AN ABSTRACT OF THE THESIS OF

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Title: EFFECT OF PRERIGOR PRESSURIZATION ON POSTMORTEM BIOCHEMICAL CHANGES IN BEEF MUSCLE.

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Prerigor pressurization of bovine semimembranosus muscle at 103.5 MNm^{-2} (15,000 psi) for two min at 35°C significantly decreased ($P<0.01$) muscle pH, and significantly ($P<0.01$) increased the rate of glycogen degradation during the first 4 hr postmortem.

Glycogen levels of pressurized muscle were significantly ($P<0.01$) lower than those of the control at 1, 2 and 4 hr postmortem. Glucose concentrations were significantly ($P<0.01$) higher in the treated muscle than in the control at all sampling periods. Until 4 hr postmortem the level of glucose-6-phosphosphate was higher in pressure-treated muscle than in the control. Lactate content increased rapidly after pressurization (1 hr postmortem), resulting in an immediate drop in pH; however, there was no significant difference between control and treated muscles at 24 hr. Pressurized muscle showed a significant ($P<0.01$) increase in lactate dehydrogenase activity over that of the control muscle at 1 hr but not at 24 hr.

Pressurization accelerated catabolism of both creatine phosphate and adenosine triphosphate which resulted in a higher ATP turnover value than that of the control. R-values (a measure of metabolic rate) were significantly correlated ($r=-0.95$) with degradation of ATP.

CPK activity was significantly higher ($P<0.01$) in pressurized muscle than in the control at 1 hr postmortem but not at 24 hr.

Concentrations of ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), bases and nucleosides (adenosine, adenine, inosine, hypoxanthine) and nicotinamide adenine dinucleotide (NAD) were determined by high pressure liquid chromatography. Pressurization completely depleted the ATP supply and increased the IMP concentration at 1, 4 and 24 hr postmortem. At 24 hr postmortem, control samples had more ADP whereas the AMP concentration was higher in the pressurized samples at 1 hr postmortem but not at 24 hr. Concentrations of bases and nucleoside metabolites and NAD did not differ between treatments.

Effect of Prerigor Pressurization on Postmortem Biochemical Changes in Beef Muscle

by

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CONTRIBUTION OF AUTHORS

Dr. W. H. Kennick provided the pressure chamber for use throughout the study.

Dr. E. A. Elgasim helped in supplying samples for the experiment and in operating the pressure chamber.

Dr. D. Goldman provided technical assistance in the operation of the high performance liquid chromatography equipment.

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EFFECT OF PRERIGOR PRESSURIZATION ON POSTMORTEM BIOCHEMICAL CHANGES IN BEEF MUSCLE

INTRODUCTION

Tenderness is an important textural characteristic which has a considerable influence on consumer acceptance of meat. A major problem of the fresh meat industry is the frequent occurrence of beef carcasses having tough meat that require a longer period of aging to achieve an appropriate level of tenderization. Lengthy aging periods are expensive since they increase the energy costs of refrigeration. Prolonged aging often results in excessive loss of weight by evaporation and, in some cases, microbial spoilage may occur in the form of surface slime. Problems mentioned above stem from the adverse economic conditions of the past decade of sharply rising costs of energy. This has resulted in an increasing trend of more forage feeding and less feedlot grain-finishing of market beef cattle. These animals are older at time of slaughter and meat toughness usually increases with maturity. Moreover, carcasses of such animals have less fat covering and thus, more tenderness problems are likely to arise during conventional chilling in the form of cold shortening. The latter takes place when the temperature of carcasses drops below 15°C before the onset of rigor mortis. When this occurs, muscles shorten and a more severe type of rigor is initiated resulting in a significant toughening of meat. This toughness resists tenderization that occurs naturally during the normal aging process.

Within the food industry, meat processing is the leading energy consumer using more than 99 trillion BTU in 1973 (Unger, 1975). In

view of the above problems, new tenderizing techniques capable of ensuring tenderness at acceptable levels need to be developed. Hydrostatic pressurization of prerigor meat is a relatively new method that tenderizes beef significantly (MacFarlane, 1973; Kennick et al., 1980). Under this procedure the major muscle masses are removed immediately after evisceration and washing of the carcasses. The meat is then vacuum packed, pressure treated at 15,000 psi for 2 minutes at 37°C, chilled and boxed for market distribution. This procedure also may result in substantial savings in both space and energy. A 30% reduction in energy requirements for refrigeration may be realized because only the edible meat is chilled and refrigerated while bones, trimmings, etc. are channelled immediately to rendering operations. This minimization of energy consumption through utilization of prerigor pressure treatment has potential economic and energy benefits to meat industry.

The application of hydrostatic pressure on biological systems is not new (Johnson et al., 1954; Johnson and Eyring, 1970). In meat research, several investigators have employed hydrostatic pressures. MacFarlane (1973) reported that pressures of 103-138 MNm^{-2} at 30-35°C resulted in a rapid drop in muscle pH, indicating that pressurization accelerated glycolysis. Pressurization was also noted to exert a substantial tenderizing effect. Previous workers (Johnson et al., 1954; Brown, 1957; Johnson and Eyring, 1970) found that hydrostatic pressure influenced the development of tension in muscle. Tension increased or decreased depending upon the temperature at which the muscle was pressurized. In other studies, the disruptive effects

of pressurization on myofibrillar structure were observed which was not unexpected in view of the known sensitivity of the constituent proteins to disassociate upon the application of pressure (Ivanov et al., 1960; Ikkai and Ooi, 1966, 1969; Joseph and Harrington, 1966, 1967, 1968; MacFarlane, 1974; MacFarlane and McKenzie, 1976). In spite of these changes, Elgasim et al., (1982) recently reported prerigor pressurization did not detrimentally affect the functional property of emulsification capacity of muscle proteins. Horgan (1981) found that pre- and post- rigor pressurization caused loss of extra ATPase (Ca^{2+}-dependent) activity while basal ATPase (Mg^{2+}-dependent) activity was relatively unaffected. However, proteolytic degradation of some sarcoplasmic reticulum (SR) proteins, including the 100,000 dalton ATPase and calsequestrin, was observed. The yield of SR was greatest in those muscles showing higher calcium uptake activities and having high concentrations of extra ATPase. It was postulated that destruction of SR by pressurization released Ca^{2+} into the sarcoplasm which, in part, accounts for the enhanced rate of glycolysis.

The mechanism(s) by which pressurization improves meat tenderness has not been established conclusively. Bouton et al. (1977) proposed that pressure causes the myofibrillar proteins to dissociate, and when accompanied by heat, the proteins denature so they are unable to reassociate upon release of pressure. Late in 1980, Bouton et al. indicated that aging of meat disrupted structures that were also disrupted by the pressure-heat treatment. However, this treatment had no effect on the myofibrillar components of aged meat. MacFarlane and

Morton (1978) reported the absence of the M-line, loss of integrity and aggregation of the I-band filaments as a result of post rigor pressure treatment. In an attempt to reveal the importance of both the I-band and M-line proteins in respect to mechanical properties of post rigor muscle, MacFarlane et al. (1981) reported a loss of integrity of I-band filaments and a reduction of material from the region of the M-line with the post rigor pressure treatment. Their study supports the theory for contraction state toughness proposed by Voyle (1969) in which increasing toughness is caused by an increasing incidence of sarcomeres in which thick filaments have been compressed onto the Z-line, thus removing the I-band as a zone of weakness. In pre-rigor pressurization, MacFarlane et al. (1976) proposed two explanations for the tenderizing effect on muscle. Either the myofibrillar structure was disrupted by myosin filaments of the severely contracted muscle being forced into the Z-line or the actin filaments were weakened by a pressure-induced F-G transformation of actin. Bouton et al. (1977) reported that prerigor pressurization caused semitendinosus muscles to contract by 40% and maximum tenderization was achieved when the samples were heated at 45°C for 45-180 minutes immediately before application of the pressure-heat treatment (150 MNm^{-2} at 60°C for 30 min.). They concluded that pressure-heat treatment primarily affected the myofibrillar structure. Again, MacFarlane and Morton (1978) suggested a weakening of thin filaments and M-line bridges when combined with a pressure induced contraction, facilitated disruption of prerigor pressure-treated muscle. Recently, Elgasim and Kennick (1982) studied the effect of

prerigor pressurization on meat microstructure. They found several physical changes which include separation of sarcolemmal and endomysial sheaths, contraction bands, disruption of myofibrillar structure, and increased interfibrillar and intermyofibrillar spaces. At subcellular levels, disappearance of glycogen granules, appearance of swollen mitochondria and sarcoplasmic reticulum were noted. They concluded that the interactions between the chemical and physical effects should account for the tenderizing effect of the pressure treatment.

Knowledge of biochemical changes is very important because postmortem metabolism plays a major role in the conversion of muscle to meat and in determining the subsequent meat quality. The synthesis of adenosine triphosphate (ATP) through the tricarboxylic acid (TCA) cycle in muscle ceases after death due to termination of blood supply. At first, the level of ATP in muscle is maintained by conversion of adenosine diphosphate (ADP) to ATP at the expense of creatine phosphate (CP), but when CP is exhausted, the ATP level falls. The loss of ATP also triggers the anaerobic conversion of glycogen to lactate with the result that after 24 hours the pH declines to about 5.5 (Bendall, 1973; Penny, 1980). However, when the level of ATP can no longer be maintained from CP and glycogen sources, due either to the amount being reduced to a critical level or to a decrease in the activity of the enzymes associated with glycolysis, the muscles start to contract and attain an inflexible rigid state, known as rigor mortis (Bendall, 1973).

A number of factors determine the overall rate of glycolysis. These include the type of carcass, antemortem treatments, the physiological and hormonal status of the animal (Honikel, 1976; Wismer-Pederson, 1976; Bendall, 1979), the temperature at which the carcass is held (Okubanjo and Stouffer, 1975; Bendall, 1978; Honikel and Hamm, 1978; Nuss and Wolf, 1981; Jolley et al., 1981), subcutaneous fat cover (Smith et al., 1976), nutritional status of the animal (Asghar and Yeates, 1979) and prerigor treatments of carcasses (Hamm, 1982).

The rate and extent of ATP breakdown has great influence on meat quality. ATP can be sequentially degraded to ADP, adenosine monophosphate (AMP), inosine monophosphate (IMP), and inosine which is eventually converted by a much slower process into hypoxanthine. The concentration of ADP, nicotinamide adenine dinucleotide (NAD), and NADH decreases, while that of IMP, inosine, hypoxanthine (Tsai et al., 1972), lactic acid, orthophosphate, and reducing sugars increases in postmortem muscle (Bodwell et al., 1965). Two important features underlie nucleotide metabolism in muscle postmortem. Firstly, stiffening of muscle postmortem is related to a decrease in ATP content (Bendall, 1973). Secondly, there are indications that nucleotides contribute to flavor perception and desirability. In this respect, IMP and inosine have been implicated as compounds which contribute to the flavor of meat (Batzer et al., 1962; Kuninka et al., 1964; Shimazono, 1964; Dannert and Pearson, 1967). It is possible that pressurization may change the concentrations of these flavor compounds.

Early postmortem changes in muscle influence the tenderness and water holding capacity (Hamm, 1982). Hydrostatic pressurization of prerigor muscle has profound effects on physico-chemical properties of meat. A rapid decline in muscle pH and higher tenderness ratings for the pressure-treated meat have been reported by MacFarlane (1973) and Kennick et al. (1980).

In view of previous investigations on the pressurization of meat, it is apparent that pressurization of prerigor muscle can markedly influence the contraction state of the muscle and biochemical reactions thereby affecting eating quality and acceptability of meat. Since very little research has been completed on the biochemical aspects occurring and resulting from the prerigor pressurization of meat, the present work was undertaken with the following objectives:

1. To evaluate some of the changes occurring in the glycolytic metabolites and lactate dehydrogenase activity of pressure-treated beef muscle.
2. To determine the effect of high pressure treatment on the degradation of ATP, CP and the activity of creatine phosphokinase.
3. To establish quantitative information about the concentration of the nucleotides and similarly related compounds at various times postmortem in prerigor pressurized muscle.

II. EFFECT OF PRERIGOR PRESSURIZATION ON POSTMORTEM BOVINE MUSCLE LACTATE DEHYDROGENASE ACTIVITY AND GLYCOGEN DEGRADATION

EXPERIMENTAL

Sample Preparation and Treatment

Muscle samples were obtained from eight steers (approx. wt. 470-550 kg) of Good and Choice USDA quality grades, slaughtered at the Oregon State University Meat Science Laboratory, using normal commercial procedures.

Prerigor semimembranosus (SM) muscle was excised from one side of each carcass 30-40 min. after slaughtering, vacuum packed in Cryovac bags and placed in the pressure chamber (30.5 cm diameter and 60.9 cm long). The chamber was filled with warm water (37°C), tightly closed, and a pressure of 103.5 MNm^{-2} (15,000 psi) was applied and maintained for 2 min according to the procedure of Kennick et al. (1980). The matching muscle (untreated) on the opposite side of each carcass was left on the carcass and stored along with the treated sample at 1±1°C. Samples from both treatments were removed at 1, 2, 4 and 24 hr postmortem and were immediately frozen in liquid nitrogen and stored at -40°C for analyses of metabolic intermediates. Samples for determination of lactate dehydrogenase (LDH) activity were removed from fresh muscles of both treatments at 1 and 24 hr postmortem.

Analysis of Metabolic Intermediates

Three grams of pulverized muscle (Borchert and Briskey, 1965) was homogenized with 30 ml of 0.6 N perchloric acid in a Tissumizer

(Tekmar) for 60 sec at 4°C. After centrifugation (12,000 x g) for 20 min at 0°C, the resulting supernatant was filtered through Whatman No. 1 filter paper to remove any floating material. After 1 ml of supernatant was removed for glucose determination as described below, the remaining supernatant was neutralized to a methyl orange end-point with 5 M K_2CO_3. The resulting potassium perchlorate was allowed to precipitate for 20 min in an ice bath. The supernatant was filtered through Whatman No. 5 filter paper and used for subsequent enzymatic analyses of glycolytic metabolites.

Glucose-6-phosphate (G-6-P) and lactate contents were determined by the methods described by Lang and Michal (1974) and Gutmann and Wahlefeld (1974), respectively.

For the glucose determination, 0.2 ml of unneutralized acid supernatant was assayed by a colorimetric procedure utilizing peroxidase/glucose oxidase enzymes (Sigma Chemical Company, 1982).

Glycogen was isolated by treating pulverized samples with the potassium hydroxide-ethanol procedure dexcribed by Dalrymple and Hamm (1973). The isolated glycogen was solubilized in distilled water and the amounts determined by the method described by Dubois et al. (1956) using phenol-sulphuric acid.

Measurement of pH

The pH of samples was measured with a Corning pH Meter, Model 125, fitted with an Orion Combined Glass Electrode. The glass electrode was inserted into a freshly made incision (approx. 3-5 cm deep) each time pH was taken, at 1, 2, 4 and 24 hr postmortem.

Measurement of Lactate Dehydrogenase Activity

Fresh muscle samples were homogenized in 0.01 M Tris-HCl buffer containing 0.15 KCl and 2 mM EDTA, pH 7.4. The homogenates were centrifuged at 105,000 x g for 30 min at 4°C. Supernatants were diluted 100-fold with cold buffer immediately before use. LDH activity was determined according to the method described by Bergmeyer and Bernt (1974) except that the substrate concentration was 2mM.

Statistical Analysis

Data were subjected to analysis of variance to calculate standard errors and least significant differences (LSD) at the 1% level of significance (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Data of postmortem pH decline in pressurized and control SM muscle are shown in Table 1. Treated muscle had significantly ($P< 0.01$) lower pH values than the controls at 1, 2 and 4 hr but not at 24 hr postmortem. These chagnes in pH followed a pattern similar to those reported by MacFarlane (1973) and Kennick et al. (1980) in their studies on the pressurization of muscle.

The rapid drop of pH in pressure-treated muscle was accompanied by decreasing levels of glycogen while levels of glucose and G-6-P increased in the first 4 hr postmortem period (Figure 1). Glycogen levels were significantly lower ($P<0.01$) at 1, 2 and 4 hr postmortem in the pressurized muscle than in the control. At 24 hr postmortem there was no significant difference between treatments. The overall breakdown of glycogen from 1 to 24 hr postmortem was much slower in the pressurized sample (8.58 μmoles of glucose/g tissue) than in the control (31.15 μmoles/g tissue).

Higher levels of G-6-P were found in the pressurized muscle than in the control at 1, 2 and 4 hr postmortem (Figure 1). The concentration of G-6-P did not increase in the pressure-treated muscle after 4 hr postmortem since the values at 4 and 24 hr postmortem were essentially identical. However, the level of G-6-P increased steadily in the control muscle after 2 hr postmortem. The profile of G-6-P concentration (Figure 1) for the control muscle is similar to that reported by others (Cassens and Newbold, 1966; Newbold and Scopes, 1967; Nuss and Wolfe, 1981).

Table II.1. Effect of prerigor pressurization on pH of bovine semimembranosus muscle.

Hours Postmortem	Mean[a] pH		
	Control	PRP[b]	LSD[c]
1	6.73	5.78	0.21
2	6.37	5.76	0.21
4	6.06	5.69	0.21
24	5.71	5.61	

[a] Mean of seven animals

[b] PRP = Prerigor Pressurization (103.5 MNm^{-2}, 37°C, 2 min).

[c] Least significant difference at $P<0.01$

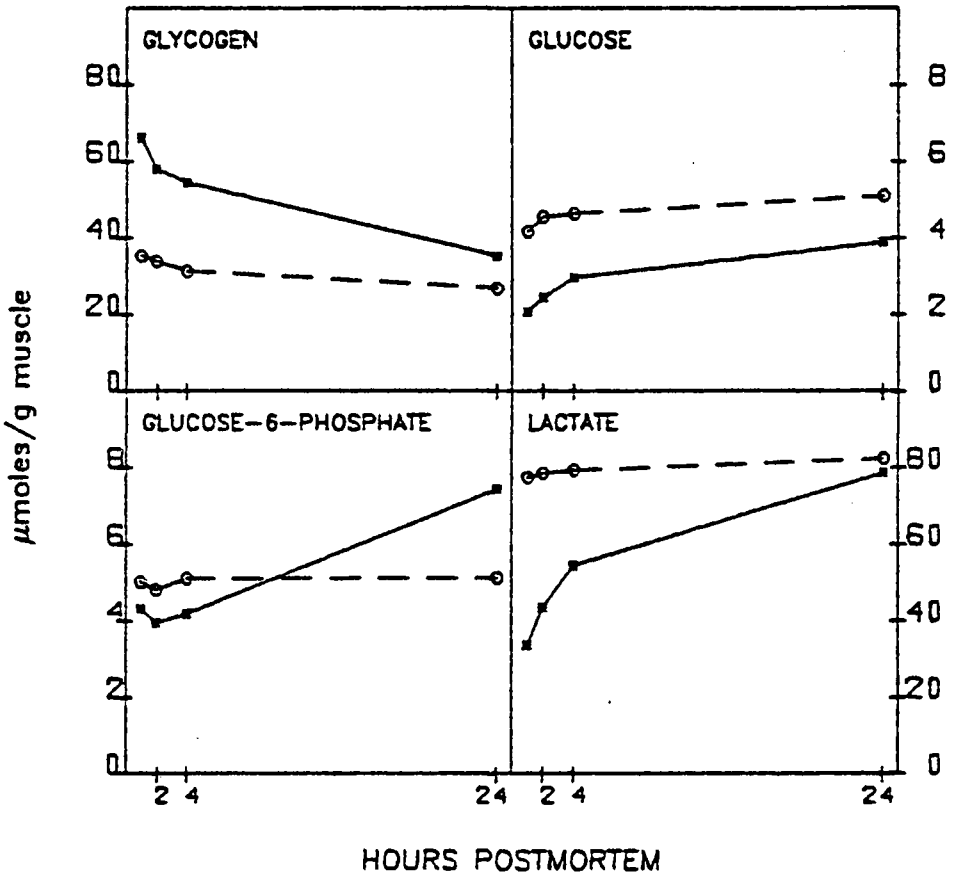

Figure II.1. Postmortem changes of glycolytic intermediates in prerigor pressurized (broken line) and control (solid line) muscles. Glycogen is expressed in glucose units.

The decrease in glycogen level in combination with an accumulation of G-6-P immediately after pressurization indicates stimulation of the phosphorylase system. This stimulation could be initiated by an accelerated release of calcium ions from the sarcoplasmic reticulum and/or mitochondria as may occur in PRP muscle (Horgan, 1981; Elgasim and Kennick, 1982). In addition, the activation of phosphorylase might also be due to the faster disappearance of adenosine triphosphate (ATP) postmortem (Kastenschmidt et al., 1968; Fischer and Hamm, 1980). According to the results discussed in Section III (Figure III.1 and III.2), the concentrations of creatine phosphate (CP) and ATP had decreased very rapidly by 1 hr postmortem in the pressurized muscle.

Glucose levels increased with time postmortem in both control and pressure-treated muscles (Figure 1). However, glucose concentrations were significantly higher ($P < 0.01$) in the treated samples throughout the 24 hr postmortem period. It is likely that the amylolytic route accounted for some portion of glycogen degradation in pressure-treated muscle. In a study concerning the technological significance of free glucose and sugar phosphates in dehydrated meat, Sharp (1962) reported that the postmortem breakdown of residual glycogen can be performed by a system alternate to the glycolytic cycle. This alternate system, which includes amylo-1, 6-glucosidase, resulted in the accumulation of free glucose postmortem.

The control muscle had a lactic acid concentration of 33.51

moles/g at 1 hr postmortem (Figure 1) while the pressurized muscle had a value of 77.41 μmoles/g at the same time frame. The value for the latter was 2.31 times greater than that of the control. A further but much slower rate of increase in lactic acid content was observed up to 24 hr postmortem in the pressurized muscle, whereas that for the control increased sharply. These findings are consistent with the glycogen and pH data discussed earlier.

The activity of LDH was measured because of its importance in lactic acid formation, which induces the fall in pH postmortem. Values of LDH activity are presented in Figure 2. Pressurized muscle had significantly greater ($P < 0.01$) LDH activity than the control at 1 hr postmortem. At 24 hr, LDH activity values were similar for both treatments. The enzyme was uniformly reactive at both times postmortem in each treatment. The reason for the increased LDH activity in pressurized muscle at 1 hr postmortem is not clear. There have been several reports on the binding-solubilization of skeletal muscle LDH. Solubilization was enhanced by an increase of NADH or NADPH (Hultin et al., 1972). On the other hand, enzyme binding to muscle particulates could exert an important influence on the function of the pathway. In association with an accelerated glycolytic rate on electrical stimulation of muscle, Clarke et al. (1980) reported a significant increase in the binding of certain glycolytic enzymes, one of which was LDH. It is, however, not clear whether the increased enzyme binding was the cause of the increased glycolytic rate in the electrically stimulated muscles, even though kinetic properties of several key glycolytic enzymes are known to be modified significantly

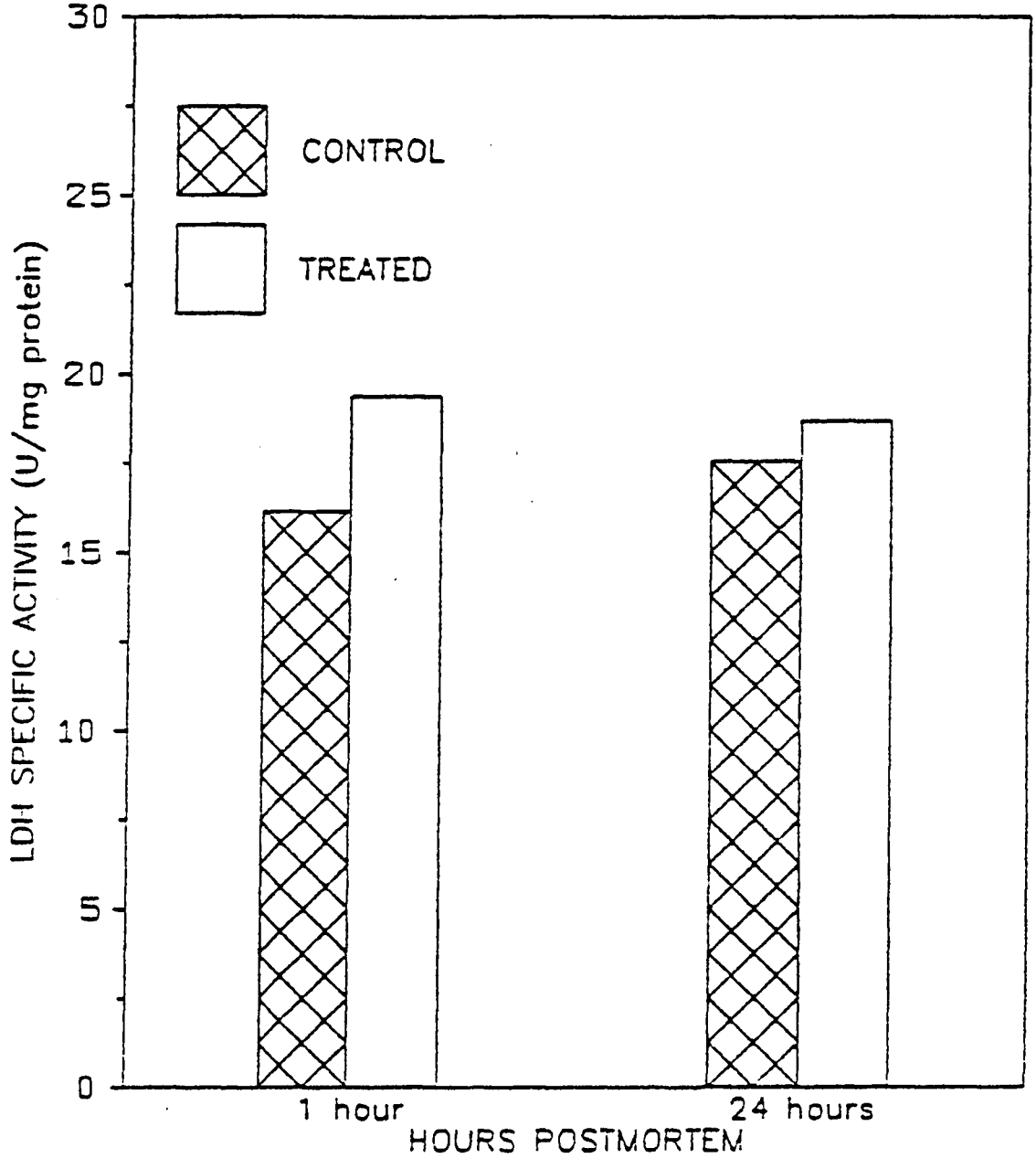

Figure II.2. Specific activity of lactate dehydrogenase in pressure-treated and control muscles.

on binding to subcellular particulate matter (Walsh et al., 1977; Dagher and Hultin, 1975; Karadsheh and Uyeda, 1977). Although our experiment does not establish any binding effect, the present data suggest that this effect together with the concentration of metabolites should be given serious consideration in future research when the glycolytic system of pressurized muscle is analyzed.

III. INFLUENCE OF PRERIGOR PRESSURIZATION ON POSTMORTEM BEEF MUSCLE CREATINE PHOSPHOKINASE ACTIVITY AND DEGRADATION OF CREATINE PHOSPHATE AND ADENOSINE TRIPHOSPHATE.

EXPERIMENTAL

Source of Muscle

Prerigor semimembranosus (SM) muscles were obtained from 8 steer carcasses of Good and Choice USDA quality grades (approx 470-550 kg) processed at the Oregon State University Meat Science Laboratory. The muscles were excised from one side of each carcass, vacuum packed in Cry-O-Vac bags and then pressurized at 103.5MNm^{-2} (15,000 psi) for 2 min at 35°C (Kennick et al., 1980). The matching muscle on the companion side was left intact on the carcass and conventionally chilled at 1°C until sampled at appropriate times postmortem.

ATP and CP Determinations

Samples were excised for ATP, CP and R-value analyses at 1, 2, 4 and 24 hr postmortem, frozen in liquid nitrogen and stored at -40°C. Three grams of frozen-pulverized muscle (Borchert and Briskey, 1965) were extracted with 30 ml of 0.6 M perchloric acid in a Tissumizer (Tekmar) operated intermittently for 60 sec at 4°C. After centrifugation at 12,000 X g for 15 min at 0°C, the clear supernatant was neutralized to a methyl orange endpoint with 5 M K_2CO_3. The neutralized extract was allowed to precipitate for 20 min at 0°C prior to filtering (Whatman No. 1 filter paper) for use in assay mixtures.

ATP was determined with the procedure described by Lamprecht and Trautschold (1974). The glucose concentration of the assay mixture was reduced to 25 mM to retard the rate of a "creep" reaction which slowly produces NADPH that interferes with the assay (Tarrant and

Mothersil, 1977). CP was measured by the method described by Lamprecht et al. (1974). Each assay was conducted in duplicate.

Absorbance Ratios (R-values)

The perchloric acid extract prepared for enzymatic assay (0.1 ml) was diluted with 4.9 ml 0.1 M phosphate buffer (pH 7.0) and the absorbance was determined at 250, 258 and 260 nm (Honikel and Fischer, 1977). R-values were calculated as the ratio of absorbance readings at either 250 nm:260 nm or as the 250nm:258 nm absorption ratio. These values are related to the estimation of the inosine/adenosine ratio and to rigor onset in muscle. In the case of postmortem changes in muscle it represents the degree of breakdown of ATP to inosine monophosphate and inosine (Honikel et al., 1980; Calkins et al., 1982; 1983).

Measurement of Creatine Phosphokinase Activity

Homogenates were prepared from fresh muscle at 1 and 24 hr postmortem. Tissue samples were homogenized (1 min) in 10 volumes of 0.01 M Tris-HCl buffer containing 0.15 M KCl and 2 mM EDTA, pH 7.4, and centrifuged at 105,000 x g for 30 min at 4°C. Supernatants were diluted 100-fold with cold buffer before measuring enzyme activity. CPK was determined according to the method described by Forster et al. (1974).

Measurement of pH Value

The pH of the samples was measured at 1, 4 and and 24 hr postmortem using a Corning pH Meter (Model 125) fitted with an Orion combined glass electrode. The glass electrode was inserted into a freshly made incision each time the pH was taken.

Statistical Analysis

Analysis of variance was used to calculate standard errors and least significant difference (LSD) at the 1% level. Correlation coefficients were also included (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

The concentration of ATP and CP as a function of time in pressurized and control muscles are shown in Figures 1 and 2. Pressurization effectively lowered the ATP concentration in the SM muscle (Figure 1). ATP concentration fell very rapidly to a value of 1.95 μM/g at 1 hr postmortem in the pressurized muscle. Concentrations of ATP were significantly ($P<0.01$) lower in the pressurized samples than in the control samples at 2 and 4 hr postmortem. Similarly, rapid depletion of CP occurred in the pressurized muscle (Figure 2). CP concentration was significantly ($P<0.01$) lower in pressurized muscle than in control at 1 and 2 hr postmortem. At 24 hrs only 0.38 μM/g remained in both the PRP and control muscles.

The rate of ATP turnover is important because it reflects the total energy released during contraction. It was calculated (Honikel and Hamm, 1978) as follows:

ATP turnover = $\Delta\{CP\} + 2\Delta\{ATP\} + 1.5\Delta[lactate]$ where $\Delta\{CP\}$ and $\Delta\{ATP\}$ are the decrease of CP and ATP concentrations in the tissue and $\Delta\{Lactate\}$ corresponds to the formation of lactate. Values for the latter were reported in Section II (Figure II.1). In the present experiment, it was not necessary to consider the formation of ATP from CP and ADP by the CPK reaction at the beginning of postmortem measurements (1 hr postmortem) because CP was <3.0 μM/g (Figure 2). Therefore, $\Delta\{CP\}$ can be neglected as soon as the CP level falls below about 3 μmoles/g (Bendall, 1973). Initially, the rate of ATP turnover was very high (37.8 μmoles/g/min) in pressurized muscle and was almost 4-fold higher than that observed by Bendall *et al.* (1976) in

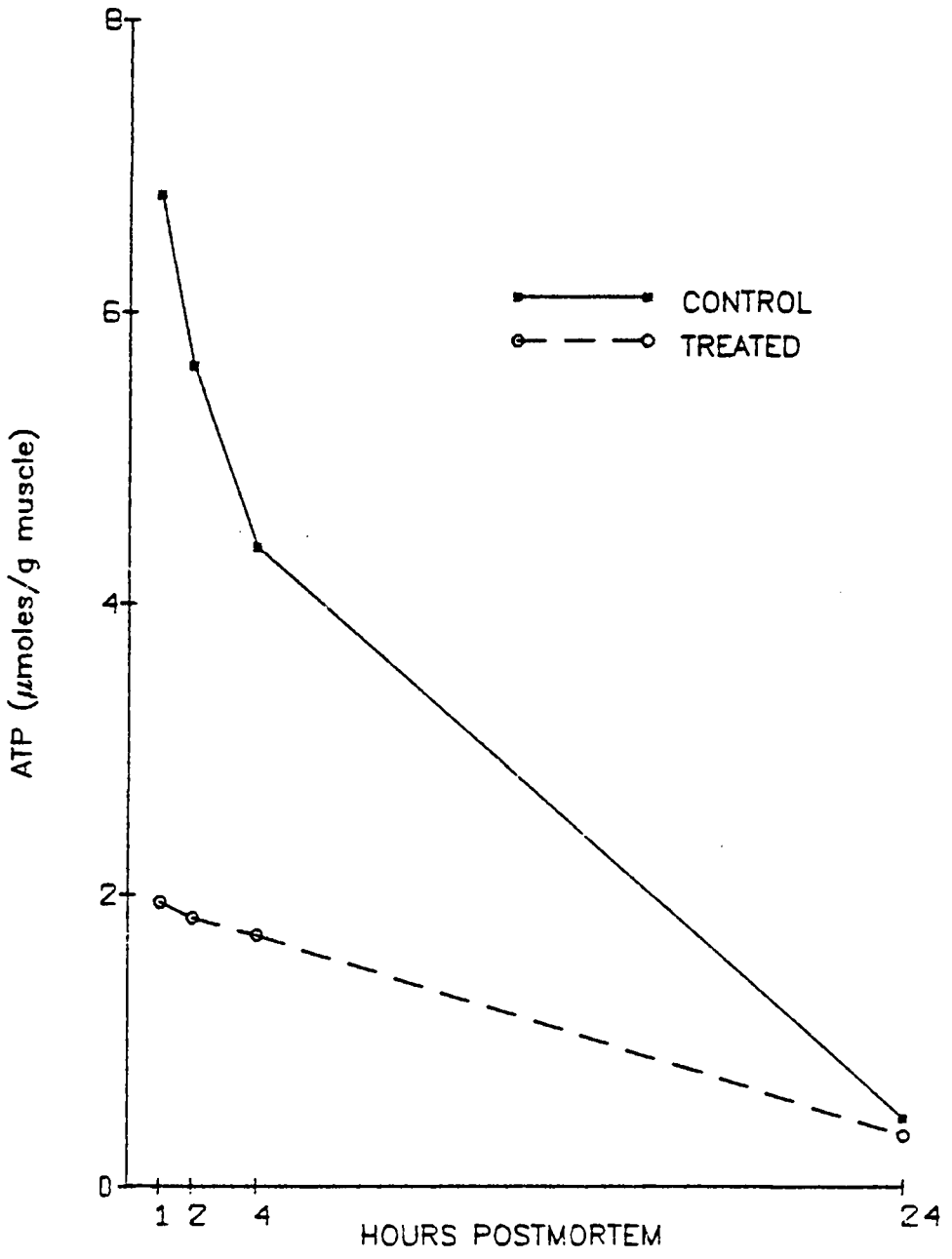

Figure III.1. Postmortem degradation of ATP in control and pressurized semimembranosus muscle. Each point is the average of 8 determinations.

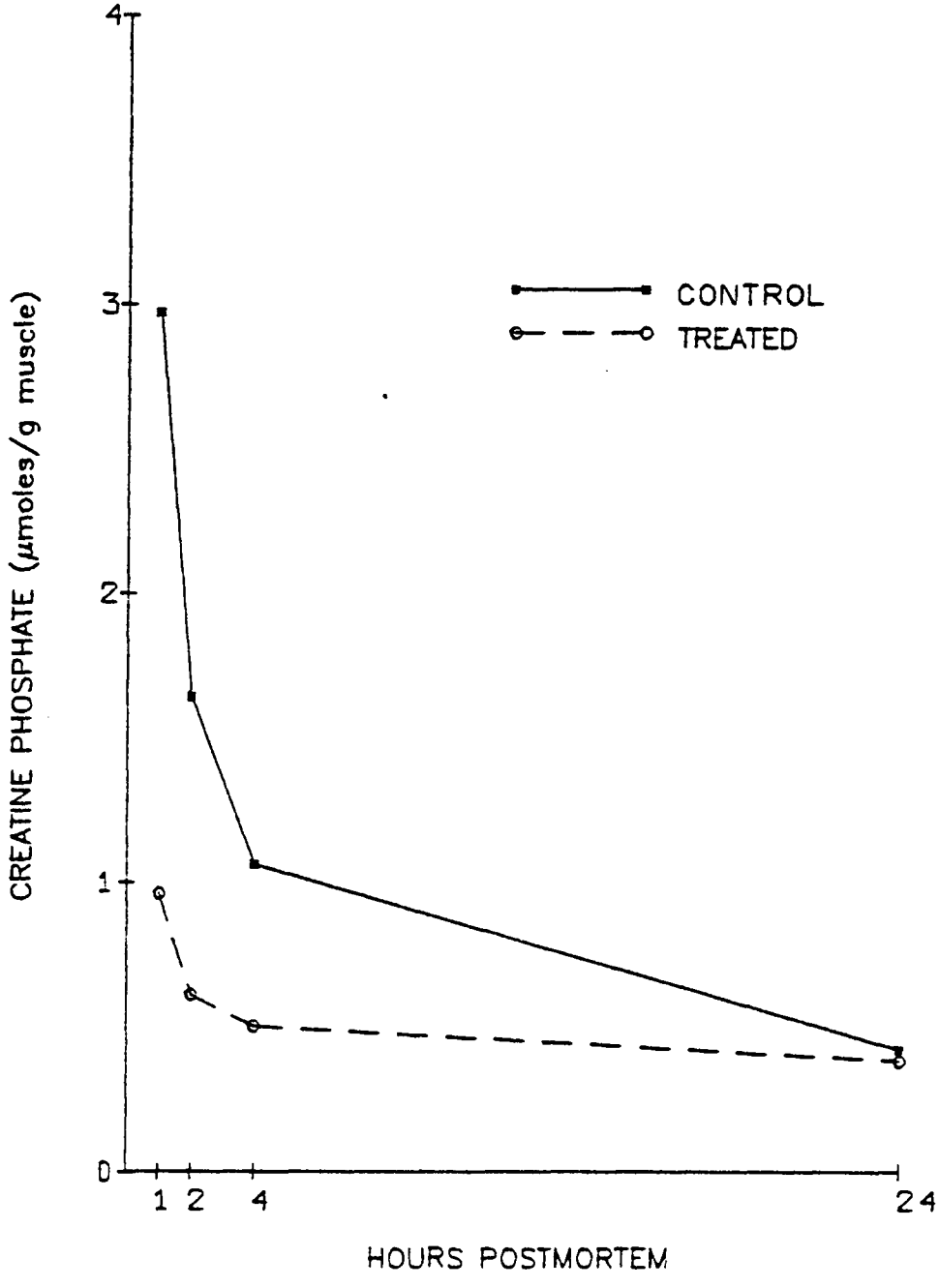

Figure III.2. Postmortem degradation of CP in control and pressurized semimembranosus muscle. Each point is the average of 8 determinations.

electrically stimulated muscle (10.2 μM/g/min). It is interesting to note that this value is 100 times greater than the rate with which the "non-contractile" myosin ATPase splits ATP in muscle held at 25°C (∿0.34 μM/g/min) as reported by Newbold and Scopes (1971) and Bendall (1973). It should be mentioned that pressure treatment (1000 kg/cm$^{2)}$ increased the activity of Ca^{2+}-ATPase of heavy actomeromyosin and subfragment 1 (Ikkai and Ooi, 1969). Between 1 and 4 hr postmortem the turnover of ATP was extremely low (Table 1), being only 1/1890 of the rate immediately after pressurization. The low rate of ATP turnover after pressurization was apparently due to the decrease in ATP concentration (28% of the initial level) and to the onset of rigor mortis (pH 5.78). Honikel et al. (1981) reported the onset of rigor to occur at pH ∿5.9.

A simple and rapid method based on the ratio of absorbance values at 250 nm:258 nm for detecting the early onset of rigor mortis in beef was reported by Khan and Frey (1971). Similarly, Honikel and Fischer (1977) explained the principle behind the use of absorbance ratios to indicate the stage of rigor mortis and were successful in relating R-values to PSE (pale, soft, exudative) and DFD (dark, firm, dry) pork. The adenine nucleotides (ATP, ADP and AMP) exhibit abosrbance maxima near 260 nm whereas those of IMP and inosine are at 248-249 nm. Hypoxanthine absorbance peaks near 250 nm. Since the adenine nucleotides are converted to IMP, inosine and hypoxanthine in postmortem muscle, the R-value (ratio of absorbance at two wavelengths) of an extract containing these compounds is an indicator of the relative amounts of adenine nucleotides to inosine compounds (Honikel and Fischer, 1977).

Table III.1 - Comparison between postmortem turnover of ATP in pressurized muscle and other published values.

State of Tissue[a]	pH of Tissue	Turnover of ATP[b]	Reference
Pressurized	5.78	37.80[c]	Present work
Pressurized	5.76	0.02[d]	Present work
Intact	6.37	0.29	Present work
Intact	6.70	0.15	Hamm (1977)
Electrically stimulated	6.48	10.20	Bendall et al. (1976)
Ground	6.10	0.48	Hamm (1977)
PSE (SS Pietrain)	6.90	4.70	Bendall (1973)

[a]All samples were beef except PSE (pale, soft, exudative pork).

[b]ATP turnover in μM/g/min.

[c]ATP turnover in muscle immediately after pressure treament (103.5 MNm^{-2}, 37°C, 2 min). Mean results from eight animals.

[d]ATP turnover of pressurized muscle in the range of 1-4 hr postmortem.

Honikel et al. (1981) reported a set of criteria for conditions indicative of rigor mortis in beef neck muscles based on the decrease in extensibility of muscle fibers as described by Bendall (1973). These conditions specify that postmortem pH decreased to values around 5.9, the R-value reached a level around 1.10, and ATP was present at a level of about 1μM/g. Based on the results obtained in this study (Figure 1 and Table 2), pressurized muscle had a more rapid metabolic rate and thus, entered rigor mortis soon after pressurization. The non-pressurized muscle, on the other hand, had an R-value of 1.05, an ATP level of 4.38 μmole/g and a pH of 6.06 which indicated that the control muscle was still in the prerigor state at 4 hr postmortem (Figure 1 and Table 2). Also, the relationship between R-value and ATP content was linear ($r=-0.95$) for the control during the 24 hr postmortem. Thus the R-value appears to be a reliable method for detection of rigor mortis.

CPK activity of both the control and pressurized samples at 1 and 24 hr postmortem is shown in Figure 3. These data demonstrate that the rapid drop in CP during pressurization was accompanied by a significant ($P<0.01$) increase in the activity of CPK at 1 hr postmortem. At 24 hr, however, CPK activity of the treated muscle decreased by 25% from that at 1 hr postmortem. A significant correlation coefficient ($r=0.55$, $P<0.05$) between CP level and enzyme activity suggests that the substrate level is important to trigger CPK activity in postmortem metabolism. CPK comprises about 10% of beef sarcoplasmic proteins (Scopes, 1964) and is found in different areas of the cell including mitochondria, microsomes (Klein, 1965; Klingenberg and Pfaff, 1966) and in the myofibrillar area,

Table III.2 - pH and R-values for pressurized and non-pressurized beef muscle at various times postmortem.

Hours Postmortem	R-Value[a] 250/260 nm Control	250/260 nm PRP[b]	250/258 nm Control	250/258 nm PRP	pH Control	pH PRP
1	0.91[c] *	1.46[c]	0.90 *	1.34[c]	6.73 *	5.78[c]
2	0.98[c] *	1.46[c]	0.99[c] *	1.34[c]	----	----
4	1.05 *	1.49[c]	1.05[c] *	1.34[c]	6.06 *	5.69[c]
24	1.43	1.50[c]	1.31	1.35[c]	5.71	5.61[c]

[a] R-values were calculated as the ratio of absorbance at different wavelengths (250:260 or 258 nm).

[b] PRP = Prerigor pressurization (103.5 MNm^{-2}, 37°C, 2 min).

[c] Means in the same column bearing same superscript letter are not different ($P>0.01$).

* Mean values for the control samples are significantly different ($P<0.01$) from those for PRP samples.

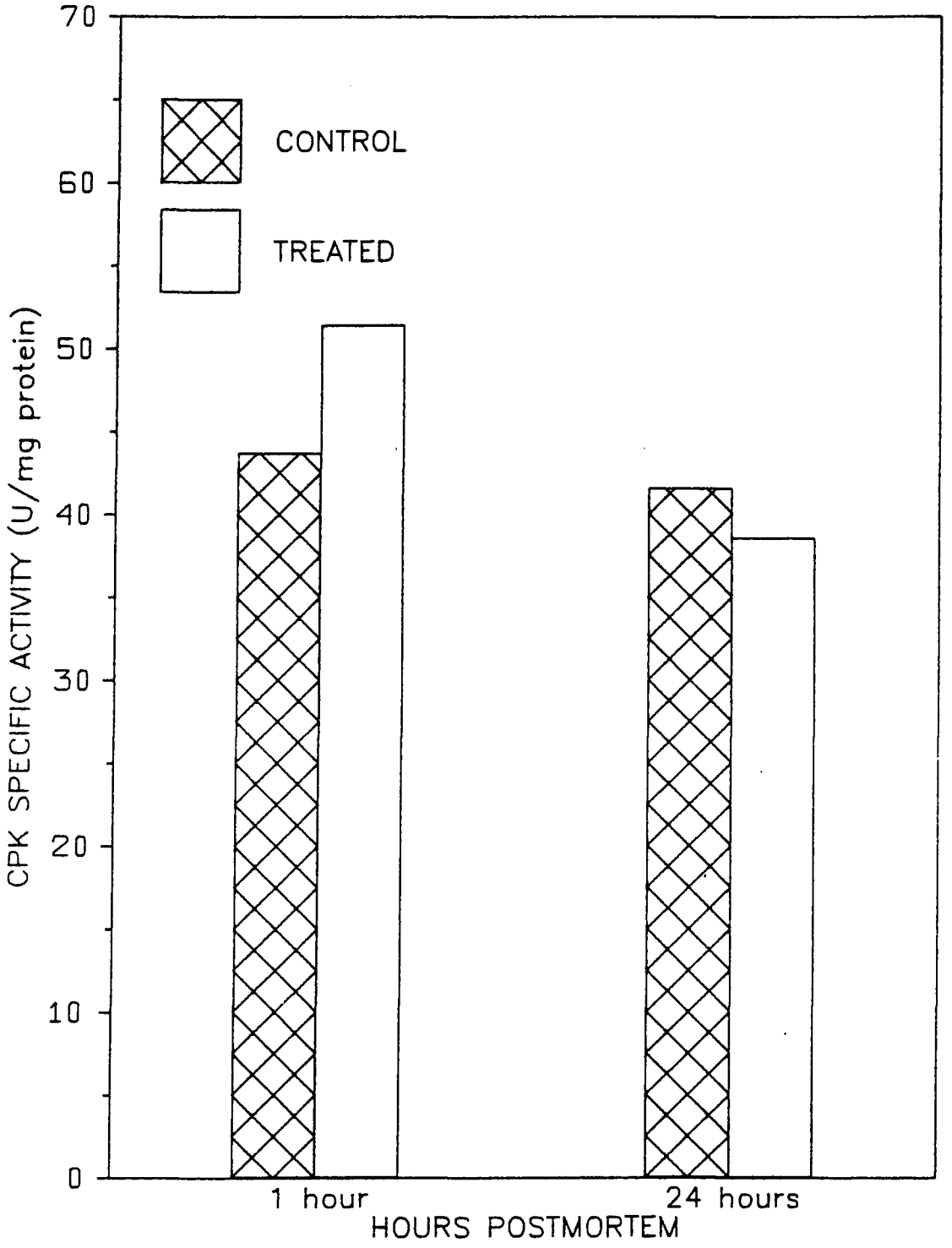

Figure III.3. Block diagram of the CPK specific activity in control and pressurized semimembranosus muscle (n=10 animals).

specifically in the M-band of skeletal muscle (Eppenberger and Heizmann, 1976). The latter authors also noted the presence of the debranching enzyme and phosphorylase b. Pressurization (100 MNm^{-2} at 25°C) of sheep SM muscle resulted in physical fracture of muscle fibers, weakening of M-band and extensive structural disruption with formation of the contraction band during the prerigor treatment (Macfarlane and Morton, 1978). From our experimental results it seems likely that CPK present in the M-band was mobilized when muscle was pressurized.

IV. HPLC DETERMINATION OF ATP METABOLITES IN PRERIGOR PRESSURIZED BEEF MUSCLE.

EXPERIMENTAL

A Varian Model 5000 high performance liquid chromatography (HPLC) with a Varian UV-5 detector operating at a wavelength of 254 nm was used. Peak areas were electronically integrated using a Varian Vista 401 Data System. The HPLC was fitted with a Whatman precolumn gel which is essential to prolong life of the anion exchange resin (Atwood et al., 1979). The analytical column, 30 cm x 4 mm inner diameter, was packed with Partisil-10 SAX with a Haskel pump.

Chromatographic Conditions

Three isocratic separations were employed. Buffer A, (0.01 M KH_2PO_4, pH 4.4) was used to separate bases, nucleosides and nucleoside monophosphates. Buffer B (0.16 M KH_2PO_4, pH 3.2) was used to separate nucleoside diphosphates. Buffer C (0.5 M KH_2PO_4, pH 3.2) was used to separate nucleoside triphosphates. At least three consecutive samples can be run with Buffer A or Buffer B before the retained compounds need to be washed off with Buffer C. All buffers were filtered through a 0.22 μm membrane filter (Millipore) before using. The flow rate was 1.5 ml/min and all analyses were run at 21°C.

Preparation of Samples for Injection

Prerigor semimembranosus (SM) muscle was excised from three carcasses and pressurized (103.5 MNm^{-2}, 37°C, 2 min) according to the procedure of Kennick et al. (1980). Meat samples were collected at various times postmortem, frozen in liquid nitrogen, pulverized to a powder (Borchert and Briskey, 1965) and stored at -40°C for 2 months

before the nucleotides were extracted. The control samples were treated similarly but were not pressurized. The nucleotides were extracted by homogenizing 1 g of tissue powder in 10 ml of 0.5 N perchloric acid for 30 sec at 4°C. Homogenates were filtered to remove the precipitated protein. The procedure of Khym (1975), employing a 0.5 M tri-n-octylamine/Freon 113 solution, was used to extract the acid from the nucleotides. Equal volumes of tissue-extract samples and amine/Freon solutions were mixed in screw cap centrifuge tubes. The contents were centrifuged to promote layer separation. Three layers were visible: the top layer was the aqueous phase which contained the nucleotides, the middle layer consisted of a perchlorate-tri-n-octylamine complex and the bottom layer was Freon 113. Twenty µl of the top layer was injected onto the HPLC. No pH adjustments were necessary since the neutralized extracts were always between pH 4 and 6 (Chen et al., 1977).

Identification and Quantitation

Identification was made solely on the basis of the retention times of standards. The reference compounds, dissolved in water were applied to the column singly or in various simple mixtures until elution positions were established in each buffer system. In some cases, peak identity was confirmed by rechromatography of the samples with the added standard. After determination of concentrations of the standards by spectrophotometry, 20 µl of an appropriate dilution was injected onto the HPLC and the peak areas used for calibration. Concentrations of ATP and its degradation products were calculated from the integrated peak areas of the 254 nm absorbing materials

eluting from the column. The concentrations were expressed as µmoles/g grozen wet tissue weight.

Statistical Analysis

Since the design of the experiment utilizes a paired comparison, the data were analyzed by the paired t-test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Separation of nucleotides by HPLC is commonly performed using ion exchange columns. However, the use of phosphate buffers in gradient elution causes problems with irreproducible baseline shifts due to UV impurities. Some improvement was noted using HPLC grade phosphate salts (Fisher Scientific) but the problem persisted. For this reason, the gradient elution procedure of Currie et al. (1982) was not used. Instead we employed isocratic separation of nucleotides in three different phosphate buffer concentrations to overcome the problem of baseline irregularities. This method is simple and reliable. Taking into account the time needed for column equilibration, the analysis time per sample was about one hour, which is similar to that needed with gradient anion exchange methods. In addition, small amounts of compounds can be quantitated since the baseline is very flat and stable.

Typical chromatograms of various postmortem muscle samples are shown in Figures 1, 2 and 3. The most striking difference between PRP and control muscles was that the pressurized samples had no ATP but higher levels of IMP as compared to non-pressurized samples at 1, 4 and 24 hr postmortem. Levels of ATP and IMP were significantly different ($P<0.05$) between PRP and control samples at each of the three postmortem periods (Table 1). The pressure treatment apparently altered the activity of ATPase, myokinase and 5'-AMP deaminase systems which resulted in the accelerated formation of IMP. Scopes (1971) concluded from experiments with model systems that the rate of ATP loss in bovine muscle postmortem was primarily due to the activity of

Table IV.1. - Postmortem changes in the concentrations[a] of nucleotides and related compounds in prerigor pressurized and control beef semimembranosus muscle.

Muscle Treatment	Hours Postmortem	ATP	ADP	AMP	ITP	IMP	Bases[b] + Nucleosides	NAD
PRP[c]	1	0.00(0.00)	1.30[d](0.59)[e]	0.20(0.04)	0.00(0.00)	4.69(0.36)	4.62(0.28)	0.71(0.40)
	4	0.00(0.00)	0.74(0.23)	0.17(0.02)	0.00(0.00)	4.66(0.72)	5.11(0.78)	0.65(0.07)
	24	0.00(0.00)	0.56(0.10)	0.04(0.001)	0.00(0.00)	5.05(0.40)	5.80(0.70)	0.41(0.01)
Control	1	6.13(1.50)	1.20(0.22)	0.05(0.04)	0.09(0.02)	0.26(0.11)	4.14(0.34)	1.37(0.16)
	4	1.73(0.66)	2.06(0.27)	0.10(0.01)	0.04(0.02)	1.71(0.27)	4.71(0.46)	1.21(0.30)
	24	0.04(0.01)	0.64(0.03)	0.07(0.01)	0.00(0.00)	3.58(0.36)	5.13(0.57)	0.56(0.10)

[a] μmoles/g of wet tissue.

[b] Bases + nucleosides = adenosine + adenine + inosine + hypoxanthine.

[c] PRP = prerigor pressurization (10.3 MNm^{-2}, 37°C, 2 min).

[d] Mean values (N=3).

[e] Parenthetical values are standard deviations.

AMP deaminase (5'-adenylate aminohydrolase) in the following manner

$$AMP^{2-} + H^{+} + H_2O \longrightarrow IMP^{2-} + NH^{+}_4.$$

Similarly, Hamm (1977) found that changes in the rate of ATP loss were accompanied by a corresponding change in the formation rate of IMP while the AMP concentration remained relatively constant. In the present work, AMP levels were fairly similar in both treatments except at 1 hr postmortem (Table 1). The relatively constant amount of AMP at different times postmortem is in agreement with the observation of Honikel and Fischer (1977) that AMP is deaminated almost instantaneously to IMP. ADP concentration was higher in the control samples at 4 hr postmortem. However, ADP was not completely degraded and the levels were similar for both treatments at 24 hr postmortem (Table 1). This finding parallels observations of other authors (Dovark, 1958; Newbold and Scopes, 1967; Valin and Charpentier, 1969; Hamm, 1977) who noted that the level of ADP remained constant at about 0.3 to 0.8 μmoles/g wet tissue. Valin and Charpentier (1969) reported that this residual ADP was completely bound to myofibrillar proteins. However, it is not known if this ADP fraction participates in the binding between actin and myosin at the rigor state of myofibrils. The PRP muscle reached this range at 4 hr postmortem.

A small amount of inosine triphosphate (ITP) was observed in the control muscle samples as a partially resolved peak at 1 hr and 4 hr postmortem but it was not evident in the PRP samples (Figures 1C and 2C). Furthermore, no inosine diphosphate (IDP) was detected in either treatment (IDP elutes at 18 min using Buffer B).

A problem arose with the separation of some bases and nucleosides (adenosine, adenine, inosine and hypoxanthine). These components

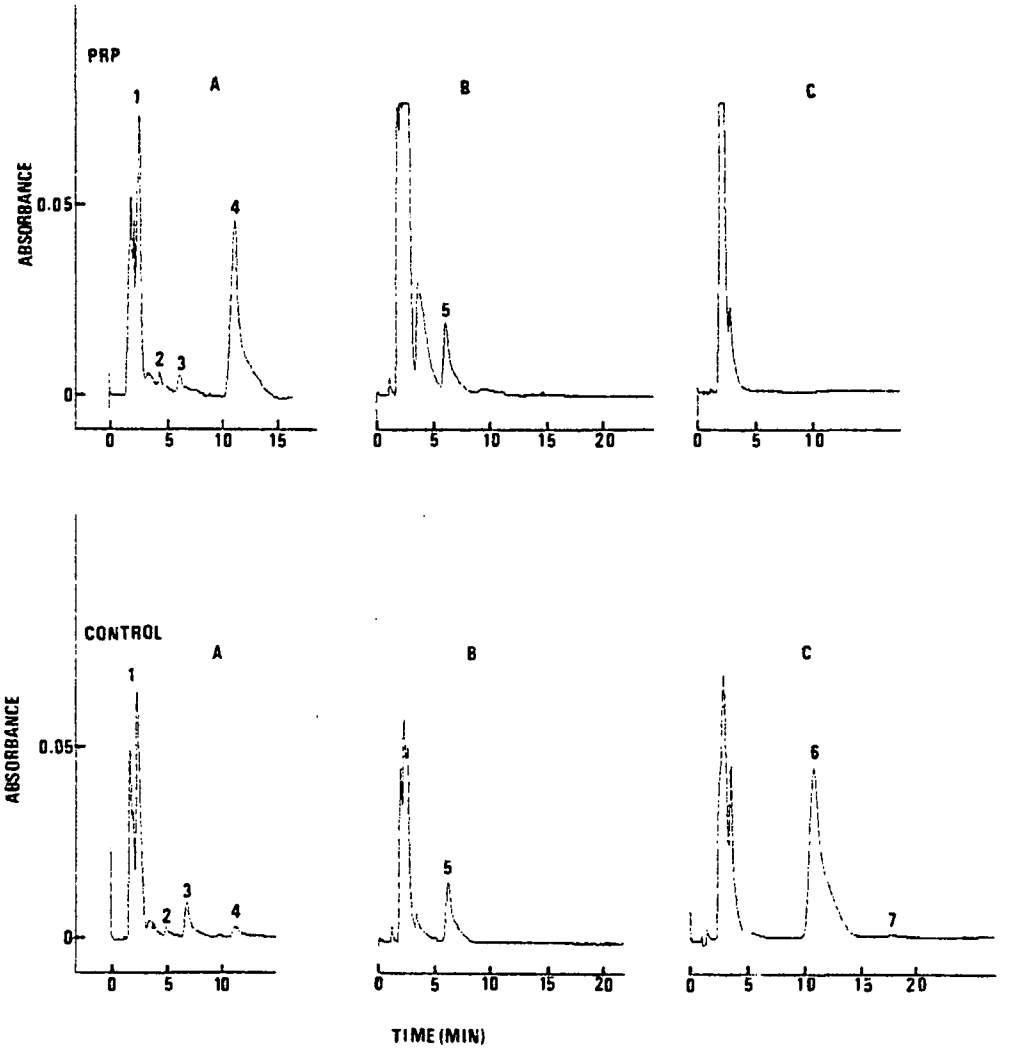

Figure IV.1. Chromatograms obtained from a 1 hr postmortem muscle extract. The upper chromatograms show prerigor pressurized muscle extract analysis, the lower chromatograms show control muscle extract analysis. The operating conditions were as follows: Buffer A, 0.01 M KH_2PO_4 at pH 4.4; Buffer B, 0.16 M KH_2PO_4 at pH 3.2; Buffer C, 0.5 M KH_2PO_4 at pH 3.2. The components identified are (1) bases and nucleosides; (2) AMP; (3) NAD; (4) IMP; (5) ADP; (6) ATP; (7) ITP.

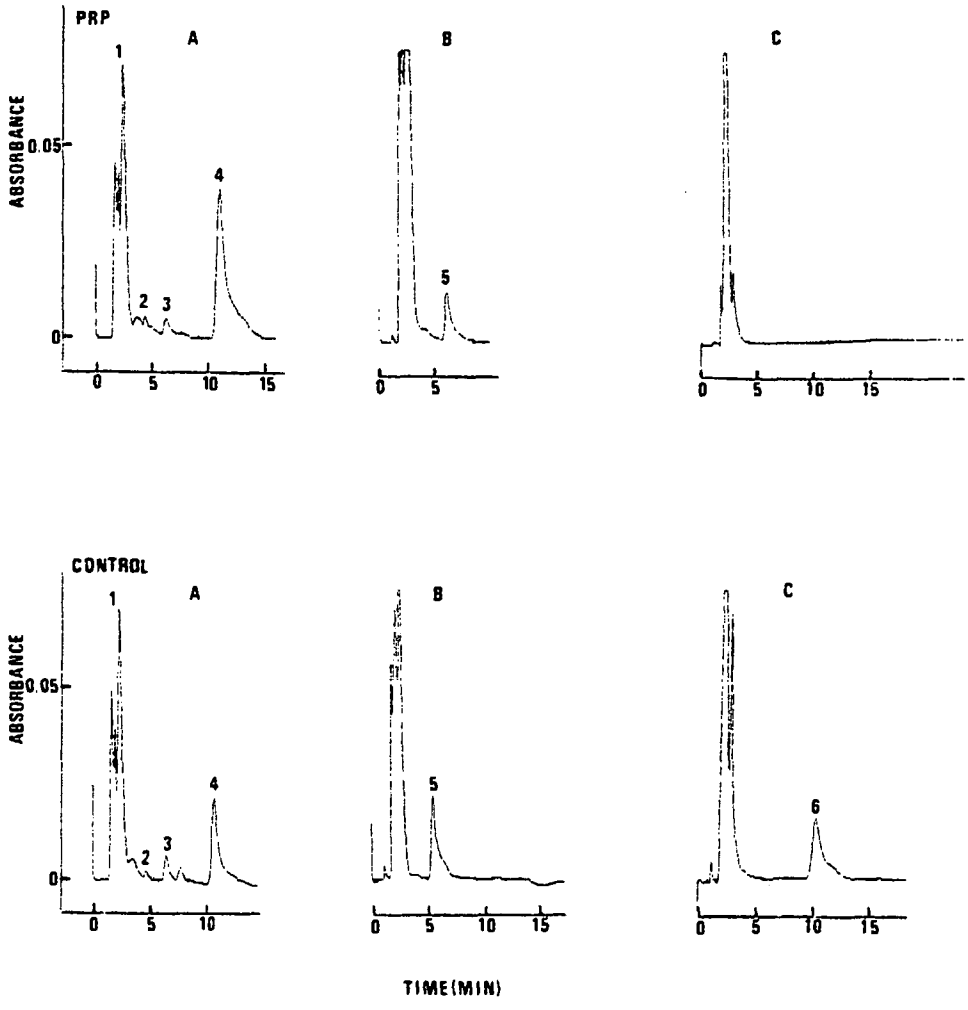

Figure IV.2. Chromatograms obtained from a 4 hr postmortem muscle extract. Chromatographic conditions and peak identifications are the same as in Figure 1.

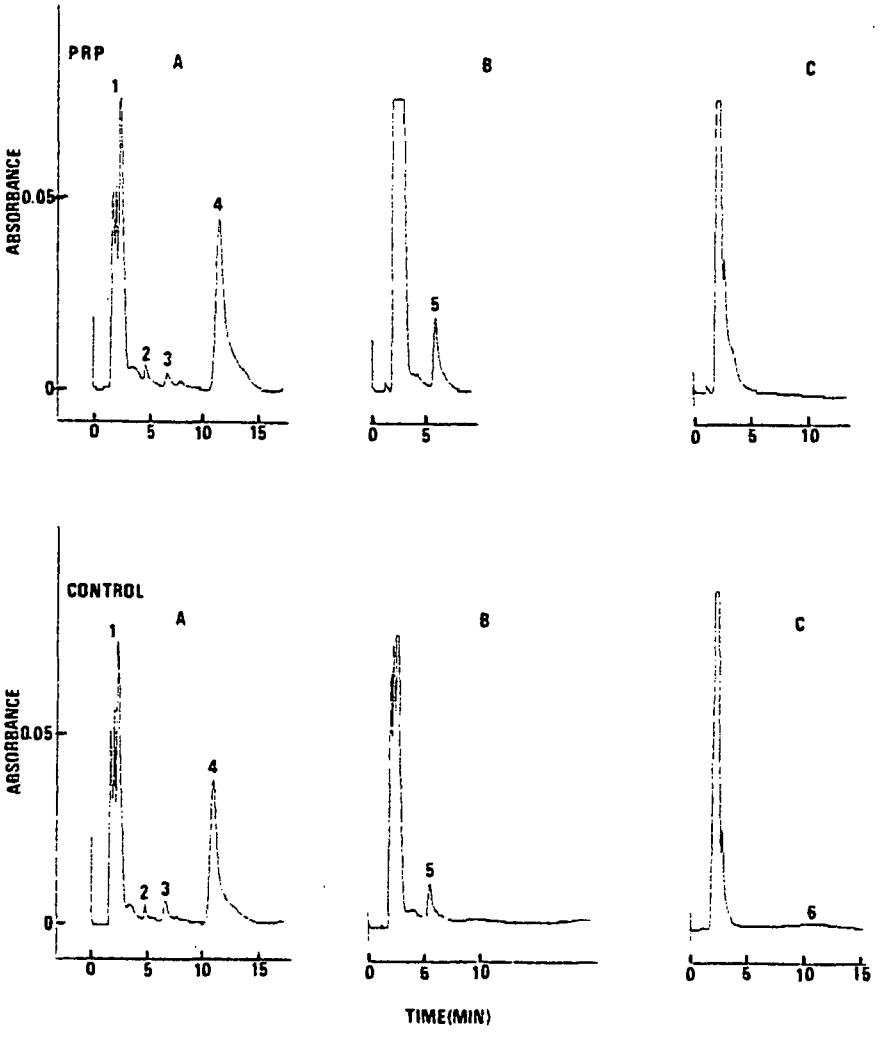

Figure IV.3. Chromatograms obtained from a 24 hr postmortem muscle extract. Chromatographic conditions and peak identifications are the same as in Figure 1.

could not be separated completely in Buffer A and tended to come out in two peaks. Fortunately, levels of these components generally remain constant in meat during the first 24 hr postmortem but increase upon aging. In the present work we computed the amounts of these components at various postmortem times from composite eluting materials as peak no. 1 (Figures 1A, 2A, 3A). PRP samples contained higher levels of these bases and nucleosides than did the controls (Table 1); however, these differences were not statistically significant ($P>0.05$). This finding indicates that the higher levels of IMP observed in PRP muscle samples were not catabolized to inosine and hypoxanthine during the 24 hr postmortem period. The postmortem metabolism of IMP to inosine and then to hypoxanthine occurs at a very slow rate. In bovine longissimus dorsi muscle, changes in the rate of ATP breakdown caused by various influences had only a slight effect on the rate of hypoxanthine formation during 72 hr postmortem (Hamm, 1977).

Although levels of nicotinamide adenine dinucleotide (NAD) were lower in PRP samples than in the controls at all times postmortem (Table1), these differences were not significant. Levels of NAD generally decrease with time postmortem. The trend of decreased NAD content postmortem was observed by Kastenschmidt et al. (1968) who found levels of NAD were much higher in 0 hr samples of slow-glycolyzing porcine muscles than in similar samples from muscles having fast rates of postmortem glycolysis.

V. CONCLUSIONS

On the basis of the studies reported herein, it was concluded that:

1. Prerigor pressurization of bovine semimembranosus muscle at 15,000 psi for two min at 35°C significantly decreased ($P<0.01$) muscle pH, and significantly ($P<0.01$) increased the rate of glycogen degradation to glucose and lactic acid during the first 4 hr postmortem.
2. The LDH activity of the pressurized samples was significantly higher ($P<0.01$) than that of the controls at 1 hr postmortem. This emphasizes the need for continuing studies to determine the mechanism by which prerigor pressurization stimulates the activity of LDH and/or other glycolytic enzymes.
3. Pressurization resulted in an accelerated decline of the energy-rich phosphate (ATP and CP) turnover. Pressurization apparently damages the control mechanisms in the muscle, allowing a sufficient sustained release of Ca^{2+} into the sarcoplasm which, in turn, stimulates both contraction and glycolysis. It is not known if some of the Ca^{2+} ions were released from mitochondria.
4. The rapid drop in CP due to pressurization was accompanied by a significant ($P<0.01$) increase in the activity of CPK at 1 hr postmortem. Whether the increase in CPK activity was due to the mobilization of the enzyme from the weakening of the M-line or of sarcoplasmic origin is not clear.
5. An overview of the HPLC data indicates that prerigor

pressurization of muscle definitely increases the rate of catabolism of high-energy adenine nucleotides. Moreover, there was a significant increase in the production of IMP in the pressurized muscle over that of the non-pressurized muscle at various times postmortem.

6. From the above findings, it can be concluded that the nucleotide related flavor of the pressurized meat is likely to differ from that of non-pressurized meat. However, additional studies are needed to clarify this point.

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