Metabolic Changes in the Lungs after Ischaemia

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SUMMARY

The effects of variable periods of ischaemia on the isolated lungs of rats and rabbits, stored for up to 6 hours at 4°C, 21°C and 37°C under standardized conditions, were investigated *in vitro* in terms of oxygen consumption, the rate of 1-^{14}C-leucine incorporation into soluble proteins, and 1-^{14}C-palmitate incorporation into total phospholipids and lipid fractions.

The endogenous oxygen uptake of rat lung slices in an air phase, from tissues stored at 4°C and 21°C under ischaemic conditions for 6 hours and at 37°C for 4 hours, was significantly different from the control values. The oxygen uptake of lungs from animals anaesthetized with pentobarbitone prior to exsanguination and stored for only 2 hours at 37°C differed significantly from control values.

Judged by the rate of incorporation of radiolabelled leucine into soluble proteins and that of palmitate into total lipids and phospholipids of lungs after storage for increasing periods at 4°C and 37°C, significant differences were already found after 1½ hours. From this observation it would appear that these parameters are very sensitive indicators for assessing irreversible lung damage due to ischaemia.

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The literature on experimental lung transplantation shows that many attempts have been made in several species without much success,[1,2] in spite of modern surgical technology. One reason for the lack of success may be irreversible metabolic damage to the lungs as a result of ischaemia. The 'survival-after-implantation' technique has been used to evaluate the methods for lung preservation,[1,2] but this approach appears to be laborious and expensive.

We believe that further research into metabolic changes in the isolated lung could provide useful information on methods for preservation, and might even solve some of the problems involved in successful lung transplantation.

The effects of ischaemia on the structure, dynamic properties and gas exchange of the lung have been studied extensively.[3,4] Von Wichert[4] attempted to relate the duration of ischaemia to metabolic changes in isolated normothermic rabbit lungs. They found a gradual decrease in glucose and adenosine triphosphate (ATP) content over a period of 3 hours accompanied by an increase in lactate concentration, although the total amount of phospholipids was not affected. Shimada *et al.*[3] studied the effects of ischaemia on isolated dog lungs, kept *in vitro* at 0° to 4°C. They demonstrated that the rates of oxidation of 1- ^{14}C-glucose and 1- ^{14}C-acetate to $^{14}CO_2$ decreased significantly after ischaemia lasting 2 and 4 hours respectively. Total glucose consumption was reduced by about 30% after 30 minutes and remained constant at that level for up to 4 hours of ischaemia.

In view of the problems encountered with lung transplantations, the present research was initiated to determine the degree of lung damage due to ischaemia. Lungs of rats and rabbits were, after isolation, stored at various temperatures and under standardized conditions for different periods of time, whereupon the rate of (*i*) oxygen utilization; (*ii*) incorporation of 1- ^{14}C-leucine into soluble proteins; and (*iii*) incorporation of 1- ^{14}C-palmitate into phospholipids of the lung was measured.

MATERIALS AND METHODS

New Zealand White rabbits (1,5 - 2,0 kg) and Long-Evans rats (180 - 200 g) were used. The rats were either sacrificed by decapitation or exsanguination by severing the abdominal aorta under sodium pentobarbitone anaesthesia (10 mg/100 g intraperitoneally) (Nembutal Veterinary; Abbott Laboratories). The rabbits were anaesthetized with a sublethal dose of 2,5% thiopental sodium (18 mg/kg intravenously). The thorax was opened quickly and the lungs were perfused *in situ* via the pulmonary artery with 15 ml (rats) and 50 ml (rabbits) isotonic saline at 4°C. The rat lungs were ventilated during perfusion. The lungs were then quickly removed and dissected free of large airways and blood vessels. Lungs with any macroscopic signs of disease were discarded.

The isolated lungs were cut into sections of ± 300 mg each. For non-ischaemic control experiments, one section was immediately chopped into 0,7 mm slices (McIlwain tissue slicer) and incubated at 37°C (see individual experiments). The other sections were stored for specific time intervals at 4°C, 21°C and 37°C in 10 ml glass containers lined with filter paper and moistened with buffered medium. At indicated intervals, the stored tissue was removed from the containers and cut into 0,7 mm slices to be used in the different incubation experiments. As far as possible, one of each pair of lungs was used for the experiment while the other provided the control.

Oxygen Consumption

The rate of oxygen consumption of normal and ischaemic lung slices, stored for up to 6 hours, was determined over 1 hour according to the direct Warburg technique[5] using a Braun's Warburg apparatus model

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V. The media used and the detailed procedure have been described.[6]

Incorporation of 1- ^{14}C-Leucine into Soluble Protein[7]

The incorporation of 1- ^{14}C-leucine into proteins extractable by low ionic strength buffers (referred to as soluble proteins) from normal and ischaemic lung tissue was determined as follows: 100 mg of slices, from both normal and ischaemic lungs, was incubated for 2 hours at 37°C in a stoppered metabolic flask containing 4 ml Krebs-Ringer bicarbonate medium[6] with glucose (10 mM) and 0,2 μmol of each of 20 amino acids. The medium was equilibrated beforehand with 95% O_2 - 5% CO_2 for 1 hour, during which time the pH was regularly adjusted to 7,4. A mixture of 1- ^{14}C-leucine (specific activity 54,4 mCi/mmol and 0,1 mCi/ml; New England Nuclear) and stock buffer solution was accurately measured into the incubation flasks to obtain approximately 500 000 cpm per 4 ml medium. Incubation proceeded in a waterbath at 37°C with a shaking speed of 90 cycles per minute. Although the incorporation of 1- ^{14}C-leucine into the soluble proteins[7] was linear over 3 hours under the present experimental conditions, we terminated the experiment after 2 hours by placing the flasks on ice. The medium was decanted, the slices were washed and transferred into centrifuge tubes containing 5 ml 0,08M KCl and 0,02M tris-HCl, pH 7,4, and homogenized by an Ultra Turrax for two 1-minute periods on ice. After 30 minutes the ice-cold homogenate was centrifuged at 10 000 *g* for 10 minutes at 4°C. The supernatant was collected, the pellet homogenized and centrifuged again. The two supernatants were pooled and the soluble proteins precipitated by addition of an equal volume of 10% TCA and left for 45 minutes on ice. The precipitated proteins were pelleted by centrifuging at 10 000 *g* for 10 minutes at 4°C, then washed with 5% TCA and again centrifuged. The pellet obtained was first extracted with ether-ethanol 1 : 1 (v/v) and centrifuged, then again extracted with ether and thereafter centrifuged at 10 000 *g* for 10 minutes at 4°C. The fat-free pellet was dried in air and then dissolved in 1 ml 1M NaOH for 12 hours at 37°C. An aliquot of 100 μl protein solution was mixed with 10 ml scintillation cocktail (Instagel; Packard) containing 250 μl 5% acetic acid to suppress excessive chemiluminescence. The samples were counted in a Beckman liquid scintillation counter with 98% counting efficiency.

The protein concentration was determined by the method of Lowry *et al.*[8] using bovine serum albumin (Miles Laboratories) as the reference standard. The amount of radioactive leucine incorporated into the soluble protein fraction was expressed as cpm per mg protein.

1- ^{14}C-Palmitate Incorporation into Lipids[9]

Exactly 30 mg each of normal and ischaemic lung slices were incubated in 3,5 ml KRB, pH 7,4, in metabolic flasks in a shaking waterbath, as described above for labelled leucine incorporation. To each flask 0,5 ml of a palmitate-albumin complex was added. The complex consisted of 36 mg cold palmitate and 6 μCi 1- ^{14}C-palmitate, both complexed after saponification to 27 ml of a 24% bovine serum albumin solution[9] (Cohn fraction V). The ratio of albumin to palmitate was 95 : 140 and the specific activity was 6 μCi/140 μmol. Although the incorporation of 1- ^{14}C-palmitate into total lung lipids was found to be linear over a period of 3 hours in the present experiments incubation was terminated after 2 hours by cooling the flasks to 4°C. The tissue slices were centrifuged and the medium decanted. The pellet, after washing, was resuspended in 6 ml chloroform-methanol 2 : 1 (v/v). The slices were then homogenized on ice in this medium with an Ultra Turrax for 3 minutes and left overnight at room temperature to extract the lipids.

Total lipids were separated from the extract by the method of Folch *et al.*[10] To achieve separation into two specific phases, 1,2 ml 0,74% KCl solution was added to the chloroform-methanol extract, mixed and centrifuged. After separation the volume of the lower phase was noted and the upper phase aspirated carefully. The interphase was washed thrice with fresh upper phase solution. Two millilitres of the lower phase was transferred to a counting vial and evaporated in a stream of nitrogen, whereafter 10 ml Instagel was added, the vial was shaken well, and the radio-activity was counted in a Beckman liquid scintillation counter. The amount of 1- ^{14}C-palmitate incorporated into the total lipids was calculated and expressed as cpm per 300 mg lung tissue.

Fractionation of Total Lipids

The extracted lung lipids together with standard reference lipid samples (Supelco) were separated by thin-layer chromatography.[11] The individual spots stained with iodine vapour were scraped off into counting vials containing 5 ml Instagel. Iodine quenching was corrected for by the external standard ratio method to obtain a counting efficiency of 90%. The incorporation of 1- ^{14}C-palmitate into the lipid fractions was expressed as cpm per mg extracted lipid.

RESULTS

The effects of ischaemia in terms of *in vitro* oxygen utilization of rat lung slices in an air phase and without substrate in the medium are summarized in Fig. 1. When the time of ischaemic storage of the lung tissue was increased, the oxygen uptake of the slices declined gradually but the rate of decrease was not directly proportional to the duration of ischaemia. The rates of oxygen uptake of ischaemic tissue stored at 4°, 21° and 37°C for 6 hours were all significantly lower than the control value ($P<0,05$), whereas after 4 hours' storage, only the oxygen uptake of tissue stored at 37°C was significantly reduced. The control values for oxygen uptake compare well with values previously reported.

The values obtained with ischaemic lungs of anaesthetized rats clearly demonstrated the additional deleterious effect[4] of pentobarbitone on the rate of oxygen uptake with time (Fig. 1).

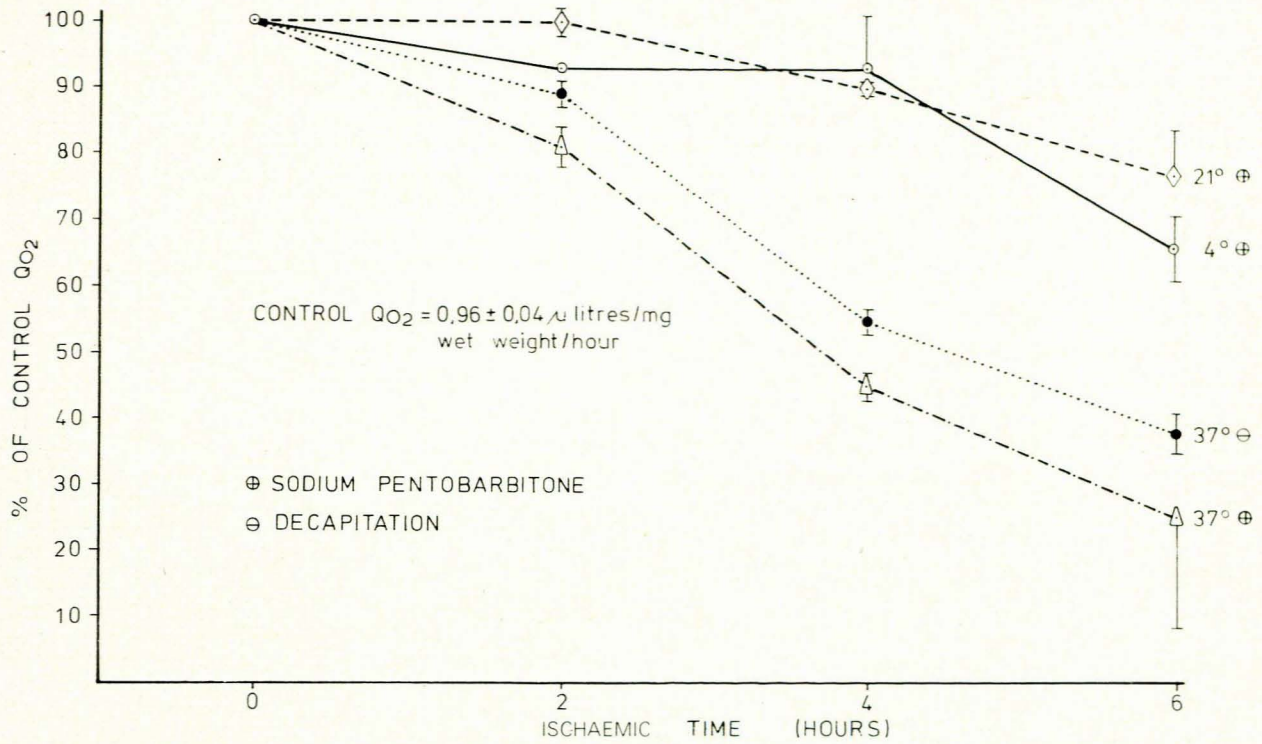

Fig. 1. The *in vitro* oxygen uptake of ischaemic rat lung slices in a Krebs-Ringer phosphate medium, pH 7,4, without exogenous substrate and in air as gas phase after storage of the lung tissue at 4°, 21° and 37°C for periods up to 6 hours. The results are expressed as percentages of control values. (Each value represents the mean of 4 triplicate determinations.)

In Fig. 2 the oxygen uptakes of ischaemic rat and rabbit lung slices in a 100% O_2 phase with 10 mM glucose as substrate are compared. The oxygen uptake of rat lung slices stored at 4° and 21°C for up to 4½ hours was not significantly different under these conditions from the control values. However, the oxygen uptake of rabbit lung, kept under similar conditions, decreased significantly after 3 hours of ischaemia. When ischaemic lungs of both species were stored at 37°C, a marked decline in oxygen uptake occurred, which was already significantly different from control values after 1½ hours ($P<0,01$) and became even more significant up to 4½ hours after storage.

In Fig. 3A and B, the effects of ischaemia after 1½ and 3 hours at 4°C and 37°C on 1- ^{14}C-leucine and 1- ^{14}C-palmitate incorporation into lung proteins and lipids respectively are recorded. The mean rate of 1- ^{14}C-leucine incorporation into the soluble proteins of control lung slices over 2 hours amounted to 18 835 ± 316 cpm/mg isolated protein. After storage of ischaemic lung for 1½ hours at 4°C, the rate of 1- ^{14}C-leucine incorporation decreased significantly ($P<0,01$), while in lung tissue kept at 37°C the reduction was even more dramatic (Fig. 3A).

The amount of soluble protein isolated from ischaemic lung tissue stored at 4°C and 37°C (Table I) and thereafter incubated for 2 hours gradually decreased with the duration of ischaemia and was significantly different from the control value obtained from the same lung tissue without storage.

TABLE I. SOLUBLE PROTEIN ISOLATED FROM ISCHAEMIC LUNG SLICES PRESERVED AT 4°C AND 37°C AND AFTER 2 HOURS' INCUBATION

Tissue	Temp. (°C)	mg protein/100 mg wet tissue	% of control	*P*
Control	—	**2,31 ± 0,11**	—	—
Ischaemic for 1½ h	**4**	**2,03 ± 0,20**	**88**	**0,01**
	37	**1,41 ± 0,14**	**61**	**0,001**
Ischaemic for 3 h	**4**	**1,93 ± 0,15**	**84**	**0,01**
	37	**1,30 ± 0,38**	**56**	**0,001**

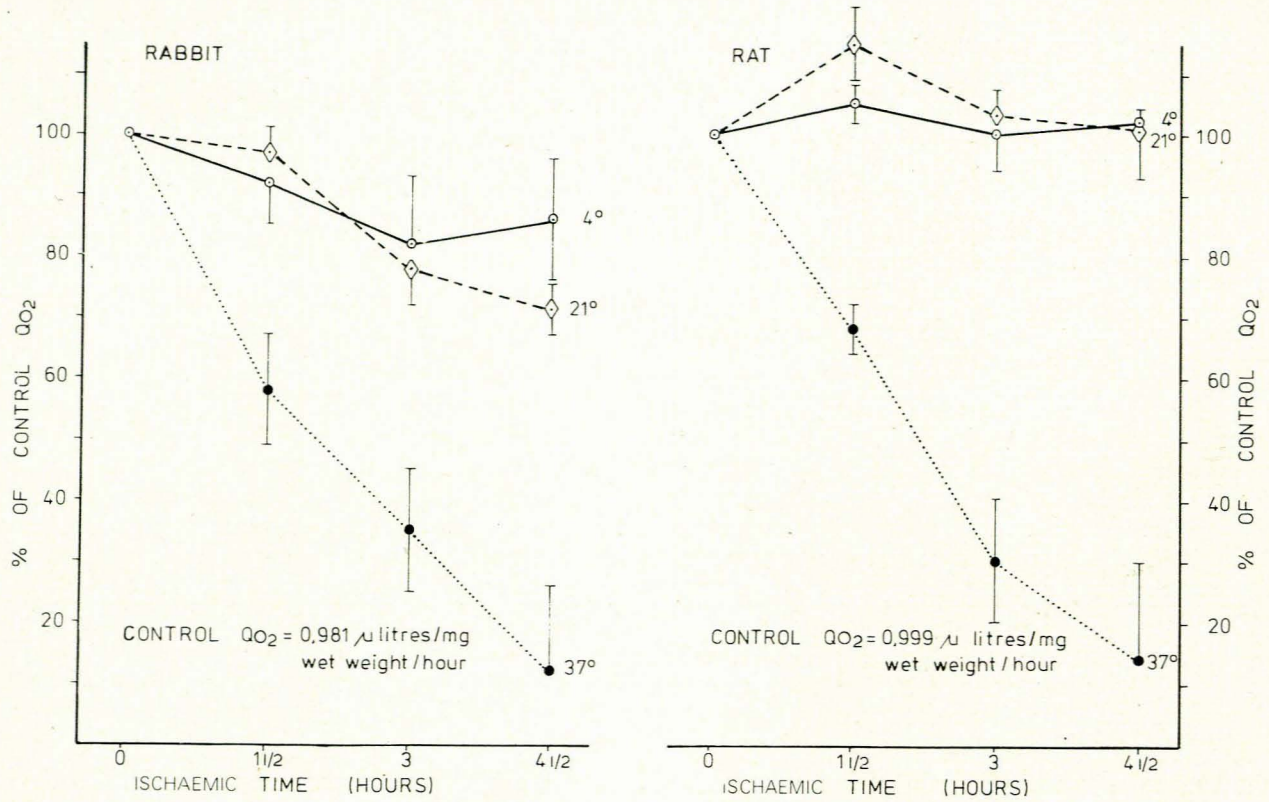

Fig. 2. The *in vitro* oxygen uptake of ischaemic rat and rabbit lung slices in a Krebs-Ringer phosphate medium, pH 7,4, with 10 mM glucose as substrate and in a 100% O_2 gas phase after storage of the lung tissue at 4°, 21° and 37°C for periods up to 4½ hours. The results are expressed as a percentage of the control values. (Each value represents the mean of 4 triplicate determinations.)

Incorporation of 1- ^{14}C-palmitate into the total extractable lipids of control lung slices over 2 hours amounted to 44 890 ± 388 cpm/300 mg tissue. The rate of incorporation into the lipids of ischaemic lung tissue stored at 4°C and 37°C for 1½ and 3 hours was significantly lower than the control value at both temperatures and time intervals (Fig. 3B).

The results recorded in Table II also demonstrated that the rate of 1- ^{14}C-palmitate incorporation into the total phospholipid fraction as well as the dipalmitoyl phosphatidyl choline fraction of the phospholipids after storage at 37°C was already markedly reduced after 1½ hours; this tendency progressed with time. By contrast, at 4°C the rate of 1- ^{14}C-palmitate incorporation into the ischaemic lung phospholipids remained normal up to 3 hours. This also applies to the amount of labelled palmitate in free fatty acid and neutral lipid fractions from ischaemic lung stored at 4°C and 37°C for up to 3 hours.

DISCUSSION

Using the rate of oxygen consumption as an index of the degree of metabolic damage induced by ischaemia at different temperatures for various periods, our findings clearly demonstrate that ischaemia at temperatures of 4°C and 21°C and lasting 4 hours did not suppress aerobic

TABLE II. 1-^{14}C-PALMITATE INCORPORATED INTO TOTAL PHOSPHOLIPIDS, PHOSPHATIDYL CHOLINE, FATTY ACID AND NEUTRAL LIPID FRACTIONS (EXPRESSED AS CPM PER 1 mg TOTAL LIPID EXTRACTED)

		Ischaemia			
		1½ h		3 h	
	Control	4°C	37°C	4°C	37°C
Total phospholipids	1 703 ± 221	1 566 ± 290	721 ± 252*	1 816 ± 216	353 ± 119*
Phosphatidyl choline	1 299 ± 147	1 205 ± 205	559 ± 195*	1 446 ± 142*	258 ± 67*
Fatty acid and neutral lipids	805 ± 169	718 ± 262	791 ± 355	978 ± 178*	1 205 ± 344

*Indicates significant deviation from control.

metabolism significantly. However, ischaemia at 37°C for 4 hours and longer depressed oxygen uptake markedly and progressively. Because the ischaemic slices used in the first experiment (Fig. 1) were not challenged with substrate after ischaemic storage, the observed rate of oxygen uptake might not reflect their maximal oxygen utilization. However, normal lung tissue utilizes oxygen at a very constant rate for up to 6 hours and longer without exogenous substrate.[12] It is known that the amount of lung glycogen is limited and the lung must therefore either metabolize fatty acids or amino acids. When exogenous glucose was added (Fig. 2) the rate of oxygen utilization was slightly, but not significantly, higher. The reduction in oxygen uptake of ischaemic rat lung tissue kept at 4°C and 21°C for up to 4 hours was never significantly different from control values, irrespective of whether glucose was available as substrate or not. However, all tissue stored at 37°C suffered significant damage after 4 hours of ischaemia, both in the presence and in the absence of glucose.

It has been shown that during ischaemia a progressive decrease in ATP synthesis in lung tissue occurs,[13] while glycolysis is increased. The resulting pyruvate is increasingly converted to lactate under the anaerobic conditions. The more glucose is metabolized anaerobically, the higher the lactate concentration becomes and the lower the intracellular pH.[13] The longer the ischaemic period and the higher the temperature during ischaemia, the greater will be the reduction in aerobic metabolism. However, it would appear from our findings that ischaemic lung tissue could be kept at 4°C and 21°C for up to 4 hours without incurring serious irreversible damage. The rate of oxygen uptake of these tissues is so close to normal that some of the changes may be reversed if the oxygen supply is restored[14] and ATP synthesis recommences. However, many lung transplants have been unsuccessful, some even after relative short periods of ischaemia.[4] This might indicate that, although short periods of ischaemia induce only a small reduction in aerobic metabolism, some other changes might be irreversible. The sensitivity of lung tissue to damage is demonstrated by the significant suppressive effect of sodium pentobarbitone anaesthesia on the oxygen utilization of ischaemic lungs at 37°C. Another important finding is that rabbit lung is more sensitive to ischaemic damage at temperatures between 4°C and 21°C than is rat lung. This might be due to the high sensitivity of rabbit lung to oxygen toxicity in a 100% oxygen phase.[6] A similar difference may exist among other species.

In the evaluation of protein synthesis by ischaemic lung tissues, a significant deviation from control values was noticed even at 4°C after 1½ hours. At 4°C, metabolism is retarded to such an extent that hardly any ATP or substrates will be used or metabolic toxic sub-

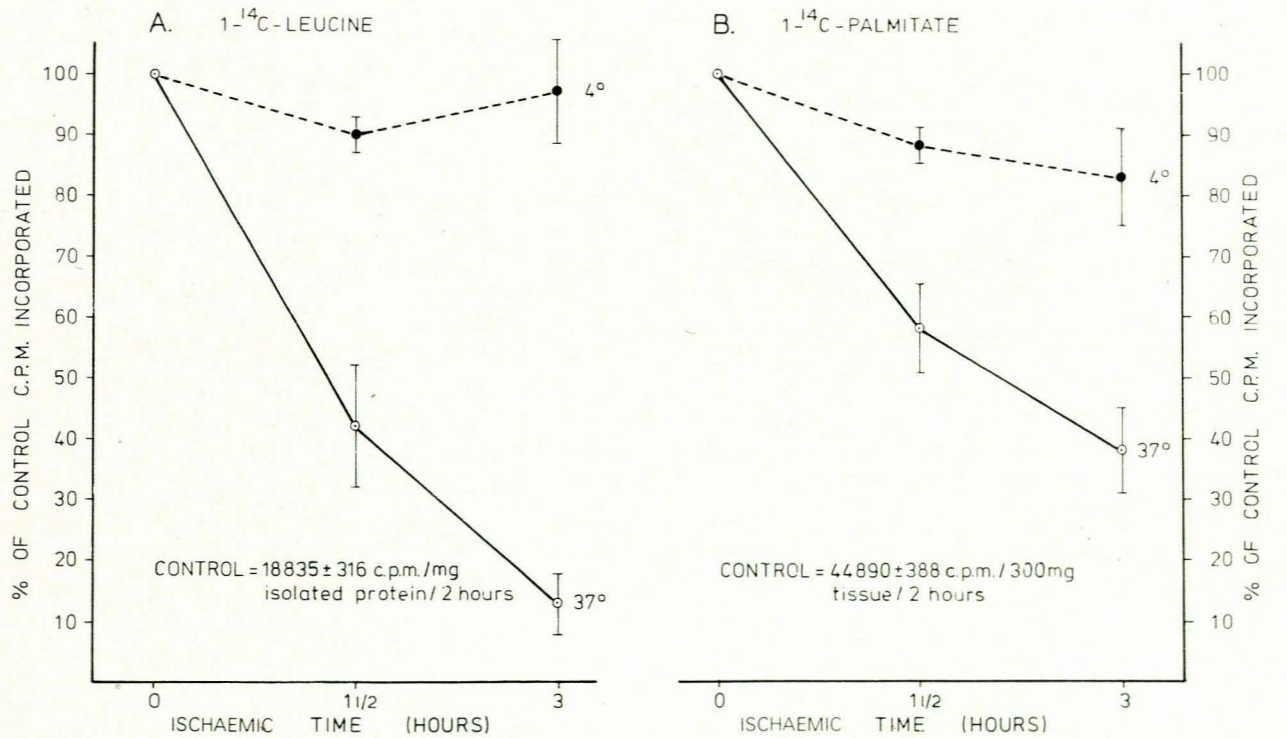

Fig. 3. The *in vitro* incorporation of 1- ^{14}C-leucine (A) and 1- ^{14}C-palmitate (B) into soluble proteins and total lipids respectively of ischaemic rabbit lung slices. The lung tissue was stored at 4° and 37°C for up to 3 hours before the rates of uptake was determined in a Krebs-Ringer bicarbonate medium, pH 7,4, with 10 mM glucose as substrate and in 95% O_2 - 5% CO_2 gas phase. The rates of incorporation of the radiolabelled substrates are expressed as a percentage of the control values. (Each value represents the mean of 3 triplicate determinations.)

stances generated. One would thus expect hardly any irreversible damage to occur at this temperature. However, protein synthesis in ischaemic tissue stored at 37°C for 3 hours is inhibited by 86%. This is certainly the most dramatic effect induced by ischaemia on lung tissue.

The decreased incorporation of 1- ^{14}C-leucine into lung protein is accompanied by a decrease in the amount of soluble, extractable protein from the ischaemic tissue, kept at 4°C and 37°C as shown in Table I. The decrease in protein content could be due to the activation of lysosomal proteases by the ischaemic conditions, with ensuing release of some intracellular proteins into the medium, or to a lesser extent to structural changes in intracellular soluble proteins due to an accumulation of calcium during ischaemia.[2]

It has been shown that ischaemia lasting 3 hours causes irreversible liver cell injury accompanied by loss of almost one-half of total phospholipids.[15] Considering the protein loss which occurred in lung tissue incubated for 2 hours and stored at 4°C and 37°C after 1½ hours, the reduction in the amount of extractable soluble protein from the ischaemic tissue at 37°C was significantly greater than that from ischaemic tissue at 4°C. This could be due to the fact that lysosomal proteases would hardly hydrolyse proteins at 4°C. Structural changes due to calcium accumulation could be expected to be very similar under our experimental conditions, and could therefore not account for the protein loss during the incubation period. Recovery experiments (not reported here) showed that almost all of the protein could be found in the incubation medium. Therefore, membrane disintegration must have occurred, resulting in the solution of cytosol proteins.

Judged by the rate of 1- ^{14}C-palmitate incorporation into the lipids of ischaemic lung tissue stored at 4°C and 37°C, a significant reduction had taken place, very similar to the retarded 1- ^{14}C-leucine incorporation into proteins. The rate of incorporation is inversely related to the temperature during ischaemia and is thus much slower when tissue is stored at 37°C than at 4°C. Whether this decreased rate of incorporation was due to a loss of total phospholipids from the ischaemic lung could not be ascertained from our results. Farber *et al.*[15] found accelerated phospholipid degradation in ischaemic liver cells, resulting from the activation of membrane-bound phospholipases. It is known that lung tissue is rich in phospholipids, but whether microsomal membrane phospholipases of lung tissue are activated by ischaemia was not determined. From indirect calculations of total lipids in normal and ischaemic lungs as used in our experiments, it would appear that the total lipid content is not affected by severe ischaemia. However, at 37°C the rate of 1- ^{14}C-palmitate incorporation into the phospholipid complex of ischaemic lungs is markedly reduced. Seeing that the type II alveolar cells are involved in phosphatidyl choline synthesis, it would appear that these cells must be particularly sensitive to ischaemic damage or that they possess a phospholipase which is activated by ischaemia. Although one could not judge the loss in total phospholipid content from our findings, the rate of 1- ^{14}C-palmitate incorporation into total phospholipids and especially into the dipalmitoyl phosphatidyl choline fraction of ischaemic lung tissue stored at 37°C was significantly reduced.

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