Some Effects of Glucose and Calcium upon the Metabolism of Kidney Slices from Adult and Newborn Rats

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Cutting & McCance (1946*a, b*) compared the metabolism of slices cut from the kidneys of newborn and adult animals. They found that the respiratory rate of kidney tissue from newborn rats changed little over a period of 2 hr., whilst that of the adult tissue fell off rapidly. Such a decline has often been reported, and Laser (1942) accepted it as a peculiarity of the kidney. Cutting & McCance noted, however, that their slices of adult tissue were visibly disintegrating after 2 hr., and had often lost 40–50% of their nitrogen. These slices were immersed in a solution buffered with phosphate at pH 7.4, similar to that described by Krebs (1933). It contained no calcium, and had about eight times the concentration of phosphate found in mammalian extracellular fluids. Cutting & McCance (1947) also studied the effect of calcium upon the respiration and the loss of nitrogen of kidney slices from adult and newborn rats. Since they believed that calcium would not remain in a solution containing phosphate at pH 7.4, they made their comparisons in a saline solution buffered with phosphate at pH 6.8 and in bicarbonate-Ringer at pH 7.4. The adult tissues lost less nitrogen in the presence of calcium, and their respiratory rate was better maintained, but was still far from constant.

The present work was undertaken to extend these observations, and a medium has been devised in which kidney slices from adult rats respired at a constant rate for 4 hr. This technical improvement has enabled the metabolism of slices from adult and newborn rats to be compared in a more reliable fashion, and in general the previous results have been fully confirmed. The new medium is an artificial extracellular fluid containing glucose, Ca^{++} and phosphate in physiological amounts at pH 7.4. It contains, however, no added bicarbonate. Its buffering capacity is less than that of the solutions which have generally been employed, but it gave satisfactory control of pH because kidney produces less acid than do the tissues upon which much of the early manometric work was done. Furthermore, it was hoped by using a smaller concentration of phosphate buffer to mitigate the difficulties which arise from the low solubility of the phosphates of calcium. Some relevant physicochemical data are briefly discussed in an appendix.

EXPERIMENTAL

Preparation of media

A saline solution was prepared by mixing 0.154 M-NaCl (232 ml.), 0.154 M-KCl (8 ml.), 0.154 M-MgSO₄ (2 ml.) and 0.110 M-CaCl₂ (6 ml.). The mixture was brought cautiously to pH 7.4 with 0.1 N-NaOH. 12 ml. of M/15-phosphate buffer (pH 7.4), prepared by mixing solutions of Na₂HPO₄ and KH₂PO₄ as described by Hawk, Oser & Summerson (1947, p. 636), were then added; pH was controlled both by capillators and by the glass electrode. To avoid precipitation the Ca and phosphate were not brought together in less than the final volume, but the completed solution was stable.

As extracellular fluids contain about 100 mg./100 ml. of glucose, this seemed a natural addition to make to the saline. Accordingly, 0.4 ml. of 5% glucose was added to each 19.6 ml. of the buffered saline immediately before use. The resulting medium contained approximately, in mmol./l., Na⁺, 140; K⁺, 5; Ca⁺⁺, 2.5; Mg⁺⁺, 1; Cl⁻, 144; P, 3 and SO₄⁻⁻⁻, 1; giving a total ionic strength of 0.158. It will be referred to as 'medium A 1'. This solution contained the equivalent of the total Ca of serum, but it was anticipated that some Ca⁺⁺ would be fixed by protein from the slices. In some experiments a similar solution (A 2) was used which contained twice as much phosphate buffer and one half as much 0.11 M-CaCl₂, giving the same ionic product, but twice the buffering capacity. No appreciable difference was found between the results of experiments in which A 1 and A 2 were used, and these results have been grouped together under the general heading 'medium A'. Several incomplete forms of this new medium were also employed in comparative studies. Medium B was the same as A 1, except that the CaCl₂ was omitted. Medium C was medium A 1 with the glucose omitted. Medium D was the same as C except that the CaCl₂ was also omitted. It gave results similar to those obtained by Cutting & McCance (1946*a*) with their phosphate solution. Since it was found that increasing the concentration of phosphate up to 18 mM. made no essential difference in the absence of Ca, the results of a few experiments using the original solution prepared by Cutting & McCance have been grouped among those under the heading 'medium D'.

In a footnote seen when this work was almost completed, Hawk *et al.* (1947, p. 301) recommended a similar solution without the Mg. A few experiments since then have suggested that respiration is better maintained when Mg is included, and Kuyper (1945) found that Mg increased the solubility of the Ca phosphates, so that its presence seems desirable.

Technique

This was in the main as described by Cutting & McCance (1946*a*), using Barcroft's differential respirometers. Adult rats were killed by a blow on the head, newborn ones by

decapitation; the kidneys were immediately removed and placed in medium *C*, in which the slices were prepared. Usually these were cut free-hand (Dixon, 1943), but the kidneys of some newborn rats were sliced by the method of Deutsch (1936), as modified by Cohen (1945). The slices were more easily cut by this method, but in the Barcroft flasks they seemed to lose more N, and not to maintain their respiration quite so well. The tissue (50–70 mg.) was bathed in 2.7 ml. of fluid in each manometer flask. The slices were roughly and quickly weighed on a glass-fibre microbalance similar to the much more sensitive quartz-fibre balance described by Lowry (1941). The centre tubes contained 0.3 ml. of 10% KOH and the usual roll of filter paper. The flasks were gassed with pure O₂ in the bath at 38°, a point discussed later. Readings usually commenced about 45 min. after the death of the animals and were repeated every 15 min. Although physiologically objectionable, continuous removal of CO₂ is necessary in order to follow the variation of respiration with time. Warburg (1923) gave an estimate of 23 mm. Hg for the pressure of CO₂ at the centre of a slice 0.47 mm. thick respiring in O₂. As the slices in this work were about 0.3 mm. thick, the cells were exposed to a pressure of CO₂ which was below that in their normal surroundings (40–50 mm. Hg according to Campbell, 1931), but which was not negligible. The adequacy of the buffer was checked by adding phenol red at the end of every experiment.

Nitrogen was determined by the micro-Kjeldahl method. The tissue fragments were transferred to a Pyrex tube, and the fluid remaining in the flask was added to 10% trichloroacetic acid (1 ml.) in a centrifuge tube. The tissue, the precipitate and the supernatant fluid (first evaporated to small bulk) were then separately digested for 12–24 hr. with H₂SO₄ containing copper selenide. The NH₃ formed was released by steam distillation, trapped in 2% boric acid coloured with bromocresol green, and titrated with 0.0143 N-H₂SO₄. The three analytical figures gave the total amount of N in the slices in each flask, and the amounts lost to the medium as 'protein' N and 'non-protein' N.

RESULTS

Kidney slices from adult rats

Respiration. Table 1 shows the average respiratory rates of adult rat kidney slices during the first hour

Table 1. *Average respiratory rates of adult rat kidney slices during the first hour in various media at pH 7.4*

Medium	Glucose	Ca	Respiratory rate	No. of exps.
			Mean ± s.d. (μl. O ₂ /mg. total N/hr.)	
A	+	+	137 ± 7	7
B	+	–	157 ± 14	5
C	–	+	140 ± 11	8
D	–	–	128 ± 13	6

Significance of differences

	<i>t</i>	<i>P</i>
A and B	2.8	<0.02
A and D	1.45	0.15
B and C	2.15	0.05
B and D	3.2	0.01
C and D	1.6	0.15

in the four media at pH 7.4. They only varied between 128 and 157 μl. O₂/hr./mg. total N, but it will be seen that Ca tended to increase the respiratory rate, and glucose increased it considerably. The latter effect was not observed, however, when glucose was added to a medium which contained Ca. The scatter shown by the standard deviations was partly due to grouping together results obtained from the kidneys of many rats. The same effects were also observed when slices from the kidneys of the same rat were compared in the different media.

Fig. 1. Respiratory rates of adult rat kidney slices in media A, B, C and D (see p. 68, col. 2, para. 2) at pH 7.4. Average respiratory rates expressed as percentage of O₂ uptake during first hr. Arrows show standard deviations. Numbers of experiments shown in brackets.

More striking differences were revealed when the experiments were continued for 4 hr., and Fig. 1 shows how the respiratory rates fell off in the different media. The ordinates are averages of the figures obtained by expressing the O₂ uptake in a given 60 min. as a percentage of that in the first hour of the same experiment. They have been plotted at the mid-points of the 60 min. periods, and the value of 100% for all initial periods has been plotted at 30 min. Standard deviations of the results in media A and D have been indicated by arrows, and the number of experiments from which each average was calculated is shown in brackets. It will be seen

that in the new medium, containing both glucose and Ca, there was no significant falling off in respiratory rate, for the slight decline in the averages was always less than the standard deviation. When both glucose and Ca were omitted, the respiration fell off 40–50 % in 4 hr., but either glucose or Ca could reduce this decline to about 20 %.

The slices still appeared normal to the naked eye after 4 hr. shaking in any of the media which contained Ca, and slices which had stood in medium C for 3–4 hr. at room temperature, and had then been kept in it overnight in the refrigerator at 4°, also appeared normal. In nine experiments, in which slices stored in this way were suspended in medium A in the Barcroft flasks 24 hr. after they had been prepared, the average Q_{O_2} was 93 % (S.D. 5) of that found for slices from the same kidneys within an hour of the death of the animal. The respiration of these slices fell off about 10 % in 2 hr., which was a smaller decline than that found by Cutting & McCance (1946a) for fresh slices in their phosphate solution without Ca. Slices which had been kept overnight in solutions containing no Ca were found to have lost most of their respiratory activity.

Loss of nitrogen. Table 2 shows the percentages of N which were lost by slices of adult rat kidneys after 4 hr. observation in the Barcroft flasks in

In a number of experiments, each made on slices from the kidneys of one adult rat, the losses of N were determined after different periods in the Barcroft flasks. Six manometers were prepared, gassed together in the bath, and then dismantled one at a time, starting at the end of the period of equilibration, so that the amounts of N in the slices and in the medium could be determined. Fig. 2 shows the

Fig. 2. Adult rat kidney slices in presence of Ca and glucose, at pH 7.4. Respiratory rate in each half-hour period expressed as percentage of O_2 uptake during first hr.; losses of N as percentage of total amount of N originally present. P (arbitrary time scale) represents zero losses when the slices were put into the flasks. Data plotted at Q were obtained by shaking slices by hand for 5 min. at room temperature.

Table 2. Average amounts of nitrogen lost by slices of adult rat kidney after respiring for 4 hr. in various media at pH 7.4

(All losses are expressed as % of the total N originally present.)

Medium	Glucose	Ca	No. of expts.	Losses of N		
				As 'non-protein'.	As 'protein'.	Total
				Mean $\pm$ S.D.	Mean $\pm$ S.D.	
A	+	+	7	8.2 $\pm$ 0.7	17.0 $\pm$ 1.2	25.2
B	+	-	6	10.6 $\pm$ 1.0	28.0 $\pm$ 5.6	38.6
C	-	+	11	9.4 $\pm$ 1.1	23.1 $\pm$ 4.2	32.5
D	-	-	4	10.3 $\pm$ 0.5	28.3 $\pm$ 2.5	38.6

Significance of differences in total N lost

	t	P
A and B	9.2	<0.001
A and C	3.4	<0.01
C and D	3.0	0.01

different media at pH 7.4. It will be seen that although glucose in the absence of Ca had been found to stimulate respiration, and to reduce its decline (Table 1 and Fig. 1), it had no effect upon the loss of N. Ca always reduced the loss of N, but did so more effectively when glucose was supplied in addition, and in this case the falling off in respiratory rate was abolished (Fig. 1).

results of one of these experiments in medium A at pH 7.4. The respiratory rate in each half-hour period is shown as a percentage of the rate during the first hour. (The apparent fall in the seventh period was probably caused by faulty re-equilibration after the manometer fluid had reached the ends of the scales.) The loss of N which had occurred by the end of the equilibration period has been plotted at zero time. The slices had then been in the flasks for about 20 min., and the shaking motor had been running for 10 min. It was assumed that no N had left the slices when they were first put into the flasks, and a point P indicating a zero loss has been plotted to the left of the origin, but not to a strict time scale. The additional data plotted at Q were obtained by shaking some slices by hand for 5 min. at room temperature. As shown in Fig. 2, 12.5 % of the N originally present in the slices was removed in 5 min. shaking, 14 % had been lost before manometer readings began, and a further 11.5 % was lost during the next 4 hr. at a diminishing rate. Meanwhile, the

initial rate of respiration had persisted. Fig. 3 shows for comparison the results of a similar experiment in which the slices were suspended in a phosphate solution actually prepared by Cutting & McCance. This time the pH was 6.8, which reduced the amount of N lost in 4 hr. from 39% for a similar medium at pH 7.4 (Table 2) to 29%. This effect of pH, discovered by Cutting & McCance, made it possible to

Fig. 3. Adult rat kidney slices in absence of Ca and glucose, at pH 6.8. Respiratory rate in each half-hour period expressed as percentage of O_2 uptake during first hr.; losses of N as percentage of total amount of N originally present. P and Q as in Fig. 2.

compare the loss of N in the new medium (A 1) with quantitatively similar losses of N in a medium lacking Ca and glucose. It will be seen that although approximately the same total amount of N was lost in each case, the time relations were quite different. Without Ca or glucose (Fig. 3) there was a loss of 14% before readings began, and another 15% was lost at an increasing rate during the next 4 hr. At the same time the respiratory rate rapidly declined, and the slices had visibly disintegrated.

Kidney slices from newborn rats

These were all studied in the new medium (A 1) at pH 7.4, so that their metabolism could be compared with that of adult tissue in the same medium.

Respiration. The average respiratory rate during the first hour was found to be $106 \mu\text{l./mg. total N/hr.}$ (s.d. 8 in seven experiments). This is very significantly less than the figure of $137 \mu\text{l./mg. total N/hr.}$ given in Table 1 for adult tissue ($t=6.3$;

$P < 0.001$). The rates in the second, third and fourth hours were 96, 94 and 89% of the initial rate, with s.d.'s of 7, 10 and 13 respectively. The scatter and the tendency for the O_2 uptake to decline were both increased by the inclusion of results from slices cut by Cohen's (1945) method.

Loss of nitrogen. The average loss of N after the respiration had been measured for 4 hr. was 20.3% (s.d. 1.4 in seven experiments). This was very significantly lower than the figure of 25.2% given in Table 2 for adult tissue in the same medium ($t=6.4$; $P=0.001$). Furthermore, as some of the slices had been cut by Cohen's (1945) method, this difference was probably underestimated. In an experiment of the kind illustrated in Figs. 2 and 3, slices cut free-hand from the kidneys of one whole litter of newborn rats were found to have lost 12% of their N before readings began, and they lost a further 5% during the next 4 hr. (cf. 14 and 11.5% in Fig. 2).

DISCUSSION

Most of the respiratory rates have been expressed in $\mu\text{l. } O_2/\text{mg. total N/hr.}$ The final dry weight would not have been a suitable basis for comparing the respiration of slices in the different media because they lost different amounts of material. It would have been equally unsatisfactory for comparing the respiratory rate of adult tissue with that of infant tissue, which contains a higher proportion of water. For this purpose the initial moist weight, advocated by Bach (1944), would have been no better, and nitrogen can be determined more precisely. It might appear even better to give an instantaneous rate of respiration per unit of nitrogen remaining at any moment. There is, however, no simple means of determining this, and if it had been done the tissues respiring in medium A would have been credited with an increasing respiratory rate.

Glucose was chosen as substrate because it is generally available in the body, and can serve the cells as a source of glycogen and of three-carbon compounds which are not normally found outside cells in comparable concentrations. A high concentration of lactate or succinate might alter the course of the metabolic reaction chains, and give information about the quantity of one enzyme system in the cells rather than about respiration as a co-ordinated activity. According to Shipley (1944), the amounts of tissue used in these experiments could have produced about 1 mg. of glucose in 4 hr., a quantity comparable with that supplied in the medium (2.7 mg.), so that little effect on the oxygen uptake might have been expected to follow the addition of glucose. It was in fact found that, in the presence of calcium, glucose had no effect upon the initial respiratory rate, but the later decline in respiration and the increased loss of nitrogen which occurred

when glucose was omitted from the medium may have been related to the exhaustion of the glucose and glycogen originally present in the cells, and to the breakdown of proteins for gluconeogenesis. This response of the slices to the presence or absence of glucose is interesting in connexion with the demonstration of Jiménez-Díaz & Souto-Candeira (1948) that the kidneys in the body add glucose to the blood when the blood sugar level is low, but take up glucose and store it as glycogen when the blood sugar is high.

The respiration of the adult kidney slices in the absence of glucose and calcium fell off only about half as much as Cutting & McCance (1946*a*) had found, even when a solution prepared by them was used. However, whereas they had gassed their flasks at room temperature, in this work the gassing was done in the bath at 38°, and a longer time was allowed for equilibration, so that part of the decline must have been over before readings began to be taken.

The loss of nitrogen occurred in two well-defined phases. There was first a rapid loss of about 12–15 % of the nitrogen originally present in the slices. This occurred during the equilibration, and it did not appear to depend upon either the composition of the medium or the age of the tissue. The nitrogen lost was probably contained in blood and cellular debris washed away when shaking began. It is unlikely that this material contributed appreciably to the uptake of oxygen, and the presence of so much inert matter constitutes an objection to calculating the respiratory rates on a basis of total nitrogen. However, it would also have been included if the initial moist weight had been determined, and with a standardized technique it did not seem to vary enough to cause serious irregularities in the respiratory rates based on total nitrogen.

A further, much slower loss of nitrogen occurred during the next 4 hr. while the oxygen uptake was being measured, and in this phase the rate of loss depended on the composition of the medium and the age of the tissue. With adult kidney slices, the loss was least in the new medium containing calcium and glucose, and its rate decreased during the period of observation (Fig. 2). As this loss of nitrogen was not associated with a change in the respiratory rate, the nitrogen cannot have come from a part of the cells concerned with respiration, and the loss may not have been unphysiological. Recent work using metabolites which contain unusual and recognizable isotopes has shown that the apparent permanence of cellular structures is the result of nicely adjusted dynamic equilibria. Thus Shemin & Rittenberg (1944) found that half the nitrogen in the liver proteins of the adult rat was exchanged with nitrogen outside the cells in about a week, and Schoenheimer (1942) gave a table suggesting an even more rapid turnover in the kidney of the same animal. During the fourth hour of the experiment illustrated in

Fig. 2 the slices lost about 1 % of their nitrogen. Had this rate continued without further decrease, the cells would have lost half their nitrogen in about 2 days, which is of the same order as the rate of exchange deduced from work with isotopes. The rate was in fact decreasing, which might be taken to suggest the approach to an equilibrium in which nitrogen could be taken up again from the medium as fast as it was being lost. The cells were in negative nitrogen balance because the medium contained at first no nitrogen for exchange, and the result was a slow loss of nitrogen unassociated with any decrease in the rate of respiration. If such an interpretation is feasible, it is interesting to look upon the slower loss of nitrogen from the infant tissue suspended in the same medium in the light of a suggestion of Rittenberg & Shemin (1947) that young animals grow, not because their cells synthesize protein more rapidly than those of adults, but because their autolytic processes are slower.

In incomplete media which did not contain calcium the second phase of the loss of nitrogen from slices of adult tissue proceeded at an increasing rate (Fig. 3), and at pH 7.4 about twice as much was lost during this phase as was lost when calcium and glucose were present. It seems reasonable to attribute the additional loss of nitrogen that occurred in the absence of calcium to the breaking down of dying cells, for it was associated with a fall in respiration. The escape of protein through damaged cell membranes may also have contributed to it.

Changes in the permeability of the cell membranes may explain the influence of calcium on the initial rates of respiration in the presence and absence of glucose (Table 1). Cutting & McCance (1947) summarized the evidence that calcium tends to stimulate the respiration of the intact cells of some tissues, including rat kidney, but inhibits the oxygen uptake when it is allowed access to the intracellular enzymes by mincing or grinding the tissue. Although the increase in respiration shown in the comparison between media *D* and *C* in Table 1 did not reach the conventional level of statistical significance, similar results were reported by Kisch (1934), among others quoted by Cutting & McCance (1947). If calcium inhibits the enzymes, its action in stimulating respiration must be exerted from outside the cells, and the reduction in the permeability of their membranes would hinder the escape of substrates and coenzymes. It would also hinder the entry of substances from the surrounding medium, and this may explain the observation that calcium prevented any stimulation of the respiration by glucose. Dickens & Greville (1935) have described an analogous inhibition by calcium of the respiration of brain slices in the presence of glucose. The increased respiratory rate which was produced by glucose in the absence of calcium did not persist (Fig. 1), and it

was not accompanied by a reduction in the loss of nitrogen. Glucose did, however, further reduce the nitrogen loss in the presence of calcium. One might say crudely that calcium helped to hold the cells together so that they could use energy derived from the oxidation of glucose to maintain their integrity. For in medium *A* their respiration was stable and the loss of nitrogen was minimal.

The steady respiration of slices of adult rat kidney in the new medium suggests that the falling off which was found by previous workers (and which could be reproduced by omitting glucose or calcium) was a consequence of the employment of incomplete media, and ought not to be accepted as a property of the tissue.

When the new medium was used to compare the metabolism of kidney slices from adult and newborn rats, the lower respiratory rates which Cutting & McCance (1946*a*) could demonstrate only during the first half hour of their experiments were found to persist throughout 4 hr. Cutting & McCance had found that the adult tissues lost more nitrogen than the infant ones, but this might have been due to a more rapid disintegration of the cells. The fact that in the new medium, when their respiration was stabilized and they did not obviously disintegrate, the adult tissues still lost more nitrogen confirms that Cutting & McCance were really dealing with an effect of age. A striking difference in sensitivity to the composition of the environment is also clearly shown by the fact that the adult slices could maintain a constant respiratory rate only in the complete medium, whereas Cutting & McCance had found the infant tissue to be far less exacting. It is interesting that Lasnitzki (1934-5) reported a lower Q_{O_2} , a slower decline in respiration, and a relative insensitivity to the ionic composition of the medium, when kidney slices from embryo rats taken in the last third of pregnancy were compared with the corresponding adult tissue. This 'toughness' in face of unfavourable conditions appears to be rather characteristic of the tissues of newborn animals. It may be an important property of the component cells of an organism which has not yet developed homeostatic mechanisms adequate to maintain that 'fixité du milieu intérieur' which is required to enter upon a free adult existence.

SUMMARY

1. A stable medium buffered with phosphate (3mm.) has been devised for use with slices of surviving kidney tissue. Except for the absence of bicarbonate its composition resembles that of mammalian extracellular fluids.

2. In this medium slices of adult rat kidney maintained a constant respiratory rate for 4 hr., and could be stored for 24 hr. at 4° without serious loss of respiratory activity.

3. Glucose and calcium were both essential for the steady respiration of slices of adult rat kidney. When either of these was absent the respiration fell in 4 hr. to about three-quarters of its initial rate, and when both were absent the respiratory rate fell to about half its initial value in the same time.

4. Slices of adult kidney in the presence of glucose and calcium lost about one eighth of their nitrogen during the equilibration period and a similar quantity during the next 4 hr. In the absence of calcium, whether glucose was present or not, they lost the same amount during equilibration, and about twice as much during the next 4 hr. Glucose without calcium did not affect the loss of nitrogen, though it augmented the nitrogen-sparing effect of calcium.

5. When compared with adult tissue in the new medium containing glucose and calcium, slices of infant rat kidney showed a lower respiratory rate which persisted for 4 hr., and lost a smaller proportion of their nitrogen.

APPENDIX

Some data concerning the solubility of the phosphates of calcium

Mammalian extracellular fluids, according to Gamble (1947), contain about 1mmol./l. of HPO_4^{--} , for at pH 7.4 H_3PO_4 is almost entirely ionized as HPO_4^{--} and H_2PO_4^- in the proportions of 4:1. If the third dissociation constant of phosphoric acid is of the order of 10^{-12} (Sendroy & Hastings, 1926-7) only about 1/100,000 of the H_3PO_4 in a solution at pH 7.4 can be ionized as PO_4^{---} . Holt, La Mer & Chown (1925) estimated that about 7 p.p.m. of the inorganic phosphate of serum are present in this form, so that the concentration of PO_4^{---} is of the order of $10^{-3} \times 7 \times 10^{-6}$, or about 10^{-8} M. Holt *et al.* (1925) found that the stoichiometric solubility product for $\text{Ca}_3(\text{PO}_4)_2$ in a solution having the same salt concentrations as serum was $10^{-27.2}$, and Logan & Taylor (1937) and Kuyper (1945) have also reported values of the order of 10^{-26} (the concentrations being expressed in mol./l.). The medium *A* 1 was made to contain 3×10^{-3} M-P, compared with 1.3×10^{-3} M in serum and extracellular fluids. As its pH was also 7.4, its PO_4^{---} concentration may be presumed to have been about twice that of serum, i.e. about 2×10^{-8} M. This, with 2.5×10^{-3} M- Ca^{++} , would make the ionic product $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{---}]^2 = (2.5 \times 10^{-3})^3 \times (2 \times 10^{-8})^2$, of the order of 10^{-24} , so that, like extracellular fluids, the medium *A* 1 should have been highly supersaturated with respect to $\text{Ca}_3(\text{PO}_4)_2$. This salt does not, however, appear to be readily precipitated from such solutions. Holt *et al.* (1925) and Greenwald (1942) pointed out that it could be formed in one stage only by a reaction of the fifth order, and Bassett (1917) found that $\text{Ca}_3(\text{PO}_4)_2$ could not exist in contact with an aqueous phase at pH 7.4.

As in medium *A* 1 the predominant phosphate ion is HPO_4^{--} , at a concentration of approx. 2.4×10^{-3} M, the ionic product $[\text{Ca}^{++}] \times [\text{HPO}_4^{--}]$ in this medium is $2.5 \times 10^{-3} \times 2.4 \times 10^{-3} = 6 \times 10^{-6}$, which is also the value of the same product in media *A* 2 and *C*. This is about 10^{18} times greater than the ionic product for $\text{Ca}_3(\text{PO}_4)_2$. Shear & Kramer (1928) suggested that CaHPO_4 was the most likely substance to be pre-

cipitated, and that even when such solutions were apparently supersaturated with respect to $\text{Ca}_3(\text{PO}_4)_2$ they might remain stable unless precipitation of CaHPO_4 occurred. This suggests that in practice the amounts of Ca and phosphate which can be included in the same solution may be limited by the (stoichiometric) solubility product of CaHPO_4 , which, according to Shear & Kramer (1928), is of the order of 3×10^{-6} at 38° in a solution of the same ionic strength as the body fluids (the concentrations being expressed in mol./l.). If this figure is accepted, the Ca-containing media, which had an ionic product of 6×10^{-6} , would seem to have been slightly supersaturated with respect to CaHPO_4 , but they were found to be stable. It was, however, found impossible

to incorporate a corresponding amount of CaCl_2 in the solution used by Cutting & McCance (1946*a*) at pH 7.4 without a dense precipitate forming immediately. As their solution contained 10^{-2} mol. P/l., the ionic product would have been of the order of $2.5 \times 10^{-8} \times 8 \times 10^{-8}$, or 2×10^{-5} .

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