salt-free standard showed an activity about 10% higher than the other because the radiation from ^{45}Ca is weak and is absorbed by the salts. The natural activity of the K in the saline solution contributes a negligible amount to the background count.

The time course of the ^{45}Ca content of the muscle was reconstructed from the radioactivities found in the portions of test solution and in the ash.

Ca analyses were made on the muscle ashes. To hydrolyse the pyrophosphates, which interfere with the analysis, the material was first heated at 100° for 10 min with 0.1 N HCl.

The method of Ca analysis is similar to that described by FALES[5], with modifications suggested by KEYNES AND LEWIS[6]. The reagents were: (a) 0.1% murexide, (b) 1 N KOH, (c) 1 μmole/ml disodium salt of ethylenediaminetetraacetic acid (EDTA). Reagents (a) and (b) were kept in polythene bottles. Reagent (a) slowly decomposes, it was stored at 0°. To carry out the analysis about 1 ml (or less if the solution was freshly prepared) of the murexide solution was added to 25 ml water and 2.5 ml of the KOH. The mixture was shaken and used to fill matched 5 cm path cuvettes.

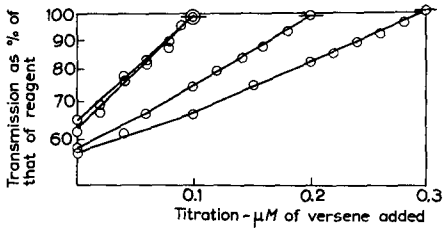

Fig. 1. Titration curves for calcium against versene using murexide indicator. The horizontal lines near 100% show the initial balance between the two cuvettes before the Ca was added. Two titrations of 0.1 μM Ca, and one each of 0.2 and 0.3 μM are shown.

The balance between the two transmissions was checked at 470 mμ using a spectrophotometer (Hilger) in which a rapid change from one to the other could be made. The coloured solution had a transmission of about 10% that of water at this wavelength. The unknown, dissolved in 0.1 ml of 0.1 N HCl was transferred with a dropper to one cuvette, an equal number of drops of the acid were put in the other. This ensures equal dilution of the colour and keeps the pH of the two sides equal which is necessary because the murexide decomposition rate depends strongly on pH. The ratio between the transmissions of solution with the added unknown and the standard was read off. The transmission rises continually on account of the decomposition of the reagent, but it is possible to find the ratio: unknown/standard in a few seconds. By using a set procedure the ratio is maintained despite decomposition. The titration of the Ca in the unknown is made by adding the EDTA reagent (c) from a microburette with addition of equal quantities of water to the standard solution. The transmission ratio is read after each addition, titration is continued until the original balance between transmissions is restored. If the balance is passed the correct reading can be found by an interpolation of a graph of (volume of EDTA) *vs.* (transmission ratio). Fig. 1 illustrates some titration plots. It is emphasised that it is not in general necessary to plot out the readings because one can estimate the amount of reagent which

References p. 87.

can safely be added from the transmission ratio. The method is suitable for quantities of 0.1 μmole or more. It is subject to the error introduced by failure of the spectrophotometer to return to an exactly reproducible balance between the two solutions; in the instrument this uncertainty was equivalent to not over 0.01 μmole Ca. The accuracy could be greatly improved by use of an automatically switched beam instrument.

The slope of the curve relating transmission to the volume of reagent added depends partly upon the exact dilution of murexide employed; it is also changed by substances which combine with calcium to form sparingly ionised compounds, for example pyrophosphates. Addition of 2.5 μmole of Mg causes a change of transmission equivalent to that given by 0.01 μmole of Ca. If the Mg content of the material to be analysed is known approximately a similar quantity of Mg can be added to the comparison solution.

Iron interferes with the determination and if the muscle contains appreciable myoglobin it is necessary to separate the calcium from the solution of ashed tissue before applying the method.

RESULTS

Exchange and extraction of Ca

Few experiments were made on the uptake of Ca. This is because direct observation of the radioactivity of the muscle gives only a measure of the superficial ^{45}Ca on account of the low penetrating power of the radiation. An uptake experiment, in which the muscle was washed for 2 min in tracer-free solution before each reading, is illustrated in Fig. 2. Initially uptake is rapid but the rate diminishes with time, in the experiment shown the radioactivities measured at 16 h and again at 20 h were equal. In a similar experiment the ^{45}Ca content became constant after only 1 h exposure.

Evidently the ^{45}Ca uptake reaches a limit. In all experiments in which muscles

TABLE I

Ca CONTENTS AND AMOUNTS OF Ca REPLACED BY LABELLED Ca

(Pair muscles shown on the same line)

Month	*Soak solution Ca concn. mM*	*Time h*	*mM/kg tissue*		*Acceleration* of output in Ca-free soln.*
			Ca content	*Labelled Ca content*	
June	0.5	16	3.7, 4.0	1.42, 1.16	+
	0.5	16	8.8, 9.0	0.81, 0.52	+
July	0.5	18	8.2	1.03	+
	0.5	16	5.6, 6.2	0.40, 0.82	Slight
	0.5	16	2.3, 3.5	0.48, 0.30	—
Sept.	0.5	3	7.7, 5.1	0.91, 0.59	—
	0.5	17	7.5, 8.4	0.73, 0.96	—
Oct.	0.5	16	2.0, 2.2	0.53, 0.44	—
Nov.	0.5	16	6.9, 6.8	0.92, 0.83	Slight
	0.5	24	5.0, 6.0	0.42, 0.42	Slight
	0.5	17	7.8, 6.3	0.47, 0.65	—
Dec.	0.5	60	5.3, 4.6	1.0, 0.6	—
	0.5	16	5.5, 6.4	0.64, 0.39	—
	0.5	60	6.1, 5.0	2.03, 1.45	—
Jan.	0.5	3	9.2	1.0	—
	1.5	16	4.7	2.2	+
	1.5	24	10.0	1.5	+
Feb.	1.5	20	10.0	1.16	+
	1.5	18	5.5	1.3	Slight
March	1.5	18	3.8	0.88	—
	1.5	18	5.2	2.05	—
April	1.5	16	7.9	2.32	—
	2.0	17	6.2	2.67	—

* + = acceleration; — = no acceleration.

References p. 87.

TABLE II

COMPARISON OF Ca CONTENTS OF MUSCLES STORED IN 1 mM Ca SALINE MIXTURE AND IN Ca-FREE SALINE MIXTURE

Time and temperature of exposure	*Ca contents mM/kg tissue*	
	Control	*Ca-free*
0° C		
16 h	3.9	3.4
16	5.9	3.0
16	5.5	4.4
24	9.2	5.6
24	7.5	4.6
24	5.6	5.0
18° C		
6 h	6.2	4.9
6	4.7	4.5
6	6.3	3.9
6	8.0	4.9
6	5.6	2.0

were exposed to tracer solution for about 16 h the exchange between solution Ca and muscle Ca was far from complete (see Table I). Prolonged exposure (60 h) did not lead to noticeably increased exchange. The quantities of ^{45}Ca taken up by the muscles are similar to the quantities which extraction in a Ca-free solution will remove (see Table II).

It is important to note that there is reason to doubt the homogeneity of the Ca in its distribution along the length of the muscle. In three experiments in which muscle ends were examined separately from the middle portion it was found that the ends contain 2–3 times as much Ca per unit weight as the middle. The ^{45}Ca in the separate

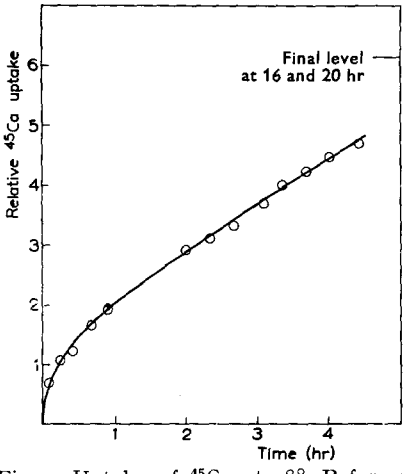

Fig. 2. Uptake of ^{45}Ca at 18°. Before each reading a 2 min wash was given. The "final" level found at both 16 and 20 h corresponded to the entry of 0.082 μM of labelled Ca. Total muscle Ca 0.50 μM.

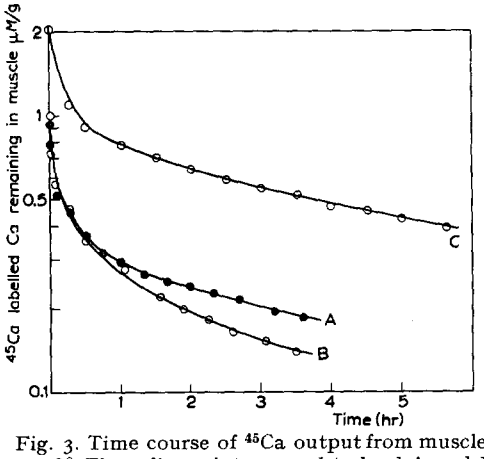

Fig. 3. Time course of ^{45}Ca output from muscles at 18°. The saline mixture used to load A and B had 0.5 mM Ca, A was exposed 16 h at 4°, and B for 3 h at 18°. C was loaded in 1.5 mM Ca saline for 16 h at 4°. Note that time of loading does not change the fraction lost in the first hour (compare A and B). Use of the stronger Ca solution increases both faster and slower fractions (curve C).

References p. 87.

portions was not distributed in the same ratio as the total Ca, the specific activity of the Ca in the ends of the muscle was less than that in the middle portion.

Time course of ^{45}Ca loss

Most observations of the ^{45}Ca loss were made using muscles which had been loaded for 16 h at 4° in the 0.5 m*M* Ca-saline solution. Muscles loaded for shorter times generally showed a lower content of the slower moving fractions (compare curves A and B, Fig. 3). Loading in 1.5 m*M* Ca-saline mixture led in some cases to a greater quantity of the tracer being in both fast and slower fractions (curve C, Fig. 3)

The output of ^{45}Ca is rapid initially but slows down after about 1 h. Although the rates appear to become constant if a short time is concerned it was consistently found that the loss in the period 4–16 h was less than that calculated from the slope of the curve between 2 and 4 h.

The initial rapid loss recalls the diffusion of Na and phosphate ions from the extracellular space of muscle (HARRIS AND BURN[7]; CAUSEY AND HARRIS[8]). There is, however, no clear separation of components attributable to extracellular fluid and cells respectively. There is a gradual change of rate such as might be expected if part of the Ca is held loosely and so becomes confused with the extracellular part and the remainder is more firmly held. Between 1 h and 4 h one can fit the output with a first order rate constant, which proves to be very variable, values range from 0.05 to 0.45 h^{-1}. This variability can be explained by the different quantities of the tracer which become bound to the tissue in a practically inexchangeable form. After an experiment lasting 4–5 h there is 10–20% of the original ^{45}Ca content remaining, and most of this cannot be removed by treatment with EDTA for 16 h. One might obtain more consistent values for a turn-over rate if a quantity, the bound ^{45}Ca, was subtracted from the total, but this would require a rather arbitrary selection of the value to be taken.

On account of the variability it was necessary to test the effects of different solutions and temperatures by interposing the changes during a single run. Changes were made 1–2 h after the commencement when the output had settled down to a relatively low value.

Temperature

Putting the muscle into portions of solution held at 0° instead of 20° reduced the rate of ^{45}Ca output (Fig. 4) by a factor of two but only after a delay of 10–20 min.

Ca-free solution

When the muscles were put into portions of solution which were initially Ca-free there was sometimes a definite acceleration of output (Fig. 5 and HARRIS[9], Fig. 1). The rate doubled occasionally but the result was variable and many trials were made in which no such acceleration could be detected. Table I includes a summary of experiments in which the Ca-free solutions were tested. There is a suggestion of a seasonal influence, but this is not definite.

Other changes of solution

The absence of Ca from the solution is only maintained if an agent is added to combine with the ion as fast as it is liberated. Addition of EDTA (1 or 4 m*M*) to the solution always evoked an acceleration of output (HARRIS[9], Fig. 2).

References p. 87.

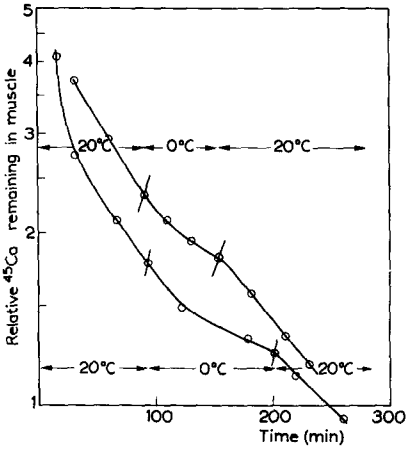

Fig. 4. The change in rate of ^{45}Ca output induced by cooling to 0° from 20°. Note the lag before the rate diminishes. The upper curve was obtained in an experiment using portions of Ca-free solution, the lower curve from one in which 1 m*M* Ca was present throughout.

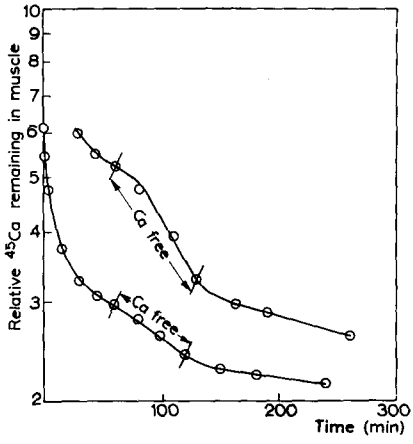

Fig. 5. The increase in rate of ^{45}Ca output which can be caused by use of Ca-free solution. Note the delay of about 10 min before the effect reaches its maximum and before it passes off in Ca-containing solution; this corresponds to the time needed for re-equilibration of the extracellular solution with the external solution. An acceleration is not always obtained in Ca-free solution.

Tests were made with altered Mg concentrations, for this ion might either substitute for Ca on adsorption sites, or might affect the activity of an enzyme. It was found that the acceleration of Ca output by Ca-free solutions was stopped by 5 m*M* Mg.

Stimulation and spontaneous activity

Some, but not all, muscles became spontaneously active while in the Ca-free solutions. This was especially so if the solutions were also made K-free. The mechanical activity was observed in specimens which did not show an accelerated ^{45}Ca output as well as in some which did. Therefore the spontaneous activity is not necessarily associated with an increased rate of ^{45}Ca output. Stimulation at either 15 or 75 impulses per min did not cause a change of output either in 1 m*M* or Ca-free solutions.

Ribonuclease and CO_2

It has been shown that the ability of *Elodea* cells to recover Ca after having been depleted in Ca-free solution was removed if they were treated with ribonuclease (LANSING AND ROSENTHAL[11]). This enzyme was tested on frog muscle by adding 0.8 mg to 2 ml portions of solution. It had no effect on the Ca output. As the enzyme has an optimum pH at about 5 the test was repeated with pure CO_2 passed into the liquid. It was necessary to test also the effect of the changed pH on the rate. It proved that use of CO_2 did increase the rate by 50%, but addition of the enzyme had no further effect.

Ionophoresis of Ca

As it seemed that part of the Ca became bound by the muscle an ionophoresis experiment was made similar to those made with other substances (HARRIS[10]). A strong ^{45}Ca

References p. 87.

solution was applied to a narrow zone (0.5 mm wide) of a sartorius and the spatial distribution of the radioactivity was found by scanning with a Geiger tube held behind a 1.5 mm slit. The distribution of measured activity is shown in curve A, Fig. 6. Current was passed between the ends of the muscle and the potential gradient (0.46 V cm^{-1}) was measured with an additional pair of electrodes. The activity distribution was measured at intervals, curve B shows the distribution after 5 h: the peak of the activity has moved by less than 1 mm in this time. Before the start of this experiment the muscle had been wiped with moistened filter paper to remove ^{45}Ca present locally in the extracellular fluid where the tracer had been applied. If this preliminary procedure was not used the distribution of the activity after ionophoresis showed two maxima. One (the greater) was near the point of application and the other moved, so that after 5 h it would have been at the position indicated by the arrow. This movement corresponds to a mobility of about 1/8 that of Ca/2 in free solution. One may conclude that most of the ^{45}Ca applied to the muscle has become incorporated in a bound or non-ionised form, while a small amount is mobile but with reduced mobility, as might result if it were in a dynamic state between ionised and non-ionised conditions. The results of the ionophoresis experiments closely resemble those obtained with phosphate (HARRIS[10]).

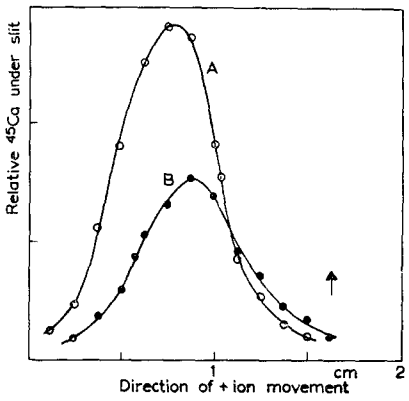

Fig. 6. The distribution of radioactivity following the application of a strip of active solution to a muscle. Curve A was taken after application and mopping with moist filter paper. Curve B as taken after 5 h ionophoresis. The arrow shows the position to which the mobile part of the Ca would have moved as calculated from a similar experiment in which the preliminary mopping had not been used. The position of the peak activity is but slightly moved by the current.

DISCUSSION

These results in common with those obtained by other methods, show that muscle Ca is not all held equally firmly. Over half the total often plays little part in exchanges with the exterior. As mentioned by GILBERT AND FENN[3] the use of complexing agents does not remove all the Ca.

There is no justification for attempting to interpret Ca movement in terms of a small number of separate first order processes because behaviour is variable and probably the result of a continuous gradation in strength of binding of the element.

The fact that only a part of the Ca will become exchanged with labelled Ca suggests that the tracer movement may not be a true measure of the behaviour of the total Ca. There is some incorporation of tracer into the "bound" fraction, as shown by the retention of ^{45}Ca by a labelled muscle even when exposed to EDTA. There is equally some dissociation of the bound Ca during the tracer experiments, this is shown if the specific activities of the Ca given up to portions of Ca-free solution are compared. It was found that the specific activity of the Ca diminished in the order: pretreatment solution; first wash; second wash; muscle. Accordingly the rate of loss of ^{45}Ca diminished with time more than did the rate of loss of total Ca.

References p. 87.

The observation that tracer loss may sometimes be accelerated in Ca-free solution would suggest that one is dealing with the dissociation of a sparingly soluble compound of which only the most accessible groups are exchanged in a Ca-containing solution, but which is progressively dissolved away in Ca-free solution if other conditions are favourable.

ACKNOWLEDGEMENTS

Part of the expenses of this work were met from a Grant from the Government Grants Committee of the Royal Society. Apparatus was provided by the Central Research Fund of London University. It is a pleasure to thank Mrs. V. BOLINGBROKE for her careful assistance with the experiments.

SUMMARY

1. The turn-over of frog muscle Ca has been studied using isotopic Ca.

2. A method of Ca analysis is described in which titration of Ca with versene is carried out in a spectrophotometer cell. The spectrophotometer permits rapid comparison to be made between the transmissions through the titration solution and a standard. This compensates for the decomposition of the murexide indicator.

3. When muscles are kept for 16 h in a solution containing isotopic Ca only 10–25 % of the Ca exchanges. Total Ca is 2–9 μmoles/g.

4. The time course of the loss of tracer Ca to a tracer-free solution is complex. A rapidly lost fraction, partly extracellular in origin, is not clearly separable from the total output. After 1–2 h the rate may be low (0.05–0.45 h^{-1}), the variability is due to part of the tracer Ca becoming incorporated in a relatively inexchangeable condition.

5. The rate of loss of the tracer Ca is reduced after a delay of 10–20 min when the tissue is cooled. The rate is sometimes increased if Ca is omitted from the bathing solution. Stimulation does not affect output.

6. Tracer applied to a point on the muscle is but slightly carried along the tissue by an electric current.

7. It is concluded that muscle Ca is held principally in a bound form, but that the strength of binding is variable.

REFERENCES

[1] G. TAUBMANN, *Arch. exptl. Pathol. Pharmakol.*, 174 (1934) 476.
[2] M. C. BERWICK, *J. Cellular Comp. Physiol.*, 38 (1951) 95.
[3] D. L. GILBERT AND W. O. FENN, *Am. J. Physiol.*, 183 (1955) 619.
[4] E. J. HARRIS, *J. Sci. Instr.*, 33 (1956) 322.
[5] F. W. FALES, *J. Biol. Chem.*, 204 (1953) 577.
[6] R. D. KEYNES AND P. LEWIS, *J. Physiol. (London)*, 134 (1956) 380.
[7] E. J. HARRIS AND G. P. BURN, *Trans. Faraday Soc.*, 65 (1949) 508.
[8] G. CAUSEY AND E. J. HARRIS, *Biochem. J.*, 49 (1951) 176.
[9] E. J. HARRIS, *J. Physiol. (London)*, 130 (1955) 26P.
[10] E. J. HARRIS, *J. Physiol. (London)*, 124 (1953) 248.
[11] A. I. LANSING AND T. B. ROSENTHAL, *J. Cellular Comp. Physiol.*, 40 (1952) 337.

Received April 21st, 1956