A cold-clamping technique for the rapid sampling of rat liver for studies on enzymes in separate cell fractions

Suitability for the study of enzymes regulated by reversible phosphorylation–dephosphorylation

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1. A technique for the rapid sampling, cooling and homogenization of rat liver is described. 2. Its effectiveness in preserving the activity status of pyruvate kinase (soluble) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) (microsomal) during sampling is assessed in comparison with that of the freeze-clamping technique and of simple excision and mincing of liver tissue before homogenization. 3. The results suggest that cold-clamping is equally effective as freeze-clamping in preserving the activity status of pyruvate kinase in liver samples obtained *in situ*, but in addition allows the subsequent separation of subcellular fractions, notably microsomes (microsomal fractions) and mitochondria. It is suggested that this property makes the technique useful in studying the activity status of enzymes (e.g. HMG-CoA reductase) the assay of which is subject to interference from the activity of other enzymes which are released from damaged organelles in crude homogenates of freeze-clamped liver samples. 4. This suggestion was tested directly; the cold-clamping technique was found to preserve a substantially higher initial/total HMG-CoA reductase activity ratio [Easom & Zammit (1984) *Biochem. J.* 220, 739–745] in subsequently isolated microsomes compared with that obtained in microsomes prepared from liver samples processed in the conventional manner. 5. The integrity of mitochondria isolated from homogenates of cold-clamped liver samples was preserved, as judged by the latency of intramitochondrial enzymes and by good respiratory control of the mitochondria. 6. Possible further areas of metabolic studies to which the cold-clamping technique could be applied are suggested.

The verification of theories of metabolic control formulated on the basis of experimental results obtained *in vitro*, and their extrapolation to the regulation of metabolic processes in the intact animal *in vivo*, present formidable experimental problems. This is particularly the case when information is required about a metabolic system which is capable of very rapid changes in response not only to normal physiological stimuli but also to the very experimental procedures used to investigate it. For example, the measurement of intracellular metabolite concentrations and of the activity of enzymes which are regulated through reversible covalent modification (e.g. phosphorylation–dephosphorylation) requires instantaneous information

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

on such parameters *in vivo*. Artefacts arise from any delay between sampling of the tissue and the cessation of metabolic activity which is likely to alter the state of the metabolic system from that existing *in vivo*. To minimize these artefacts, extensive use has been made in previous studies of the freeze-clamping technique (Wollenberger *et al.*, 1960), in which whole animals (e.g. insects) or tissues (e.g. liver, mammary gland) of anaesthetized larger animals are pressed between two metal blocks cooled in liquid N₂; this results in the very rapid cessation of metabolic activity within the tissue and thus preserves the instantaneous state of metabolite concentrations and activity status of the enzymes. Subsequent homogenization of the frozen tissue samples is performed in a medium which either destroys enzyme activity (for meta-

bolite measurement) or prevents the activity of particular enzymes (e.g. a fluoride-containing medium, which inhibits the activity of protein phosphatases); the latter procedure would be required when enzyme activities in the tissue samples are to be preserved.

In this laboratory the freeze-clamping technique has been used to study aspects of the metabolism of the liver and other tissues *in vivo*, by measurement both of metabolite concentrations and of the fraction of acetyl-CoA carboxylase (an enzyme regulated by phosphorylation-dephosphorylation) in the active form in animals in different physiological conditions (see, e.g., Zammit, 1981; McNeillie & Zammit, 1982; Zammit & Corstorphine, 1982a). However, an inevitable consequence of the freeze-clamping of liver samples is the disruption, on subsequent thawing and homogenization, of the membranes of intracellular structures such as mitochondria. Such breakdown of the compartmentalization of the liver cell impairs subsequent measurement of enzyme activity if there is mutual interference, in the resulting crude homogenate, between enzymes which *in vivo* occur in different cell compartments.

In the course of studies on the effects of different physiological conditions on the fraction of the enzyme HMG-CoA reductase in the active form in the liver *in vivo*, we encountered such interference between the activities of HMG-CoA reductase (microsomal) and HMG-CoA lyase (mitochondrial) in homogenates of freeze-clamped liver samples (Easom & Zammit, 1984). Consequently we have developed a method for the rapid (<5s) sampling *in situ*, cooling and homogenization of rat liver samples (without freezing) and with maintenance of mitochondrial integrity. The resulting homogenates were used to prepare different cell fractions by differential centrifugation. In the present paper the new technique is described, its validity is assessed, and possible further areas of metabolic studies to which it could be applied are suggested.

Materials and methods

Materials

The source of rats and their maintenance were as described previously (Zammit, 1980, 1981).

NADP⁺, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type VII, from baker's yeast), pentobarbital, phenylmethanesulphonyl fluoride, Tris and imidazole buffers were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Acetone, toluene and A.R.-grade inorganic chemicals were obtained from Fisons Scientific Apparatus (Loughborough, Leics. LE11 0RG, U.K.). 3-Hydroxy-3-methyl[3-¹⁴C]glutaric

Fig. 1. Details of construction of stainless-steel blocks used for obtaining samples of rat liver by cold-clamping (a) Face of one block: 1, cutting edge; 2, gap; 3, groove. (b) Transverse (equatorial) section through the two blocks with clamps shut. (c) Top view of clamps to show mounting of blocks on handles.

acid and DL-[2-³H]mevalonic acid lactone were obtained from Amersham International plc (Amersham, Bucks., U.K.). 3-Hydroxy-3-methyl[3-¹⁴C]glutaryl-CoA was synthesized by the method of Goldfarb & Pitot (1971). The final specific radioactivity of the product was 0.30 Ci/mol.

Liver protein phosphatase was prepared from rat livers by the method of Brandt *et al.* (1975) up to and including the first DEAE-cellulose step (Zammit & Corstorphine, 1982a). The final preparation was stored at -20°C in small batches, which were dialysed for 3 h against a buffer containing 50 mM-imidazole/HCl, pH 7.4, 250 mM-KCl and 1 mM-EDTA before being used.

Catalytic subunit of cyclic AMP-dependent protein kinase, phosphoenolpyruvate, ADP and lactate dehydrogenase, were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.).

Description of clamps

The clamps used consisted of two circular stainless-steel blocks (diam. 5 cm) carried at the ends of the two arms of tongs which could be freely shut and opened. Each block had a raised cutting edge (height 1.5 mm) down one side and extending approx. $\frac{2}{3}$ of the circumference (Fig. 1) and a groove of the same, but reciprocal shape (1.5 mm deep) symmetrically opposite. The raised edge and the groove on each block were confluent at one point, but were separated by a distance equivalent to approx. $\frac{1}{3}$ of the circumference at the diametric-

Fig. 2. Diagrammatic representation of cold-clamping of rat liver samples

(a) Lifting of left lateral lobe of liver and positioning of cold-clamps (vertical section); (b) sampling of liver; (c) flushing of liver sample into body of homogenizer with extraction medium; (d) homogenization of tissue with a Teflon/glass homogenizer (see the Materials and methods section for further details). The liver is drawn as the cross-hatched area. The scale at the bottom of the diagram gives the approximate times taken routinely to perform the different parts of the procedure.

ally opposite side of the blocks. The blocks were mounted on the arms of the tongs in such a manner that the raised edge of the one fitted into the groove of the other, with the confluent cutting edges and diametrically opposite gaps being positioned perpendicularly to the plane of closure of the tongs. In use the tongs were held with the gap pointing vertically upwards so that excess liver sample squashed between the two blocks could escape through the gap. The effect of shutting the tongs was to produce a circular cutting edge throughout the lower and lateral parts of the two apposed blocks; the clamping action simultaneously cut a sample (approx. 1 g) of the liver lobe and smeared it in a thin layer over the cooled surfaces of the blocks.

Cold-clamping of liver

A schematic representation of the procedure is given in Fig. 2. Animals were anaesthetized with pentobarbital (60 mg/kg body wt.) and allowed to stabilize for 15–20 min (Zammit & Corstorphine, 1982a). The abdominal cavity was opened and the liver was dissected free of surrounding tissue by cutting the mesenteries. The left lateral lobe of the liver was threaded 2–3 mm from the edge with a Mersilk silk suture (4/0 gauge, round bodied 16 mm needle; Ethicon, Edinburgh, Scotland, U.K.). This allowed the lobe to be lifted out vertically from the abdominal cavity. (The use of a suture rather than a pair of forceps to lift the liver lobe was necessitated by the interference of forceps with the action of the cutting edges of the clamps; routinely no bleeding of the liver or any other adverse effects of the technique were apparent.) The lobe was clamped between ice-cooled stainless-steel blocks (see above). The shut clamps containing the liver sample were immediately held over the mouth of a plastic funnel inserted into the top of the body of a Teflon/glass homogenizer. The clamps were

opened and the liver sample was washed directly into the homogenizer by squirting 25 ml of ice-cold extraction medium over the inner surfaces of the metal blocks. The tissue was then homogenized for 5 s (three passes) with a motor-driven pestle (1000 rev./min). The entire procedure was completed in less than 10 s.

Extraction media and treatment of homogenates

Unless otherwise indicated, liver was sampled and homogenized as described above. In experiments in which mitochondria were isolated, the initial homogenates were prepared in a medium containing 250 mM-sucrose, 1 mM-EGTA and 5 mM-Tris/HCl, pH 7.4 at 0°C. Homogenates were centrifuged at 600g for 10 min, and the resulting supernatant was centrifuged at 7500g for a further 10 min. The mitochondrial pellet was resuspended in the same medium and sedimented again at 7500g (10 min). The final mitochondrial pellet was resuspended in 1–2 ml of extraction medium.

In experiments in which microsomes (microsomal fractions) were isolated, homogenates were prepared in a medium containing 50 mM-sucrose, 100 mM-KF, 10 mM-Tris/HCl, 10 mM-EDTA and 0.5 mM-phenylmethanesulphonyl fluoride, pH 7.4 at 0°C. They were subsequently treated as described by Easom & Zammit (1984). HMG-CoA reductase activity was measured in the isolated microsomes; pyruvate kinase activity was measured in the post-mitochondrial supernatant.

Freeze-clamped samples were homogenized with a Polytron disintegrator (Kinematica, Basle, Switzerland).

Assay of HMG-CoA reductase and pyruvate kinase activities

HMG-CoA reductase activity was measured as described by Easom & Zammit (1984). Pyruvate kinase activity was measured as described by

Zammit & Newsholme (1978). Phosphorylation of pyruvate kinase in the fluoride-containing post-mitochondrial supernatants (100 μ l) was performed by addition of a mixture (100 μ l) containing 10 mM-ATP, 5 mM-MgCl₂, 100 μ M-cyclic AMP and 2 units of catalytic subunit of cyclic AMP-dependent protein kinase. In preliminary experiments the mixture was incubated at 37°C and samples were taken at intervals and assayed for pyruvate kinase activity (with 0.6 mM-phosphoenolpyruvate), until a constant minimum activity was obtained. Routinely phosphorylation was performed for 20–30 min. The homogenates were then used to determine the activity of pyruvate kinase at various concentrations of phosphoenolpyruvate; results pertaining to these homogenates are referred to as 'phosphorylated'.

Measurement of mitochondrial oxygen consumption

Measurement of State-3 and State-4 rates of mitochondrial respiration with glutamate and malate as substrates was performed at 37°C as described by Zammit (1980). The incubation medium contained 0.18 M-sucrose, 32 mM-KCl, 8 mM-Tris, 1 mM-EGTA, 10 mg of albumin/ml, 2.5 mM-K₂HPO₄, 2 mM-glutamate and 2 mM-malate. The final pH was 7.4 at 37°C.

Measurement of protein concentration

Protein concentrations in the various fractions of the homogenates were assayed by the method of Lowry *et al.* (1951).

Results and discussion

The cold-clamping technique was developed to enable the measurement of 'initial' and 'total' activities of HMG-CoA reductase in rat liver microsomes (see Easom & Zammit, 1984), the initial activity being that activity of the enzyme expressed *in vivo*, whereas the total activity represents that of the fully dephosphorylated enzyme *in vitro* (cf. studies on acetyl-CoA carboxylase: McNeillie & Zammit, 1982; Zammit & Corstorphine,

1982*a,b*). Therefore, in order to test its effectiveness in minimizing the artefacts inherent in the conventional manner of preparing microsomes (see the introduction), two criteria were used. Firstly, a comparison was made between the initial/total-activity ratio for HMG-CoA reductase in microsomes obtained from livers of the same animals but prepared either after mincing and homogenization of the tissue in a conventional manner or by using the cold-clamping technique. Secondly, the same study was performed on another enzyme which undergoes reversible phosphorylation and dephosphorylation, namely pyruvate kinase, but in addition a third sample of the liver from the same animal was freeze-clamped. Therefore the latter experiment afforded a direct comparison between the artefacts introduced in measuring initial/total-activity ratios in cold-clamped and minced samples, and enabled us directly to compare the effectiveness of cold-clamping in relation to that of freeze-clamping in arresting any artefactual changes in initial/total-activity ratios of enzymes regulated by phosphorylation–dephosphorylation during sampling.

Since the primary aim of using cold-clamping instead of freeze-clamping was to retain mitochondrial (and other membranous structure) integrity, the intactness of mitochondria was also assessed.

Initial/total-activity ratio for HMG-CoA reductase

Rats for these experiments were sampled 6 h into the dark phase of the light/dark cycle (see Easom & Zammit, 1984). The left lateral lobe of the liver was cold-clamped, whereas the right lobe was excised and minced on an ice-cold Petri dish. Both samples were homogenized in an identical manner in a fluoride-containing medium (see the Materials and methods section), and all subsequent treatments for the two homogenates and microsomal pellets were also identical. The results of four such experiments are given in Table 1. It is evident that initial/total-activity ratios were always appreciably lower in homogenates of minced liver samples than of cold-clamped samples. Moreover, this

Table 1. Initial (I) and total (T) activities of HMG-CoA reductase (μ mol of mevalonate formed/min per mg of microsomal protein, at 37°C) in microsomes isolated from homogenates of cold-clamped and minced rat liver samples. See the Materials and methods section for experimental details.

Expt. no.	Cold-clamped samples			Minced samples		
	HMG-CoA reductase activity		I/T ratio (%)	HMG-CoA reductase activity		I/T ratio (%)
	Initial	Total		Initial	Total	
1	0.87	1.39	62.6	0.58	1.34	43.0
2	1.85	2.38	77.8	1.36	1.97	68.7
3	1.22	1.63	68.7	0.73	1.79	40.5
4	1.52	1.95	77.6	1.17	2.25	52.0

Table 2. Comparison of kinetics of pyruvate kinase activity at low phosphoenolpyruvate concentrations in homogenates prepared from freeze-clamped, cold-clamped and minced liver samples

Activities at three suboptimal phosphoenolpyruvate concentrations are expressed as a percentage of the maximal activity measured with 3 mM-phosphoenolpyruvate (average activity 46.6 μ mol/min per g of liver at 25°C). The activities of the enzyme in homogenates of freeze-clamped samples which were incubated with a protein-phosphorylating mixture are also given.

Expt. no.	Phosphoenolpyruvate concn. (mM)	Pyruvate kinase activity (% of maximal)			
		Freeze-clamped	Cold-clamped	Minced	Phosphorylated
1	0.6	8.8	10.0	7.8	4.3
	0.8	17.3	19.2	12.3	11.6
	1.0	23.8	23.0	16.8	—
2	0.6	11.6	11.6	9.4	6.8
	0.8	19.1	18.1	13.8	—
	1.0	33.3	32.1	24.3	—
3	0.6	17.2	14.0	5.6	4.4
	0.8	28.6	23.1	13.3	5.7
	1.0	33.2	27.4	22.2	—

difference was solely due to a lower 'initial' HMG-CoA reductase activity in minced samples than in cold-clamped samples (0.96 ± 0.18 and 1.34 ± 0.22 nmol/min per mg of microsomal protein at 37°C respectively; $P < 0.005$, paired t test). The total HMG-CoA reductase activities for microsomes prepared from both types of samples were identical (1.84 ± 0.20 nmol/min per mg of microsomal protein). These experiments illustrated that the longer delay between sampling of the liver and homogenization introduced by mincing of the tissue resulted in a marked decrease in 'initial' activity of HMG-CoA reductase. It is suggested that this decrease is an artefact; other factors that may artefactually depress the initial activity further are discussed by Easom & Zammit (1984).

Preservation of pyruvate kinase kinetics

In these experiments the liver of a rat was sampled in three ways: (i) one lobe was freeze-clamped, (ii) a second lobe was cold-clamped, and (iii) a third was excised and minced (for practical reasons cold-clamping was always performed on the left lobe, but the use of the other lobes was randomized so as to eliminate the possibility of variations between lobes, although no such variations were ever observed). All subsequent treatment of the tissue was identical, except that freeze-clamped tissue was homogenized with a Polytron tissue disintegrator (see the Materials and methods section). The activity of pyruvate kinase of the three samples was measured at several suboptimal concentrations of phosphoenolpyruvate (see Table 2) and at 3 mM-phosphoenolpyruvate, which was ascertained to measure maximal activity of the enzyme. In addition, the activity of pyruvate kinase in a portion of the freeze-clamped sample homogenate which had been incubated with a protein-phosphorylating mixture (see the Materials

and methods section) was also measured at the same phosphoenolpyruvate concentrations to obtain information about the kinetics of the fully phosphorylated enzyme with respect to phosphoenolpyruvate concentration. The results of three such experiments are given in Table 2; activities of pyruvate kinase at suboptimal phosphoenolpyruvate concentrations are expressed as a percentage of the maximal activity. It is evident that, whereas the kinetics of pyruvate kinase at suboptimal phosphoenolpyruvate concentrations were very similar in homogenates of freeze-clamped and cold-clamped samples, those for the minced liver samples were very different, and were much more similar to those of the fully phosphorylated enzyme. These results demonstrated that cold-clamping preserved almost intact (compared with freeze-clamping) the activity state of pyruvate kinase *in vivo*, and therefore appeared to be an equally effective sampling technique in minimizing the artefactual apparent increase in phosphorylation of pyruvate kinase that occurred in the time taken to mince the tissue. If the rapidity of change in phosphorylation status of pyruvate kinase during sampling is representative of that of other enzymes that are regulated by phosphorylation-dephosphorylation, the above results would suggest that cold-clamping is as valid a technique as freeze-clamping for the sampling of tissue *in situ* in studies on the effects of different physiological conditions on the activity state of these enzymes *in vivo*. Both techniques are superior in this respect to excision and mincing of the liver followed by homogenization.

Assessment of mitochondrial integrity in cold-clamped samples

Two criteria were used to assess the mitochondrial integrity preserved in homogenates of cold-clamped liver samples.

(i) Latency of mitochondrial enzymes. The activities of both citrate synthase and glutamate dehydrogenase were almost completely latent in homogenates of cold-clamped rat liver samples. Routinely approx. 3% of the total activity of both enzymes was overt in the unsonicated whole homogenates and in the postmitochondrial supernatants (results not shown). Therefore the integrity of the mitochondrial inner membrane was largely preserved with respect to accessibility of the respective substrates to the two enzymes, and of the leakage of glutamate dehydrogenase from the mitochondrial matrix.

(ii) Respiratory control of mitochondria. This was assessed by measuring State-3 and State-4 rates of respiration for mitochondria isolated from cold-clamped liver samples with glutamate and malate as substrates. Both the absolute rates of oxygen consumption (56.8 ± 8.0 nmol of O_2 /min per mg of mitochondrial protein at 37°C , $n = 6$) and the State-3/State-4 ratio (average 7.5, for six determinations; range 4.0–12.5) were similar to values obtained routinely in this laboratory for mitochondria isolated after conventional liver sampling, mincing and homogenization (see, e.g., Zammit, 1980; Robinson & Zammit, 1982).

General considerations

The cold-clamping technique described for rat liver in the present paper provides rapid sampling, cooling and homogenization of liver, with apparent retention of structural and functional integrity of the mitochondria. The technique appears to be sufficiently rapid to prevent any appreciable change in the phosphorylation status of at least one cytosolic enzyme, pyruvate kinase. It also yields high initial/total-activity ratios for another (microsomal) enzyme regulated by phosphorylation–dephosphorylation, HMG-CoA reductase. Therefore the method is suited to the study of changes in initial/total-activity ratios for enzymes which cannot be assayed in crude homogenates (obtained after freeze-clamping) owing to interference from the activity of other enzymes released from different (disrupted) organelles. In the accompanying paper (Easom & Zammit, 1984) we describe a study in which the technique was used to study the diurnal variation in initial/total-activity ratio of HMG-CoA reductase, which cannot be assayed in crude homogenates of liver because of interference from the mitochondrial enzyme HMG-CoA lyase (see the introduction and Easom & Zammit, 1984).

The retention of good respiratory control and full latency of inner-membrane-matrix enzymes by mitochondria isolated from homogenates of cold-clamped samples extends the possible fields of

hepatic metabolic studies to which the cold-clamping technique could be applied. Thus it should be possible henceforth to study metabolic processes in isolated mitochondria obtained after rapid-cold-clamping and differential or gradient centrifugation. One example of a potential application of the new technique is that of the regulation of overt carnitine palmitoyltransferase activity, which has been found to undergo time- and temperature-dependent changes in its affinity for malonyl-CoA (Zammit, 1983, 1984). The cold-clamping technique could be useful in preserving the relative amounts of different-affinity forms of this enzyme which may occur in the liver *in vivo*. Similarly the ability rapidly to prepare different cell fractions from cold-clamped rat liver samples could enable the sampling of liver *in situ* and measurement of the relative amounts of soluble and membrane-bound forms of enzymes, the regulation of which may be mediated partly by reversible interconversion between soluble and membrane-bound forms (Wilson, 1978), e.g. CTP:cholinephosphate cytidylyltransferase (Vance & Pelech, 1984).

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