A Cryobiopsy Technique for Assessing Metabolite Levels in Skeletal Muscle (33790)

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The *in vivo* concentrations of adenosine triphosphate (ATP), phosphocreatine (PC), and lactic acid are difficult to assess in skeletal muscle. Even when animals are anesthetized, intubated with endotracheal tubes, and given positive pressure respiration during and after administration of *d*-tubocurarine chloride, subsequent biopsy samples are especially variable (1) in concentration of PC and lactic acid. A considerable decrease in PC and increase in lactic acid may occur during the excision of a sample and prior to cessation of its metabolic activity by freezing in liquid nitrogen or isopentane. The use of a scalpel causes twitching in the muscle, which is accompanied by some breakdown of ATP (2, 3) and a concomitant utilization of PC in the resynthesis of the ATP (4). Additionally the amount of time required to excise a muscle sample and terminate its metabolic activity, is somewhat variable.

The present cryobiopsy technique makes it possible to (a) eliminate trauma, and resultant twitching and bleeding, (b) terminate metabolic activity of the tissue before taking the tissue from its blood supply, (c) provide a relatively large sample size, and (d) conduct the operation by personnel untrained in surgical procedures.

It is the purpose of this manuscript to describe the cryobiopsy technique as well as to compare PC, ATP, and lactic acid levels in skeletal muscle samples taken by cryobiopsy with those taken through normal biopsy procedures.

Description of the cryo-probe. The Freon cooled cryo-probe herein described is a modification of the original probe developed for other applications (5). The purpose of the original development was to provide the surgeon with a less costly, inherently safer cryo-probe than the liquid nitrogen units now available.

In this cryo-probe the liquid Freon (Freon-22) is moved by gravity from the supply bottle to the probe (Fig. 1). The

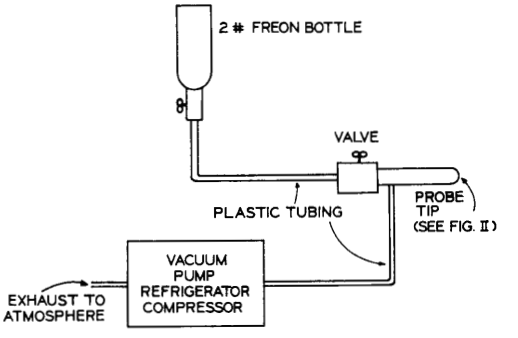

FIG. 1. Diagram of equipment placement.

probe contains an on–off valve which allows the liquid Freon to flow through a centrally located hypodermic tubing (0.026 in. i.d.) to the probe tip. The hypodermic tubing (not the on–off valve) controls the rate of Freon flow by imposing a large pressure drop on the liquid Freon (Fig. 2). This drop in pressure

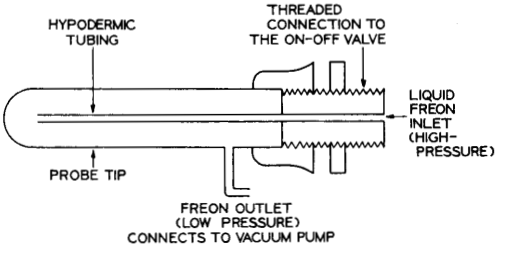

FIG. 2. Diagram of cryo-probe.

represents the pressure differential from the supply bottle to the probe tip (a Freon-22 bottle temperature of 70°F yields a pressure of 140 psia).

Pressure in the probe tip is controlled, which also fixes the temperature. Exhausting to the atmosphere yields a probe temperature

of —40°F for Freon-22, and if, as was done for all the work reported herein, a vacuum pump maintains a pressure of 3 psia, the probe will be at —95°F. Varying the tip pressure thus allows a wide selection of temperatures, and therefore the size of the cryobiopsy sample that adheres to the probe.

The temperatures obtained by this process using Freon-22 are not unusual, rather they are quite typical of refrigerants. Freon was selected for its safety features. It is not flammable (as are propane and methyl chloride), nor is it toxic (as are ammonia and sulfur dioxide).

Time of application of the probe also affects the depth of freezing. Observations show that freezing penetration is quite rapid for the first 10 sec and then drops off rapidly. For the purpose of assessing metabolite levels, rapidly frozen tissue is preferred, and the preferential method of increasing sample size is to lower probe tip pressure (temperature) rather than lengthen the time of freezing.

Description of technique. All animals selected for cryobiopsy should be anesthetized and cleaned. Whether the animal will be permitted to recover from the operation or be dispatched, will influence the degree of equipment and facility sterilization that will be required. When large animals are used, hair can be easily shaved from the sample site. A small incision can be made in the skin. If the animals have large quantities of subcutaneous fat, this latter tissue should also be pierced to allow ready access to the surface of the muscle.

The probe is manually inserted into the tissue and the flow of Freon is initiated. If the probe is operated in this position for 10–25 sec at —95°F, a frozen cylinder of tissue, about twice the diameter of the probe, will adhere to the probe. A knife, fixed to the probe can be rotated around the outer surface of the frozen tissue. Because of mechanical adherence of ice to the probe, the frozen cylinder of tissue can be easily removed from the muscle. The probe with the frozen tissue attached is then further cooled by submersion in liquid nitrogen. Subsequently the frozen tissue is fragmented and powdered (6) for extraction. Various dimensions of probes can be used to obtain different quantities of tissue.

Utilization of cryobiopsy technique. When a muscle becomes anoxic, it changes from partially aerobic to completely anaerobic metabolism and produces lactic acid via glycolysis. It is also known that after muscle tissue is removed from an animal this production of lactate will continue until the supply of glycogen is exhausted, or glycolysis is inhibited. Simultaneously PC depletion occurs and the ATP concentration diminishes rapidly. When a biopsy is taken by conventional methods (7) it is subjected to excision stimulation and excision anoxia before it can be frozen. Consequently, a sample obtained through conventional biopsy may be greatly altered in PC and lactate. In view of this difficulty we developed the herein described cryo-probe technique to facilitate the estimation of the true *in vivo* levels of PC, ATP, and lactic acid in skeletal muscle.

To test the effectiveness of this cryo-probe technique, we compared all samples with samples simultaneously excised by conventional means in the opposite muscle. Four stress-susceptible (8) pigs were used in these initial trials. All animals were anesthetized with 0.35 ml/kg of body weight of a 50/50 mixture of Dial with Urethane and Nembutal. The pigs were allowed to remain in an anesthetized state for 15 min before any biopsies were taken and periods of at least 10 min were permitted before successive biopsies. In all instances cryobiopsy and conventional biopsy were simultaneously taken from the same location in opposite longissimus muscle.

The conventional method of biopsy can be simply described as follows: An incision was made through the skin and fat over a particular area of the longissimus. A 1-in. cube of tissue was excised with a scalpel and immediately immersed in liquid nitrogen.

All frozen tissues were powdered for extraction according to Borchert and Briskey (6) and the enzyme assays for lactate were carried out according to Hohorst (9), phos-

phocreatine according to Lamprecht and Stein (10), ATP according to Lamprecht and Trautschold (11), and as modified by Kastenschmidt *et al.* (12). The results from the analyses of these samples are shown in Table I.

TABLE I.

Metabolite (μm/g of tissue)	Biopsy technique[a]		
	Conventional		Cryobiopsy
ATP	4.8	($p < 0.02$)[b]	5.2
PC	9.8	($p < 0.01$)	16.5
Lactic acid	14.0	($p < 0.01$)	5.4

[a] Paired observations.

[b] Significantly different, at designated levels of probability, from samples taken by cryobiopsy.

The ATP and phosphocreatine levels were significantly higher in the samples taken through cryobiopsy, while lactic acid concentrations were significantly lower. Even in the brief period involved in the biopsy by conventional procedures, the samples apparently suffer sufficient excision anoxia to double the production of lactic acid and breakdown sizable quantities of phosphocreatine and significant amounts of ATP.

It is readily apparent that the utilization of this cryobiopsy technique enables the researcher to obtain tissue samples which reflect levels of high energy phosphates and lactic acid more nearly like those in the resting tissue than previously obtainable by conventional methods. The samples are frozen as the blood supply is cut off, thus eliminating any period of anoxia during which the tissue could metabolize anaerobically. Twitching and bleeding has been almost completely eliminated when the cryo-probe is properly used. The cryo-probe is easy to use and the biopsy can be performed by a relatively untrained operator. Furthermore, this equipment is relatively inexpensive to construct and operate and nonhazardous during use.

Although we have used this new technique to determine *in vivo* levels of ATP, phosphocreatine, and lactic acid, in the pig, it can easily be modified to take samples from animals of any size. By varying the size of the probe, samples of nearly any size may be removed. Furthermore, because there is no residual bleeding at the biopsy site, this technique can be used to obtain samples from animals during experimentation which are not to be sacrificed. The use of the cryo-probe is recommended when assays are to be conducted for muscle constituents which are affected by either stimulation or anaerobic metabolism.

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