XCII. MANOMETRIC STUDIES ON THE EFFECT OF TISSUE EXTRACT UPON CALCIUM PRECIPITATION

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DURING the course of researches on the influence of calcium upon the anaerobic fermentation of tumour tissue [Lasnitzki & Rosenthal, 1935] an interesting side observation was made. The investigations were carried out by Warburg's method [1926], the calcium being added to the medium after the tissue had fermented for a while in potassium- and calcium-free Ringer's solution. It was found that the calcium remained in solution in relatively high concentration, while under the same conditions, but in absence of tissue, it gradually precipitated as carbonate, carbon dioxide being thereby developed. It is only because of this effect of the tissue that it was possible to investigate the influence of higher concentrations of calcium upon fermentation. Soon it was observed that stabilization of calcium also occurred if the tissue, after 1 hour's shaking in potassium- and calcium-free Ringer's solution, was removed from the medium, and the calcium subsequently added.

These findings appeared to be of particular interest, in so far as they suggested that the manometric method may be suitable to study in a simple but accurate manner the inhibition of calcium precipitation under physiological conditions by the action of certain substances, especially those contained in animal tissues. From this point of view further investigations have been carried out in a similar way to test whether the observed effect can be obtained with extracts from normal tissue in different dilutions. Before, however, considering the results of these investigations we have to discuss in some detail the application of the manometric method to our subject.

DEVELOPMENT OF CO₂ AS A MEASURE OF CALCIUM PRECIPITATION

As in the fermentation experiments, the manometer vessel consisted of a cylindrical flask with a side-bulb. 2 ml. of a solution of 0.12 *M* NaCl and 0.0275 *M* NaHCO₃ were pipetted into the main receptacle, 0.2 ml. of a solution of 0.12 *M* NaCl and 0.198 *M* CaCl₂ into the side-bulb. (The side-bulb of the thermobarometer contained instead 0.2 ml. (0.12 + 0.198) *M* NaCl.) After the solutions had been saturated with about 5% CO₂ in nitrogen, the manometers were shaken in the thermostat at 37.5° until temperature and pressure were in equilibrium. Then, after the first reading, the solution in the side-bulb was mixed with that in the main receptacle. The concentration of calcium chloride now amounted to 0.018 *M*² and that of the bicarbonate to 0.025 *M* (*pH* ~ 7.47). The shaking

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² This concentration of CaCl₂ is ten times as great as that in a normal Ringer's solution used for metabolism experiments.

of the manometers was then continued, and the readings repeated from time to time ($K_{\text{CO}_2} \sim 1.5$).

Fig. 1 shows a typical example of the course of the CO_2 development after the addition of CaCl_2 (mean values of two parallel estimations). It will be seen

Fig. 1. CO_2 development in a solution containing 0.018 M CaCl_2 and 0.025 M NaHCO_3 , and saturated with about 5% CO_2 in nitrogen, at 37.5° .

that the development is at first slow, and then proceeds with greater rapidity.¹ The series of reactions taking place may be expressed as follows:

For each calcium ion bound one molecule of CO_2 is thus set free. 0.1 mg. of precipitated calcium corresponds therefore to 56 $\mu\text{l.}$ of CO_2 (0° , 760 mm. Hg).

In this pH range, the concentration of the CO_3 ions is not nil but, nevertheless, is so slight that in fermentation measurements it can be ignored. It can be brought down practically to nil if the reaction medium is saturated with CO_2 . Then, as is to be expected, there is no notable CO_2 development after the addition of CaCl_2 . On the other hand, if the reaction medium is saturated with pure nitrogen the concentration of the CO_3 ions increases markedly. Thus in this case the rapidity of the CO_2 development is very great at first, but gradually becomes less, apparently as a result of the lowering of the calcium and bicarbonate concentrations. Such a diminution should also be apparent in the case of Fig. 1 after a more lengthy observation time. The question arises, however, as to why the maximum speed of the CO_2 development is only gradually reached here. The explanation may be found by considering that the precipitation of the calcium carbonate takes place in two stages: firstly the formation of very small crystal-germs² and secondly the development of these germs into larger crystals. The last process represents a surface reaction the speed of which must naturally rise with the increase of the total crystal surface, and therefore the amount and size

¹ The quantity of CO_2 liberated in this case may be considered as typical. But it sometimes happens that the CO_2 development is weaker or even practically absent within the given (short) period of observation. This is probably due to presence of a minimum amount of organic impurities.

² That part of the CO_2 development which is due to the formation of crystal-germs must be extremely small and can, therefore, be neglected.

of crystals already formed. The lessening of the calcium and bicarbonate concentrations acts in an opposite direction, and the balance finally leads to a lowering of the rate of CO_2 development. On saturating with pure nitrogen this stage is apparently already reached during the first time interval.

In each case the process must come to an end after a certain time. Then the bicarbonate must be reduced to a small amount, and the residue of the free Ca ions, being in equilibrium with this amount of bicarbonate and the amount of CO_2 present, should approximately agree with the formula of Rona & Takahashi [1913].

THE EFFECT OF TISSUE EXTRACT

The liver of the rat was chiefly used for these investigations, but in one experiment rat's kidney was also used. The fresh organs were, as far as possible, rendered free from blood and finely minced. 1 g. of the mince was suspended in 45.5 ml. of 0.9% NaCl solution, the whole shaken for 10 min. and then filtered. As desired, further dilutions (1 to 10, 1 to 100, 1 to 1000) with 0.9% NaCl solution were prepared from the filtrate. 1 ml. of the original or diluted extract was pipetted into the main receptacle of the flasks, and 1 ml. of solution containing 0.55 *M* NaHCO_3 and sufficient NaCl to make a concentration in the mixture of 0.12 *M* was added. As control, 1 ml. of 0.9% NaCl was used

Fig. 2. CO_2 development under conditions as given with Fig. 1. *a*, with liver extract 1:10³; *b*, with liver extract 1:10³; *c*, control.

instead of the extract. The side-bulb again contained 0.2 ml. of a solution of 0.12 *M* NaCl and 0.198 *M* CaCl_2 . The thermobarometer was prepared as above. After the solutions had been saturated with about 5% CO_2 in nitrogen, the procedure was as above. When the solution in the side-bulb was mixed with that in the main receptacle the concentration of calcium chloride became again

0.018 *M* and that of the bicarbonate again 0.025 *M*. In the final dilution the ratio (amount of extracted tissue in g.) : (volume of fluid in ml.) was for the original extract 1 : 10², and for the preliminary dilutions 1 : 10³, 1 : 10⁴ and 1 : 10⁵.

Fig. 2 illustrates the result of a typical experiment with liver tissue. While the control shows a normal course of CO₂ development, we find in both cases where liver extract was used that there was also, at first, development of a small quantity of CO₂, but that CO₂ development soon ceased. The results in the two parallel tests with the extracts 1 : 10² and 1 : 10⁴ were similar to those in the two tests here considered with the extracts 1 : 10³ and 1 : 10⁵. The experiment with kidney, carried out in the same way, gave similar findings, Fig. 3 showing the

Fig. 3. CO₂ development under conditions as given with Fig. 1. *a*, with kidney extract 1 : 10³; *b*, with kidney extract 1 : 10⁵; *c*, control.

results obtained with the two extracts 1 : 10³ and 1 : 10⁵. Thus we see that a tissue extract, even in fairly high dilution, is capable of preventing calcium precipitation under the given conditions. This is at least true when the observation time is short. Needless to say, at the end of the experiments CaCO₃ crystals could only be detected in the control vessels.

The small quantity of CO_2 liberated from the solution after the addition of calcium may be due, not to precipitation of calcium carbonate, but to a slight acidification resulting from the very weak hydrolysis of calcium chloride: it would consequently follow that the value of the control for the same interval would depend only to some extent on the precipitation of calcium carbonate. In support of this interpretation is the fact that, in the tests with extract, the observed rise in pressure apparently reached its maximum at the beginning of the interval.

In accordance with other well-known phenomena of a similar nature the most likely explanation of the inhibitory effect appears to be that it is due to an adsorption of certain colloidal constituents of the extract (probably chiefly proteins) at the surface of the crystal-germs of CaCO_3 whereby the development of these germs into larger crystals, that is to say the fixation of further Ca and CO_3 ions, is prevented. Thus the Ca ions must be held in solution in a state of supersaturation. This will probably last until the quantity of crystal-germs has increased so that the amount of organic colloids becomes insufficient, when calcium precipitation must take place. The higher the dilution of the extract and the greater the concentrations of the Ca and CO_3 ions the shorter the time elapsing. As far as CO_3 ions in particular are concerned it was found that when the reaction medium was saturated with pure nitrogen, and the CO_3 ion concentration accordingly raised, the (more dilute) extract was entirely unable to inhibit calcium precipitation.

A marked inhibitory effect was further obtained in an experiment with human serum, carried out in the same way as those described above. In the tests with serum, the final dilution of which was 1:100, there was less than 1 μl . CO_2 developed after 3 hours, while in the two control tests there was an average of about 270 μl ., the initial CO_2 development being ignored in both cases. Thus it appears that tissue extract and highly diluted serum act in a similar manner.

SUMMARY

In a solution containing 0.018 M CaCl_2 as well as bicarbonate and CO_2 in physiological concentrations, and kept at body temperature, a precipitation of calcium carbonate takes place, the amount of which can be measured by the amount of CO_2 developed, estimated manometrically. It is shown, by means of this method, that extracts of liver and kidney tissue are capable of preventing calcium precipitation, at least for a short time. Fairly high dilutions of the extracts retain this power. Highly diluted serum appears to behave similarly.

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