Metabolic Alterations in Tissues Perfused with Decalcifying Agents

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It has long been known that calcium plays an important role in the adhesiveness of normal metazoan cells. By the use of calcium-free incubation media, it has been possible to obtain isolated viable cells from a variety of plant and embryonic animal tissues (Rinaldini, 1958). Anderson (1953) described a method for obtaining isolated cells from adult rat livers in high yield and apparently intact morphologically. The method consisted essentially of perfusion of the liver with a calcium-sequestering agent (citrate or EDTA) followed by gentle mechanical separation of the cells. Metabolic studies of such cells, however, have shown them to differ markedly from tissue slices in various respects (Laws & Stickland, 1956; Lata & Reinertson, 1957; Kalant & Young, 1957; Branster & Morton, 1957; Zimmerman, Devlin & Pruss, 1960), thus throwing doubt on their functional, if not their morphological, integrity. In contrast, Ehrlich ascites-tumour cells exposed to the same decalcifying agent showed no alteration of succinoxidase activity (Kalant & Young, 1957), although Wu (1959) reported that EDTA increased the leakage of glycolytic enzymes from mouse ascites-tumour cells. Because of these findings, and the reports (Coman, 1944; Lansing, Rosenthal & Kamen, 1948; DeLong, Coman & Zeidman, 1950) on the differences between normal and malignant tissue with respect to calcium-binding, it seemed of interest to explore further the connexion between calcium and normal tissue structure and metabolic patterns.

The work reported here indicates that sequestration of calcium from normal adult mammalian tissues with citrate or EDTA results in a gross alteration of cell-wall permeability which in turn causes the observed changes in metabolic behaviour in vitro. Evidence has been presented by Leeson & Kalant (1961) that this treatment of the tissue does not result in mechanical breakage of the cell membranes.

METHODS

Perfusion of liver. Decalcified liver and kidney tissues were prepared by a modification of the perfusion technique of Anderson (1953), by using warmed perfusing fluids introduced via the portal vein (Branster & Morton, 1957). This modification resulted in easier and more complete perfusion than the original method.

In the present work, various modifications were tried, some of which are described in the Results section, but the final procedure was as follows. Rats of about 300 g. were anaesthetized with intraperitoneal pentobarbital sodium (0.5 mg./100 g. body wt.), and in each case the abdominal cavity was opened and the liver and portal vein were exposed. A polyethylene cannula filled with 0.4% sodium chloride containing heparin was inserted into the portal vein, and a ligature, previously placed loosely around the vessels of the porta hepatis, was then tightened about the cannula, thus blocking the flow of mesenteric and pancreatic venous blood and hepatic arterial blood into the liver. Until the moment of ligation, hepatic arterial flow was uninterrupted and the liver did not become anoxic. Perfusion via the cannula was started immediately, and the inferior vena cava was nicked below the liver to permit drainage. The thorax was opened, and the vena cava clamped just above the diaphragm. The perfusion fluid, previously saturated with oxygen, was passed from an elevated reservoir through a glass coil immersed in a water bath maintained at 39-40°, and then through a polyethylene screw-valve into the perfusion cannula. By the use of a 75 cm. head of perfusing fluid above the polyethylene valve, and a suitable setting of the valve, the flow rate was controlled at approximately 6 ml./min. This flow was too slow to distend the liver by itself. However, digital pressure on the abdominal vena cava dammed back the perfusion fluid and produced slow distention, which could be employed intermittently to flush the liver out thoroughly without injuring it. Manometric measurement indicated that the hydrostatic pressure within the liver during such distension was not more than 15 cm. water.

Immediately on completion of the perfusion, the liver was removed and placed in a small volume of ice-cold Krebs-Ringer phosphate solution (Krebs & Henseleit, 1932). When chilled, it was used for the preparation of slices or isolated cells.

Perfusion of kidneys. In the experiments on perfused kidneys, the procedure was as described above, except that the perfusion cannula was inserted into the lower part of the abdominal aorta, directed cephalad. The aorta was ligated around the cannula below the point of origin of the left renal artery, and was clamped above the origin of the right renal. Perfusion was commenced immediately, the flow being almost exclusively through the two renal arteries and kidneys into the vena cava, which was nicked to permit drainage.

Tissue slices. These were cut by means of a Stadie-Riggs tissue slicer from blocks of tissue kept in ice-cold Krebs-
Ringer phosphate medium until cut, and the slices were kept similarly chilled until used.

Preparation of isolated cells. The method employed was only slightly modified from that of Anderson (1953). The chilled perfused tissue was quickly cut into pieces about 1-2 mm.² with scissors, and transferred to a glass homogenizer tube with approximately 40 ml. of cold oxygenated perfusion medium. A loosely fitting Perspex pestle (0.5 mm. clearance) was worked slowly up and down by hand about fifteen times until all the tissue appeared to have been dispersed. The suspension was decanted through a triple thickness of surgical gauze, which retained unbroken fragments of tissue, fibrous strands etc., the cell suspension passing through into a silicone-treated Pyrex round-bottomed centrifuge tube surrounded by crushed ice. When necessary, preliminary centrifuging at 50g for 1 min. brought down remaining fragments of tissue, or large clumps of cells, from which the cell suspension could be separated by decantation into another tube. Centrifuging of free cells was carried out in a refrigerated centrifuge at 50g for about 4 min., as recommended by Longmuir & ap Rees (1956). This sedimented the liver or other tissue cells without packing them too tightly and left the red blood cells and cellular debris in suspension. After the centrifuging, the supernatant, containing red blood cells, nuclei and other subcellular fragments, was removed by suction with a fine-tipped bulb syringe. The cells were resuspended by gentle swirling with a quantity of the medium to be used for the final suspension. The precipitation and resuspension were repeated twice, giving a very clean suspension consisting essentially of single cells with some clumps of two or three cells.

Kaltenbach’s (1954) method was also tested, but the modification of Anderson’s (1953) method outlined above was employed in the experiments reported here as it proved superior in our hands.

Respiration studies. Tissue samples were incubated at 38° in 20 ml. Warburg flasks. The media, volumes, gas phases and times of incubation were varied in the different experiments as explained in the Results section. In experiments with tissue slices, the slices were removed from the Warburg flasks at the end of incubation and transferred immediately to micro-Kjeldahl digestion flasks for nitrogen determination. In experiments with cell suspensions, triplicate samples of the suspension were digested, and the mean nitrogen content was used. Oxygen uptakes are in most cases expressed as µl. of O₂/mg. of N/10 min.

Preparation of mitochondrial suspensions. In some experiments, mitochondria were separated from the isolated liver cells as follows. The cells were suspended in 0.25 M sucrose solution and broken by means of a Potter-Elvehjem type of tissue grinder, fitted with a Teflon pestle with a clearance of 0.006 mm. (A. H. Thomas Co., Philadelphia, Pa., U.S.A.). Unbroken cells were removed by centrifuging at 50g for 5 min. at 0°, and retained for nitrogen measurements. The supernatant suspension was transferred to a Spinco model L refrigerated ultracentrifuge, and the mitochondria were isolated and washed as described by Green, Loomis & Auerbach (1948).

From the nitrogen contents of the various fractions a proportionality factor was obtained permitting comparison of the respiration of the cell suspensions with that of the isolated mitochondria. For example, in one experiment 25-0 ml. of a cell suspension, with a nitrogen content of 2-18 mg./ml., was used for the preparation of mitochondria. The residue of unbroken cells after homogeniza-

RESULTS

Comparison of isolated cells with tissue slices. To verify and extend the findings reported by Kalant & Young (1957), a comparison was made of the oxygen uptakes by isolated cell preparations and by slices from untreated tissues ('intact slices') in the presence of various substrates. Both liver and kidney preparations were employed, from rats and rabbits. Isolated cells were prepared from livers and kidneys which had been perfused with an oxygenated ice-cold mixture of 9 vol. of Krebs-Ringer phosphate solution and 1 vol. of 5% (w/v) sodium citrate or EDTA solution. Krebs-Ringer phosphate solution was also used as the suspension medium for the cells, and the incubation medium for both cells and slices. Under these conditions, it was readily confirmed that rat- and rabbit-liver cells showed an almost complete absence of endogenous respiration, and a much higher oxygen uptake in the presence of succinate than that shown by intact liver slices (Figs. 1b and 1c).
endogenous respiration of kidney-cell suspensions was much lower than that of intact kidney-cortex slices, but still appreciable. On the addition of succinate, the cell suspensions took up oxygen at approximately the same rate as the slices (Fig. 1a).

Intact rat-liver slices incubated in Krebs–Ringer phosphate medium showed a small but consistently reproducible increase in the rate of oxygen uptake on addition of 0.03 M α-oxoglutarate, whereas isolated liver cells in the same medium showed only a slight oxygen uptake even when the α-oxoglutarate was present from the beginning of the incubation (Fig. 2). Essentially the same results were obtained when the added substrate was pyruvate. Isolated rabbit-liver cells behaved in the same way as the rat-liver cells towards these two substrates.

In addition, rabbit-liver-cell suspensions showed no tryptophan-peroxidase activity as measured by the method of Knox & Auerbach (1955), and no induction of this activity on incubation with tryptophan in CMRL-1066 tissue-culture medium (Parker, 1961). In preliminary experiments with the same medium, induction of this enzyme in untreated liver slices was readily demonstrable by the method of Civen & Knox (1959). A single attempt to grow a rat-liver-cell suspension in tissue culture, in roller flasks containing CMRL-1066 medium, also failed.

Similar marked alterations of metabolic behaviour in vitro were obtained when various modifications were made in the perfusion procedure, in the method for preparing cell suspensions and in the incubation medium. Modifications of the technique included: the use of warmed (38°) perfusion fluid; the addition of colloids, namely 1% of casein, 1% of bovine serum albumin, or 3.5% of polyvinylpyrrolidone; the addition of lysed red blood cells; and variation of the perfusion volume and pressure. Various methods of preparing cell suspensions were tested and included: alternate suction and expulsion through a large-bore pipette (Dulbecco & Vogt, 1954); extrusion from a Perspex tissue press fitted with graded sieves (Lata & Reinertson, 1957); incubation of liver slices for 15 min. in a shaker bath at 38° in the oxygenated medium containing citrate, EDTA, or 0.01% of trypsin, with subsequent harvesting of the cells that had been shaken free from the slices. Modifications of the incubation medium included: the use of phosphate, tris and bicarbonate buffers, both iso-osmotic and of twice normal osmolality; and the addition of haemoglobin, polyvinylpyrrolidone and bovine serum albumin. None of these changes made any detectable difference to the respiration of the cell suspensions, except that the use of phosphate buffer of twice normal osmolality caused a decrease in the rate of oxygen uptake in the presence of added succinate, of the same order as that reported by Johnson & Lardy (1958) with isolated mitochondria in hyperosmotic sucrose. However, it was found that the use of a warmed perfusing solution permitted much easier and more uniform perfusion, and that the addition of albumin or polyvinylpyrrolidone during the preparation of the cell suspensions resulted in a larger yield of cells, presumably by decreasing mechanical breakage.

Respiration of liver cells in high-potassium medium. Since rat-liver-cell suspensions in Krebs–Ringer phosphate medium failed to oxidize pyruvate or α-oxoglutarate, the high-potassium medium used by Green et al. (1948) for studies with mitochondrial-cyclophorase preparations was tried. When incubated in this medium, with 0.05% of added ADP, the liver-cell suspensions showed a vigorous oxygen uptake in the presence of pyruvate (Fig. 3) and of α-oxoglutarate (Fig. 2). However, the oxygen uptake in the presence of succinate in this medium was somewhat lower than that observed in Krebs–Ringer phosphate medium, though the rates of carbon dioxide release anaerobically in the presence and absence of added fructose, used as indices of the rates of glycolysis, were approximately the same as those observed (Kalant & Young, 1957) in Krebs–Ringer phosphate medium.

Mitochondrial respiration in isolated cell suspensions. An attempt was made to determine whether the process of isolating liver cells resulted in any damage to the mitochondria. For this purpose, rat-liver-cell suspensions were made, and mitochondria isolated from them (see the Methods section), and compared with mitochondrial pre-
parisons from normal untreated rat livers. When incubated in the medium of Green et al. (1948), in the presence of α-oxoglutarate and ADP, the liver cells and their corresponding mitochondrial suspensions showed identical initial rates of oxygen uptake, of the same magnitude as those of 'normal' mitochondria, but the rate of uptake by the cell suspensions began to decrease rapidly after 30–40 min., whereas that of the mitochondrial preparations continued relatively unchanged (Fig. 4).

Oxidative phosphorylation by rat-liver-cell suspensions was investigated indirectly by observing the enhancement of respiration on addition of 5 mM-2,4-dinitrophenol. Isolated mitochondria from intact normal liver showed a prompt effect of 2,4-dinitrophenol on the rate of oxygen uptake with α-oxoglutarate as substrate; within 5 min. of the addition of 2,4-dinitrophenol, the rate of oxygen uptake rose to 218 % of its previous value. With intact liver slices oxidizing the same substrate the 2,4-dinitrophenol effect was considerably less marked, the rate of oxygen uptake increasing to 159 % of the control rate. With liver-cell suspensions incubated in the medium of Green et al. (1948) containing α-oxoglutarate (0.03 M) and added ADP, the addition of 2,4-dinitrophenol after 20 min. of incubation increased the rates of oxygen uptake to 220, 223 and 251 % of the control values in three separate experiments, but the elevated rates were maintained for only 10–30 min., whereas with the liver slices they were maintained for at least 1 hr. When the 2,4-dinitrophenol was added to a liver-cell suspension after 40 min. of incubation, it increased the rate of oxygen uptake by only 0–30 %. These results suggest that the mitochondria within the isolated liver cells are intact at least initially, as shown by a response to 2,4-dinitrophenol which is indistinguishable from that shown by washed mitochondrial suspensions prepared from intact liver. Further, the 2,4-dinitrophenol appears to have a greater effect on the mitochondria within the isolated cells than on those within the intact slices, and to damage them more rapidly, as indicated by an early cessation of respiration after the addition of 2,4-dinitrophenol.

Comparison of intact slices with slices from perfused livers. As described by Kalant & Young (1957), slices cut from livers which had been perfused with Krebs–Ringer phosphate solution containing citrate showed altered respiration in vitro resembling that of the isolated cells. This was confirmed repeatedly, and the extent of alteration of respiratory pattern was roughly proportional to the thoroughness of perfusion of the liver. In order

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**Fig. 4.** Oxygen uptake of isolated rat-liver cells, and of mitochondria from such cells, in the presence of 0.03 M-α-oxoglutarate. ○, Isolated liver cells. ○, Solid line, mitochondria; broken line, pooled washings from the preparation of mitochondria. △, Mitochondria from normal untreated liver. The incubation medium was that used by Green et al. (1948) for the mitochondrial cyclophorase system, and contained added ADP (0.05%). Closely similar results were obtained in two other experiments, except that the falling-off of respiration with cell suspensions occurred at different times.
to rule out the possibility that this altered respiratory activity might result from mechanical damage during perfusion, and to explore further the effects of decalcification, the following experiments were undertaken. Slices were cut from intact livers, and from livers which had been carefully and uniformly perfused with Krebs–Ringer phosphate medium alone, or with Krebs–Ringer phosphate containing (a) citrate, (b) citrate plus 3.5% (w/v) of polyvinylpyrrolidone, (c) EDTA, or (d) EDTA plus 3.5% (w/v) of polyvinylpyrrolidone. The slices were washed in Krebs–Ringer phosphate medium to remove the perfusing fluid and incubated in the same medium. Their oxygen uptakes were measured during control periods without added substrate, and also after addition of 0.2 ml. of either 0.3 M sodium succinate or Krebs–Ringer phosphate solution. The mean oxygen-uptake curves for the various groups are shown in Fig. 5. At the end of incubation nitrogen measurements were made on each slice, and on the medium in which it had been incubated. The results are summarized in Table 1.

Endogenous respiration was essentially the same in all groups, and was unrelated to the size of the slices as indicated by their nitrogen content. This indicated that perfusion had not caused loss of endogenous substrates, or damage to the respiratory mechanisms. Regardless of the type of treatment used, the oxygen uptake in the control flasks continued practically unchanged after the addition of Krebs–Ringer phosphate solution from the side arm, indicating that the endogenous respiration was essentially constant throughout the period of incubation.

The increase in oxygen uptake on addition of succinate ("succinate effect") was essentially the same in the slices from untreated livers and those from livers perfused with Krebs–Ringer phosphate solution (Table 1). In comparison with both the untreated and perfused control groups, there was a highly significant increase in the succinate effect in slices from the EDTA- and citrate-perfused livers.

![Oxygen uptake curves](image)

**Fig. 5.** Oxygen uptake of rat-liver slices in Krebs–Ringer phosphate medium. (a) ●, Untreated control livers; ○, livers perfused with Krebs–Ringer phosphate solution. (b) ●, Livers perfused with Krebs–Ringer phosphate + EDTA; ○, livers perfused with Krebs–Ringer phosphate + EDTA + 3.5% of polyvinylpyrrolidone. (c) ●, Livers perfused with Krebs–Ringer phosphate + citrate; ○, livers perfused with Krebs–Ringer phosphate + citrate + 3.5% of polyvinylpyrrolidone. Broken lines, endogenous respiration; solid lines, respiration in the presence of succinate (0.03 M) added at 40 min. (arrow).

**Table 1.** Effects of various treatments on the respiration and on the protein loss by rat-liver slices.

<table>
<thead>
<tr>
<th>Perfusion fluid</th>
<th>No. of slices (no. of rate in parentheses)</th>
<th>Endogenous O₂ uptake (µl/10 min./mg. of N)</th>
<th>Succinate effect (µl of O₂/10 min./mg. of N) (Y)</th>
<th>Nitrogen loss into medium (%) (X)</th>
<th>Coeff. of correlation of X and log Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (intact untreated slices)</td>
<td>35 (7)</td>
<td>13.2 ± 0.31</td>
<td>18.7 ± 1.20</td>
<td>26.4 ± 1.0</td>
<td>0.70</td>
</tr>
<tr>
<td>Krebs–Ringer phosphate</td>
<td>30 (5)</td>
<td>12.9 ± 0.57</td>
<td>20.8 ± 1.63</td>
<td>26.8 ± 1.0</td>
<td>0.72</td>
</tr>
<tr>
<td>Krebs–Ringer phosphate + EDTA</td>
<td>31 (5)</td>
<td>12.2 ± 0.46</td>
<td>32.6 ± 2.88</td>
<td>28.4 ± 0.9</td>
<td>0.80</td>
</tr>
<tr>
<td>Krebs–Ringer phosphate + EDTA + polyvinylpyrrolidone</td>
<td>30 (5)</td>
<td>12.4 ± 0.56</td>
<td>35.1 ± 4.50</td>
<td>38.7 ± 2.2</td>
<td>0.83</td>
</tr>
<tr>
<td>Krebs–Ringer phosphate + citrate</td>
<td>28 (5)</td>
<td>13.0 ± 0.75</td>
<td>54.8 ± 7.07</td>
<td>41.0 ± 1.7</td>
<td>0.97</td>
</tr>
<tr>
<td>Krebs–Ringer phosphate + citrate + polyvinylpyrrolidone</td>
<td>34 (6)</td>
<td>14.0 ± 0.79</td>
<td>40.4 ± 2.63</td>
<td>41.8 ± 2.1</td>
<td>0.96</td>
</tr>
</tbody>
</table>
(P < 0.001 in all cases). The addition of polyvinylpyrrolidone to the perfusing solution appeared to diminish the succinate effect in the latter two groups, although the statistical significance of this decrease was questionable (citrate compared with citrate plus polyvinylpyrrolidone, P > 0.02, < 0.05; EDTA compared with EDTA plus polyvinylpyrrolidone, P > 0.05). These findings suggested that perfusion with EDTA or citrate, though not affecting the endogenous respiration, either removed some inhibition of the succinate effect or permitted easier access of the succinate to the mitochondria.

Nitrogen loss during incubation was virtually identical in the slices from intact livers and from those perfused with Krebs-Ringer phosphate solution (Table 1). Slices from EDTA-perfused livers showed almost the same mean percentage nitrogen loss as those of both control groups, but the remaining three groups all showed significantly larger nitrogen losses (P < 0.001 in all cases). In both control groups the percentage of nitrogen loss was independent of the total nitrogen content of the sample (i.e. slice plus medium after incubation). Among the EDTA- and citrate-perfused slices, however, the nitrogen leakage tended to be proportionally somewhat greater from the smaller slices, i.e. those with lower total nitrogen. The magnitude of the nitrogen loss bore no relationship to that of the endogenous respiration in any of the groups, confirming that the loss of protein from the cells was not initially accompanied by any recognizable loss of endogenous substrate or damage to the oxidative systems.

Within each experimental group the log of the succinate effect was found to be positively correlated with the nitrogen loss. Coefficients of correlation (Table 1) were all significant at considerably better than the 1% probability level. Comparison of the EDTA group with the EDTA plus polyvinylpyrrolidone group, and of the citrate group with the citrate plus polyvinylpyrrolidone group, suggests some type of 'protective' effect of polyvinylpyrrolidone, since the increment in succinate effect for a given increment in nitrogen loss was smaller when polyvinylpyrrolidone was employed.

Comparison of slices from intact and perfused kidneys. No detailed comparison of the succinate effect and the nitrogen loss was undertaken with kidney slices. However, the endogenous respiration and succinate effect were studied in slices either from intact rat kidneys or from those perfused with Krebs-Ringer phosphate alone or with Krebs-Ringer phosphate containing either citrate or EDTA, and from intact and EDTA-perfused rabbit kidneys. The slices were cut from kidney cortex only. The results are shown in Fig. 6. Quite unexpectedly, the slices from citrate- and EDTA-perfused rat kidneys showed almost the same rate of oxygen uptake and succinate effect as did the control slices, whereas the EDTA-perfused rabbit-kidney slices had somewhat lower uptakes than the controls, though this difference was not statistically significant.

**DISCUSSION**

As outlined in the introduction, there is ample evidence that the metabolic behaviour of isolated cell suspensions prepared from adult rat liver, whether by the methods of Anderson (1953), of Kaltenbach (1954), of Longmuir & ap Rees (1956), or by modifications of these (Lata & Reinertson, 1957; Branster & Morton, 1957), is abnormal compared with that of untreated tissue slices. It is clear from the present work that the same is true also of cell suspensions prepared from adult rabbit liver, and Branster & Morton (1957) have shown that it is true of livers from other species.

Kalent & Young (1957) suggested that isolated rat-kidney cells also showed a greatly increased succinate effect, but made no direct comparison with kidney slices. In the present study such a comparison was made, and the results confirmed the previous suggestion. The endogenous respiration of isolated kidney cells was much lower than that of kidney-cortex slices (Fig. 1a), but oxygen uptake in the presence of succinate was virtually the same as that of the slices. The effect of succinate on respiration was therefore considerably greater for the isolated kidney cells than for the slices. Thus,
even though the difference was not as striking as that seen with liver cells, in which the oxygen uptake in the presence of succinate actually exceeded that of liver slices, the results with kidney preparations are qualitatively similar. It is reasonable to suppose that other tissues would show similar changes with this treatment.

The abnormalities or disturbances of activity *in vitro* shown by preparations of tissues perfused with EDTA or citrate solutions are numerous. The metabolic functions affected indicate disturbances of most or all of the cellular organelles. Thus the failure of oxidation of α-oxoglutarate and pyruvate suggests some disturbance of mitochondrial function. The loss of tryptophan-peroxidase activity suggests impairment of microsomal function. The sharp decrease of glycolysis indicates a disturbance of enzyme systems contained in the cytoplasmic fraction. These disturbances, however, may result not from damage to the organelles or enzymes themselves but from loss of electrolytes or other necessary cofactors from the intracellular milieu, as suggested by Branster & Morton (1957). Electron-microscopic evidence presented by Leeson & Kalant (1961) indicates that the organelles in such tissue are not grossly damaged morphologically. The present work shows that the mitochondria from isolated liver cells oxidize pyruvate and α-oxoglutarate normally when placed in a high-potassium medium with added ADP, suggesting that the impaired function of the corresponding enzymes in the cell suspensions was due to a loss of ADP and potassium or to an excess of intracellular sodium in these preparations. Conversely, the much greater stimulation of oxygen uptake by succinate observed with these preparations, and with slices from perfused organs, than with 'intact' slices suggests that the succinate has readier access to the mitochondria in the perfused tissue. Similarly, the more rapid and more marked effect of 2,4-dinitrophenol on liver-cell suspensions than on intact liver slices suggests easier access of 2,4-dinitrophenol to the mitochondria of the isolated cells.

One attempt to grow isolated rat-liver cells in tissue culture, with a normal extracellular type of medium with a high sodium and a low potassium content, failed. Garvey (1961) has described the successful growth of liver cells, prepared by raking incubated rat liver with a wire gauze, in a most unusual tissue-culture medium containing 0.05 M sodium chloride and 0.20 M potassium chloride. Though she does not comment on the ionic composition of this medium, her success with a high-potassium medium and our failure with a low-potassium medium are compatible with the findings described above, suggesting that isolated liver cells are unable to retain the normal high concentrations of intracellular potassium.

Finally, the loss of protein into the incubation medium was greater from citrate- or EDTA-treated liver slices than from the control slices, again indicating abnormally high permeability of the cell membranes. There is inevitably some cellular damage in tissue slices, even from normal untreated tissues, especially if the Stadie–Riggs microtome is used (McIlwain, 1961). Therefore it is understandable that the slices from untreated livers and from livers perfused with Krebs–Ringer phosphate solution showed an appreciable protein loss, and that a higher protein loss was generally accompanied by a greater succinate effect. The fact that the magnitude of protein loss was independent of slice size (total nitrogen) in these two groups suggests that it occurred mainly from damaged cells at the surfaces of the slices. The effect of perfusion with citrate or with EDTA, however, was to increase the nitrogen loss from slices of a given total nitrogen content, and to make this loss proportionally even greater from smaller slices. If the action of EDTA and citrate results in increased permeability of all the cells, including the deeper ones within the slices, these findings would be expected. Since the tissue blocks from which slices were cut were kept to a fairly uniform size, a difference in total nitrogen of the slices reflected a difference in thickness rather than in area. The proportionately higher losses from thinner slices might be attributable to the lesser resistance to diffusion out of the deeper cells.

Henley, Sorensen & Pollard (1959) and Henley, Wiggins, Pollard & Dullaert (1958, 1959) have examined the protein lost into the surrounding medium by cell suspensions prepared from decalcified liver, and have shown that it consists largely of enzymes from the soluble portion of the cytoplasm, including most of the lactate dehydrogenase, glucose 6-phosphate dehydrogenase and glutamate–pyruvate transaminase. Zimmerman et al. (1960) have found that cell suspensions prepared from citrate-perfused liver and kidney lose almost all their glycerophosphate dehydrogenase, aldolase and lactate dehydrogenase into the surrounding medium. These findings readily explain the observed decrease in anaerobic glycolysis, and are fully compatible with the inferences drawn above.

In electron-microscopic studies, we have encountered considerable technical difficulty in the osmic acid fixation of isolated liver- and kidney-cell suspensions, so that it cannot yet be stated with certainty that such cell suspensions are morphologically undamaged, as had been reported previously. However, repeated electron-microscopic examinations of livers perfused with Krebs–Ringer phosphate medium with and without added EDTA (Leeson & Kalant, 1961) have shown clearly that EDTA does not cause any detectable structural alteration other than separation of adjacent
liver cells from each other. Moreover, the process of perfusion does not, of itself, damage the tissue appreciably, as shown by the fact that slices from untreated livers and from livers perfused with Krebs–Ringer phosphate in the present study showed identical values for oxygen uptake and protein leakage. It is therefore clear that the differences demonstrated between slices from livers perfused with EDTA and with Krebs–Ringer phosphate solution can be explained as either direct or indirect consequences of a functional alteration in the cell membranes caused by EDTA, and not as a consequence of mechanical breakage.

The failure of perfusion of the kidney with EDTA or citrate to increase the succinate effect on respiration of kidney-cortex slices is rather puzzling, since the expected effect was found with isolated kidney-cell suspensions. The explanation is perhaps to be found in the differences in histological organization between kidney and liver. Liver cells are separated from the blood only by thin layers of sinusoidal endothelium, through which the EDTA has ready access to the cells. Distension of the liver during perfusion permits gradual separation of the adjacent parenchymal cells from each other so that the EDTA can gradually reach the whole cell interface. In the kidney, distension is prevented by the tough renal capsule and by the prominent basement membrane surrounding each renal tubule. In addition, the peritubular capillaries are separated from the tubular epithelium by the basement membrane. These factors may prevent the EDTA from effectively removing calcium or other bivalent cations from the cell surfaces during perfusion. Only when the kidney is cut into small pieces and mechanically disrupted in the hand homogenizer, as in the preparation of isolated cells, is it certain that the cell surfaces are fully exposed to the action of EDTA.

It is not yet possible to offer any detailed speculation on the nature of the functional alteration in cell membranes produced by the action of EDTA or citrate. Presumably these substances remove calcium from the cell surface or intercellular material, somehow modifying the cell membranes in a manner which gives rise to increased or abnormal permeability. Until more is known of the function of calcium, and of its chemical linkage in structures pertaining to cell membranes, there is no basis for conjecture about the action of EDTA and citrate or the reason for the apparent protective effect of the addition of 3–5% of polyvinylpyrrolidone to the perfusing fluid.

**SUMMARY**

1. Isolated parenchymal-cell suspensions, prepared from livers and kidneys of adult rats and rabbits after the perfusion of these organs with citrate or EDTA, were compared with tissue slices from unperfused organs. The isolated cells in each case showed a much lower rate of endogenous respiration *in vitro* than the corresponding slices, a lower rate of anaerobic glycolysis and a greater increase in respiration on the addition of succinate. They showed no increase in oxygen uptake on the addition of pyruvate or α-oxoglutarate unless incubated in a high-potassium medium with added ADP.

2. Mitochondria obtained from isolated rat-liver cells and incubated in the presence of α-oxoglutarate respired at the same rate as mitochondria freshly prepared from untreated normal liver. Liver-cell suspensions incubated under the same conditions showed the same initial rate of oxygen uptake as equivalent amounts of mitochondrial preparations, but the rate decreased rapidly during continued incubation of the cells. The cells showed a sharp rise in rate of oxygen uptake when 2,4-dinitrophenol was added early in the incubation, but not when it was added after 40 min.

3. Tissue slices cut from unperfused rat livers and from livers perfused with Krebs–Ringer phosphate solution showed identical rates of endogenous respiration, identical increases in oxygen uptake on the addition of succinate, and identical losses of protein into the incubation medium. Slices from livers perfused with the same solution containing citrate or EDTA showed the same rate of endogenous respiration as the controls, but a greater increase in oxygen uptake on addition of succinate, and a greater leakage of protein into the medium. The addition of polyvinylpyrrolidone to the perfusing fluid appeared to diminish these effects.

4. Kidney-cortex slices cut from rat and rabbit kidneys after perfusion with solutions containing EDTA or citrate showed essentially the same rates of endogenous respiration, and the same stimulation of respiration by the addition of succinate, as corresponding controls.

5. The results are interpreted as evidence for a marked alteration of permeability of the cell membrane after treatment with citrate or EDTA, independently of any mechanical damage to the cell. The absence of such changes in slices from perfused kidneys is attributed to the histological organization of the kidney, which may prevent free access of the chelating agent to the renal epithelium.

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**REFERENCES**


The Conversion of Adenosine 5'-Phosphate into Adenosine Triphosphate as Catalysed by Adenosine Triphosphate–Creatine Phosphotransferase and Adenosine Triphosphate–Adenosine Monophosphate Phosphotransferase in the Presence of Phosphocreatine

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Morrison & Doherty (1961) have reported that the addition of adenosine 5'-phosphate and phosphocreatine to a partially purified rabbit-muscle preparation, treated with charcoal and Dowex 1 (Cl⁻ form) to remove nucleotides, results in the formation of adenosine triphosphate and creatine according to equation (1).

\[ 2 \text{Phosphocreatine} + \text{AMP} \rightarrow 2 \text{creatine} + \text{ATP} \] (1)

Further, they found that a similar reaction was catalysed by the combined action of creatine kinase (adenosine triphosphate–creatine phosphotransferase, EC 2.7.3.2) and adenylate kinase (adenosine triphosphate–adenosine monophosphate phosphotransferase, EC 2.7.4.3). These findings raise once again the question whether or not the phosphoryl group of phosphocreatine can be transferred directly to AMP.

An enzyme catalysing such a direct transfer was reported by Banga (1943), who claimed that, together with creatine kinase, it could bring about reaction (1). But with the discovery of adenylate kinase (Colowick & Kalckar, 1943) it became generally accepted that reaction (1) was the sum of reactions (2) and (3),

\[ \text{AMP} + \text{ATP} \rightarrow 2 \text{ADP} \] (2)

\[ \text{ADP} + \text{phosphocreatine} \rightarrow \text{ATP} + \text{creatine} \] (3)

which occurred because of the presence of trace amounts of either ADP or ATP (Chappell & Perry, 1954). Molnar & Lorand (1960) showed that reaction (1) occurs even after exhaustive dialysis of crystalline preparations of creatine kinase and adenylate kinase.

Further investigations have been made of the conversion of AMP into ATP in the presence of phosphocreatine and the results of these are reported in this paper. It has been shown that reaction (1) occurs with highly purified preparations of AMP and purified preparations of the enzymes that have been treated repeatedly with charcoal.