Effect of Added Solutes on Urea Denaturation. Ethanol at 0.17 M effectively protects chymotrypsin from the effects of 0.05 M urea in frozen solutions (Table III). Calcium chloride at 0.05 M is also a protective agent. Here the effect is probably a combination of the known decreased rate of denaturation in the presence of Ca^{2+} (Martin and Frazier, 1963) and the well-known inert solute effect in frozen systems. Assuming ideal conditions, the actual concentration of urea in frozen solutions containing various other solutes A, B, . . . will be $[U][0.05\ M\ \text{urea}/(0.05\ M\ \text{urea} + [A] + [B] + \ldots)]$ where $[U]$ is the concentration of urea in equilibrium with ice at a given temperature below freezing (*i.e.*, $[U] = 8\ M$ at 12°) (Pincock, 1969). A solution of 0.05 M urea and 0.17 M ethanol would give a urea concentration of only $8 \times 0.05/0.22 = 1.8\ M$ in frozen solutions at −12°. A solution containing 0.05 M $CaCl_2$ would be as effective as a 0.15 M ethanol solute in preventing the buildup of high urea concentration in frozen solutions. On the other hand, solutes initially less than 0.05 M would not greatly affect the urea concentration in frozen solutions; *e.g.*, tyrosine at 0.015 M is not a protective agent since the urea concentration would still be about 78% of its concentration without any added solute.

CONCLUSIONS

Relative to supercooled solutions the denaturation of α-chymotrypsin in frozen solutions shows rate accelerations, a rate–temperature maximum, and a protective effect of otherwise inert solutes. Although the data could not be made sufficiently precise to allow application of a quantitative treatment, these observed features are common to reactions in frozen solutions which are governed by the concentration effect. At temperatures a few degrees below freezing, and especially with buffers or natural mixtures present, most soluble proteins are not likely to be incorporated in the solid phase. They would be subject to the concentration effect and show the general features of all frozen solution reactions. In addition to these general characteristics, many specific characteristics of enzymes such as reversible conformational changes, denaturation on dilution, thermal denaturation, and autolysis may occur as "secondary effects" in frozen solutions. Since even a single enzyme like chymotrypsin or ribonuclease shows some of these features, the concentration effects during frozen storage of complex enzyme systems would be very difficult to separate out in any detail. However, the freezing of a dilute solution is a way of subjecting an enzyme to a high concentration of solutes yet recovering the sample for easy analysis at the original dilute concentration. Such treatment might be useful for investigating rapid reversible changes brought about by high solute concentrations.

LITERATURE CITED

Belikova, E. V., Komolova, G. S., Egorov, I. A., *Izv. Akad. Nauk SSSR Ser. Biol.* **6**, 847 (1968).
Buttkus, H., Abstract, 163rd National Meeting of the American Chemical Society, Boston, Massachusetts, April 1972, AGFD no. 11.
Chadwell, H. M., Politi, F. W., *J. Amer. Chem. Soc.* **60**, 1291 (1938).
Chilson, O. P., Costello, L. A., Kaplan, N. O., *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **24**, S-55 (1965).
Martin, C. J., Frazier, A. R., *J. Biol. Chem.* **238**, 3268, 3869 (1963).
Meryman, H. T., Ed., "Cryobiology," Academic Press, New York, N.Y., 1966.
Pincock, R. E., *Accounts Chem. Res.* **2**, 97 (1969).
Pincock, R. E., Kiovsky, T. E., *J. Amer. Chem. Soc.* **88**, 4455 (1966).
Schonbaum, G. R., Zerner, B., Bender, M. L., *J. Biol. Chem.* **236**, 2930 (1961).
Schwart, G. W., Takenaka, Y., *Biochem. Biophys. Acta* **16**, 570 (1955).
Stowell, R. E., Ed., *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **24**, No. 2, Part III (1965).
Tappell, A. L. in "Cryobiology," Meryman, J. C., Ed., Academic Press, New York, N.Y., 1966, p 163.

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Rates of Postmortem Metabolism in Frozen Animal Tissues

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Rates of ATP depletion and lactate accumulation in both *pectoralis major* (breast) and *biceps femoris* (thigh) muscles of 18-month-old chickens were determined at several temperatures between +10 and −10°. Rates of these reactions were at least as fast (and in breast muscle significantly faster) at −3° (frozen) as at +10°, +5°, or 0° (unfrozen). The rate of ATP depletion in breast muscle at −3° was significantly slower following immersion in liquid nitrogen than it was following freezing at slower rates to lesser depths. A similar study was performed on *sternomandibularis* (neck) muscles of cutter-grade cows. Rates of ATP depletion and lactate accumulation were significantly faster at −3° (frozen) than at +10° or 0° (unfrozen).

As postmortem muscle goes through rigor mortis, it undergoes a series of complex biochemical and physical changes. The time changes of several chemical and physical properties of beef *sternomandibularis* muscle and horse *longissimus dorsi* muscle during rigor mortis at 37° have been reported, respectively, by Newbold (1966) and Lawrie (1953). These relationships are very similar and feature the following main points: almost immediately after slaughter, a decline is observed in pH and creatine phosphate content; adenosine triphosphate (ATP) remains virtually constant until the creatine phosphate content has been reduced substantially; and the decrease in muscle extensibility is not appreciable until ATP has

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been depleted below a certain critical level, the exact level varying with species. This relationship between loss of extensibility of postmortem muscle and decrease in ATP content also has been shown in rabbits (Bate-Smith and Bendall, 1947; Bendall, 1951), whales (Marsh, 1952), chickens (de Fremery and Pool, 1960), and pigs (Bendall *et al.*, 1963).

The effect of chilling temperatures (37 to 0°) on glycolysis in muscle tissue has been studied in rabbits (Bate-Smith and Bendall, 1949, 1956), in beef (Cassens and Newbold, 1966, 1967a,b; Marsh, 1954; Partmann, 1963), in lambs (Marsh and Thompson, 1958), in chickens (de Fremery and Pool, 1960; Partmann, 1963), in cod (Fraser *et al.*, 1961), in haddock (Sharp, 1934), and in rainbow trout and mirror carp (Partmann, 1963). From these studies it can be concluded that rates of glycolytic reactions decrease as the temperature is lowered from 37 to about 15°. However, as the temperature is lowered from about 15 to 0°, it is not uncommon for rates of glycolytic reactions to remain constant or even increase significantly (Cassens and Newbold, 1967b; de Fremery and Pool, 1960). The rate of glycolysis in unfrozen muscle is an important matter since rapid glycolysis frequently results in excessive contraction, undesirable toughness, and poor water-holding capacity (de Fremery, 1966; de Fremery and Pool, 1960; Khan, 1971; Khan and Nakamura, 1970; Locker and Hagyard, 1963; Marsh and Leet, 1966).

Reactions representing the progress of glycolysis in frozen muscle have been studied in frogs (Smith, 1929; Smith and Moran, 1930), haddock (Sharp, 1934, 1935), cod (Bito and Amano, 1960; Nowland and Dyer, 1969; Tomlinson *et al.*, 1963), chickens (Khan *et al.*, 1963; Partmann, 1963), and in beef, trout, and carp (Partmann, 1963). In the majority of these studies it was found that there exists a temperature just below the freezing point (often −2 to −4°) where the rate of ATP depletion or lactic acid accumulation attains a maximum, and that further lowering of the temperature causes rates of these reactions to decline substantially. In contrast to this behavior, Partmann (1963) found no acceleration of glycolysis just below the freezing point in chicken or beef. In frozen muscle, the consequences of various rates of glycolysis are not fully known, but it is likely that rapid glycolysis can be tolerated since the presence of ice crystals precludes contraction (Marsh and Thompson, 1958). If true, it may prove advantageous in some commercial applications to complete glycolysis rapidly at a high subfreezing temperature.

The object of this study is to determine rates of ATP depletion and lactate accumulation in both chicken and beef muscles at several temperatures between +10 and −10°. The effects of different initial freezing treatments on subsequent rates of glycolysis in frozen chicken and beef muscles also will be determined.

EXPERIMENTAL SECTION

Poultry Muscle. Eighteen-month-old Leghorn hens (raised together under identical conditions) were sacrificed by breaking their necks. Extreme care in manual restraint was used during and immediately following killing so as to minimize any death struggle. The *pectoralis major* (breast) muscles and the *biceps femoris* (thigh) muscles were removed from each side of the carcass within 15 min postmortem. The two muscles from one side of the carcass were immediately packaged in tightly-closed polyethylene bags and immersed in liquid nitrogen for at least 20 min prior to placement in Dry Ice. These two muscles served as "zero time" controls for their respective paired muscles. The two muscles from the other side of the carcass were immediately packaged in tightly-closed polyethylene bags and immersed in an ethanol-water bath at the desired temperature (±0.1°). Bath temperatures both above and below the freezing point of chicken muscle (−1.3°) were utilized.

For studies at subfreezing temperatures, the muscles were first frozen in a −20° bath (immersion for 30 min) and then transferred to a bath at the subfreezing temperature desired for reaction (−2, −3, −4, −5, or −10°). For studies at temperatures above −1.3°, the muscles were directly immersed in a bath at the desired temperature.

Fifteen chickens were used for each study at a given temperature. This enabled withdrawal of triplicate samples of both breast and thigh muscles at each of five different times. Following the desired time–temperature treatment, muscles were removed from the constant temperature bath, immediately immersed in liquid nitrogen for at least 20 min (presumably to stop further enzyme activity), and then stored in Dry Ice until analyzed.

When a muscle was removed from frozen storage, it was pulverized, deproteinized, neutralized, and analyzed for ATP and L^+ lactate as outlined in Bergmeyer (1965). Graphs were prepared of log % ATP remaining *vs.* time at a constant temperature, and the slope of the linear portion of the plot was determined by linear regression analysis (Huntsberger, 1967).

The amount of lactate accumulated (ΔL_t) in a given muscle after a given time–temperature treatment was calculated by subtracting the lactate content of the zero-time control muscle from the lactate content of the paired test muscle. At each temperature, one of the treatment times yielded a maximum mean value (mean of triplicates). This value was designated $\Delta \bar{L}_{max}$. For each temperature, graphs were made of log ($\Delta \bar{L}_{max} - \Delta L_t$) *vs.* time, and the slope of the linear portion of the plot was determined by linear regression analysis.

Beef Muscle. *Sternomandibularis* (neck) muscles were removed from freshly slaughtered (45 min postmortem) cutter-grade cattle at Oscar Mayer Inc., Madison, Wis. The muscles were labeled and wrapped in aluminum foil. Muscles to be studied at temperatures above freezing (−1.7° for beef muscle) were immediately layered on crushed ice. For studies below −1.7°, the muscles were immediately frozen in crushed Dry Ice. All muscles were then transported to the laboratory (30 min). Each neck muscle was promptly cut into four pieces of approximately equal length. The four pieces of a given muscle, designated A, B, C, and D, were packaged in tightly-closed polyethylene bags. Piece A (the "zero time" control muscle for pieces B, C, and D) was quickly frozen (if not already frozen) by immersion in liquid nitrogen for 20 min and then stored in Dry Ice. Pieces B, C, and D were transferred to a bath at the appropriate constant temperature according to the procedures described for poultry.

Neck muscles from 12 carcass halves were utilized for each study at a given temperature. Division of each muscle into four parts (a "zero time" control and three experimental samples) allowed withdrawal of triplicate samples at each of 12 different times. Muscles were removed from the constant temperature baths and analyzed for ATP and lactate as described previously for chicken muscle. In addition, the pH of each sample was measured using a glass electrode pH meter standardized against pH 6.86 phosphate buffer and pH 4.01 phthalate buffer. Samples for pH measurement were prepared by mixing 2.0 g of powderized muscle with 20 ml of 0.005 *M* sodium iodoacetate at room temperature. A graph of lactate (μmol/g) *vs.* pH was prepared and the slope was determined by linear regression analysis.

RESULTS AND DISCUSSION

Poultry Muscle. Rates of ATP depletion and lactate accumulation in both *pectoralis major* (breast) muscles and *biceps femoris* (thigh) muscles were determined at +10, +5, 0, −1, −2, −3, −4, −5, and −10°. Typical plots

of ATP depletion *vs.* time for both *pectoralis major* and *biceps femoris* muscles at several different chilling temperatures are presented in Figure 1. Linear regression equations for lines of best fit (for linear portions of the plots) are presented in the legend of Figure 1. Each regression equation was calculated from triplicate values at the three consecutive times which most closely surrounded the value of 50% ATP remaining [the approximate percentage of ATP remaining when poultry muscle undergoes a loss in extensibility (de Fremery and Pool, 1960)].

Typical plots of lactate accumulation *vs.* time for both *pectoralis major* and *biceps femoris* at several different chilling temperatures are illustrated in Figure 2. Linear regression equations for lines of best fit (for linear portions of the plots) are presented in the legend of Figure 2. Each regression equation was calculated from triplicate values at three times before the lactate content reached its maximum value.

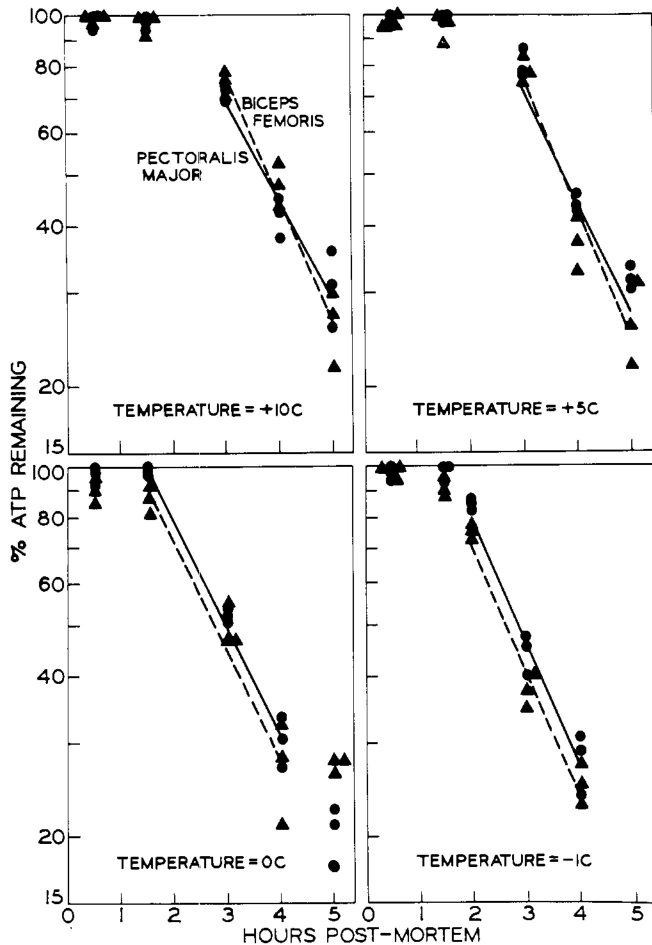

Figure 1. Rates of ATP depletion in excised poultry muscles at various temperatures. With temperature = +10°, for *pectoralis major:* $y = -0.1837x + 2.3894$; slope ±95% C.I. = −0.1837 ±0.0502; standard deviation of y at $\bar{x}$ = 0.0520. For *biceps femoris:* $y = -0.2271x + 2.5719$; slope ±95% C.I. = 0.2271 ±0.0520; standard deviation of y at $\bar{x}$ = 0.0538. With temperature = +5°, for *pectoralis major:* $y = -0.2017x + 2.4911$; slope ±95% C.I. = −0.2017 ±0.0344; standard deviation of y at $\bar{x}$ = 0.0356. For *biceps femoris:* $y = -0.2430x + 2.6023$; slope ±95% C.I. = −0.2430 ±0.0672; standard deviation of y at $\bar{x}$ = 0.0695. With temperature = 0°, for *pectoralis major:* $y = -0.2069x + 2.3159$; slope ±95% C.I. = −0.2069 ± 0.0220; standard deviation of y at $\bar{x}$ = 0.0285. For *biceps femoris:* $y = -0.2020x + 2.2568$; slope ±95% C.I. = −0.2020 ±0.0525; standard deviation of y at $\bar{x}$ = 0.0684. With temperature = −1°, for *pectoralis major:* $y = -0.2440x + 2.4030$; slope ±95% C.I. = −0.2440 ± 0.0421; standard deviation of y at $\bar{x}$ = 0.0436. For *biceps femoris:* $y = -0.2424x + 2.3469$; slope ±95% C.I. = −0.2424 ±0.0415; standard deviation of y at $\bar{x}$ = 0.0429.

The rates (slope ±95% confidence interval) of ATP depletion and lactate accumulation in *pectoralis major* muscles at the various temperatures are summarized in Figures 3 and 4. Figure 3 (rate of ATP depletion in *pectoralis major* muscle *vs.* temperature) indicates that the rate of ATP depletion: did not change appreciably between +10 and 0°; gradually increased as the temperature was lowered from 0 to −3 or −4°; and decreased sharply as the temperature was decreased from −4 to −10°. Rates of ATP depletion were significantly ($p < 0.05$) greater at −3 or −4° than at +10, +5, 0, −5, or −10°.

Figure 4 (rate of lactate accumulation in *pectoralis major* muscle *vs.* temperature) shows that the rate of lactate accumulation: tended to increase during cooling from

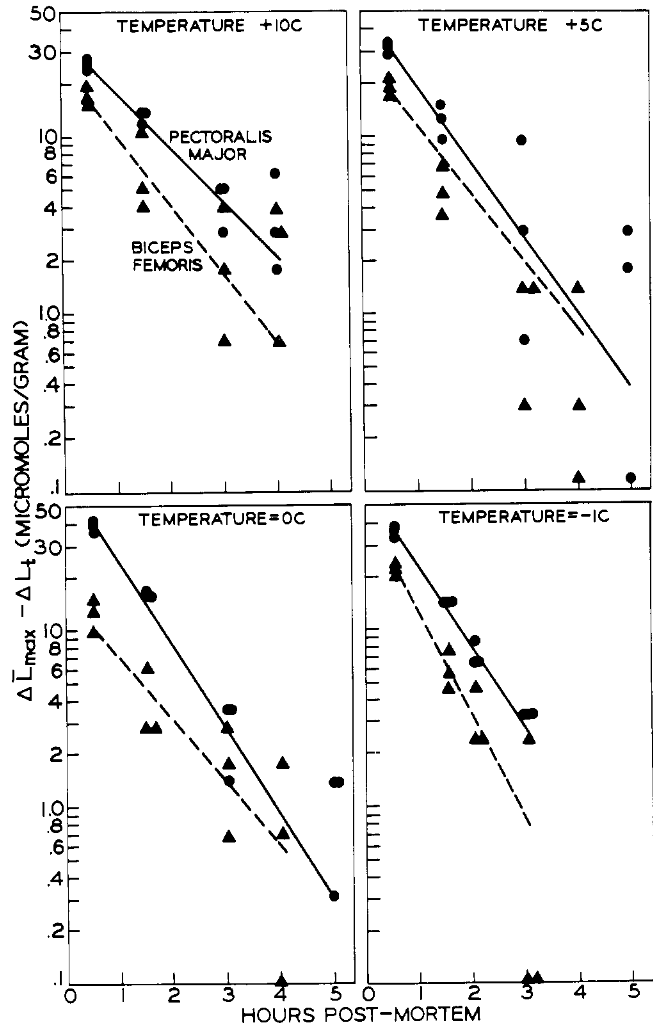

Figure 2. Rates of lactate accumulation in excised poultry muscles at various temperatures. With temperature = +10°, for *pectoralis major:* $y = -0.3140x + 1.5739$; slope ±95% C.I. = −0.3140 ±0.0625; standard deviation of y at $\bar{x}$ = 0.0814. For *biceps femoris:* $y = -0.3930x + 1.3997$; slope ±95% C.I. = −0.3930 ±0.1820; standard deviation of y at $\bar{x}$ = 0.2371. With temperature = +5°, for *pectoralis major:* $y = -0.4285x + 1.7183$; slope ±95% C.I. = −0.4285 ±0.2366; standard deviation of y at $\bar{x}$ = 0.3083. For *biceps femoris:* $y = -0.5423x + 1.5364$; slope ±95% C.I. = −0.5423 ±0.1707; standard deviation of y at $\bar{x}$ = 0.2225. With temperature = 0°, for *pectoralis major:* $y = -0.4718x + 1.8575$; slope ±95% C.I. = −0.4718 ±0.1039; standard deviation of y at $\bar{x}$ = 0.1352. For *biceps femoris:* $y = -0.3515x + 1.2014$; slope ±95% C.I. = −0.3515 ±0.1685; standard deviation of y at $\bar{x}$ = 0.2195. With temperature = −1°, for *pectoralis major:* $y = -0.4502x + 1.7890$; slope ±95% C.I. = −0.4502 ±0.0676; standard deviation of y at $\bar{x}$ = 0.0534. For *biceps femoris:* $y = -0.5560x + 1.6061$; slope ±95% C.I. = −0.5560 ±0.1338; standard deviation of y at $\bar{x}$ = 0.1058.

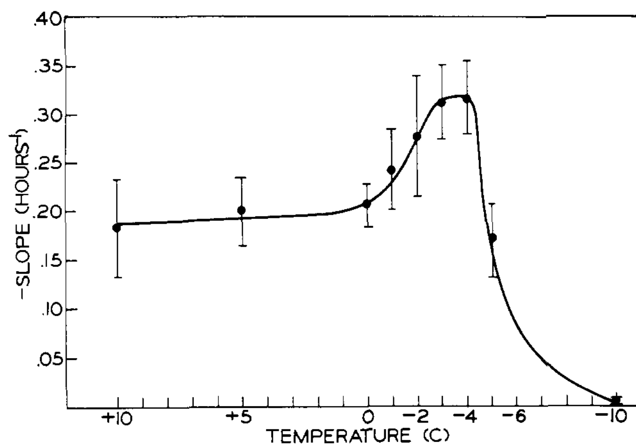

Figure 3. Rates of ATP depletion (slope ±95% C.I.) in *pectoralis major* (chicken breast) muscles at various temperatures.

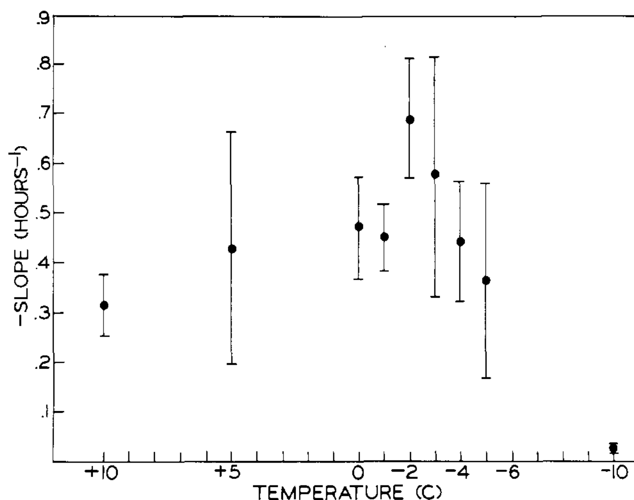

Figure 4. Rates of lactate accumulation (slope ±95% C.I.) in *pectoralis major* (chicken breast) muscles at various temperatures.

+10 to 0°; increased sharply during cooling from −1 (unfrozen) to −2° (frozen); tended to decrease during cooling from −2 to −5°; and decreased sharply during cooling from −5 to −10°. The rate of lactate accumulation was significantly ($p < 0.05$) greater at −2° than at +10, −1, −4, −5, or −10°.

The rates (slope ±95% confidence interval) of ATP depletion and lactate accumulation in *biceps femoris* muscles at the various temperatures tended to follow the same general patterns as illustrated for *pectoralis major* muscles in Figures 3 and 4.

This is the first known report of the existence of a temperature just below the freezing point where ATP depletion and lactate accumulation proceed at a maximum subfreezing rate in chicken muscle. Partmann (1961, 1963) found that the rate of ATP depletion in chicken breast muscle steadily decreased as the temperature decreased from 0 to −2, to −4, and finally to −8°. Although no definitive explanation for this discrepancy is apparent, it seems possible that differences in the species, breed, strain, or age of chickens could be responsible.

An experiment was conducted to determine the effect of the initial freezing treatment on the subsequent rate of ATP depletion in *pectoralis major* muscle of chicken in the frozen state. A reaction temperature of −3° was selected because it is apparent from Figure 3 that the rate of ATP depletion in chicken breast muscle attained a subfreezing maximum at about this temperature. Initial freezing treatments were as follows: A, immersion in liquid nitrogen for 20 min; B, immersion in a refrigerated bath at −20° for 30 min; C, placement (sample single-wrapped in a polyethylene bag) in an air-blast freezer at −23° for 1.0 hr (after which the internal temperature of the muscle was −19.4°); or D, placement (sample triple-wrapped in polyethylene bags) in an air-blast freezer at −23° for 2.0 hr (after which the internal temperature of the muscle was −18.8°).

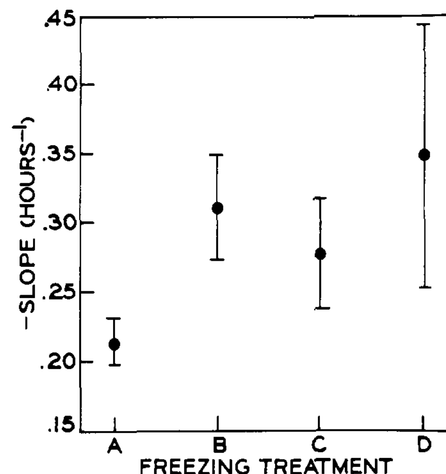

Figure 5. Effect of various freezing treatments on the subsequent rates of ATP depletion (slope ±95% C.I.) in *pectoralis major* (chicken breast) muscles at −3°. Freezing treatments: A = immersed in liquid nitrogen for 20 min (ultimate temperature at geometric center of sample was about −196°); B = immersed in a refrigerated bath at −20° for 30 min (ultimate temperature at geometric center of sample was about −20°); C = placed (single-wrapped) in an air-blast freezer at −23° for 1 hr (ultimate temperature at geometric center of sample was −19.4°); D = placed (triple-wrapped) in an air-blast freezer at −23° for 2 hr (ultimate temperature at geometric center of sample was −18.8°).

Summarized in Figure 5 are rates (slope ±95% confidence interval) of ATP depletion in breast muscle of chicken at −3° following the different initial freezing treatments described above. It should be noted that the rate of freezing involved in the four treatments decreased in the order A > B > C > D, and that the depth of freezing differed as follows: treatment A involved a greater depth than B $\simeq$ C $\simeq$ D. Thus, treatment A differed from treatments B, C, and D in both rate and depth. However, treatments B, C, and D differed only in rate. Figure 5 shows that the rate of ATP depletion at −3° following immersion in liquid nitrogen for 20 min (treatment A) was significantly ($p < 0.05$) less than that obtained following treatments B, C, or D, and the rates of ATP depletion at −3° following treatments B, C, or D did not differ significantly.

The decreased rate of ATP depletion resulting from treatment A could be the result of the rapid rate of freezing, the greater depth of freezing, or a combination of both. Since rates of ATP depletion following treatments B, C, or D did not differ significantly, it appears that the initial freezing rate, within the relatively slow values spanned by these three treatments, had no significant effect on the rate of ATP depletion in chicken breast muscle at −3°.

We are unaware of any results comparable to those reported here. However, it seems advisable to mention several other studies wherein freezing rate and/or depth influenced the native properties of proteins in tissue. Huber and Stadelman (1970) reported that freezing treatment had a highly significant ($p < 0.01$) effect on the subsequent solubility of myofibrillar proteins of chicken breast muscles frozen after aging. They found that retention of myofibrillar protein solubility was greater ($p < 0.01$) after

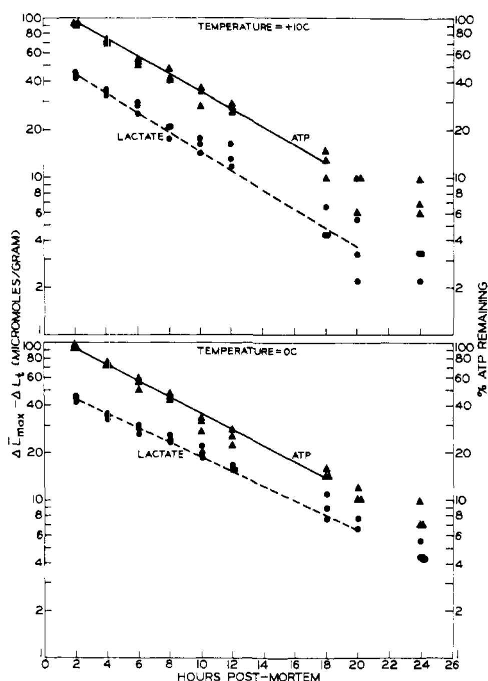

Figure 6. Rates ot ATP depletion and lactate accumulation in excised *sternomandibularis* (beef neck) muscles at various temperatures. Upper temperature = +10°; for ATP depletion, $y = -0.0538x + 2.0661$; slope ±95% C.I. = −0.0538 ±0.0038; standard deviation of y at $\bar{x}$ = 0.0423. For lactate accumulation, $y = -0.0606x + 1.7936$; slope ±95% C.I. = −0.0606 ±0.0058; standard deviation of y at $\bar{x}$ = 0.0825. Lower temperature = 0°; for ATP depletion, $y = -0.0523x + 2.0587$; slope ±95% C.I. = −0.0523 ±0.0040; standard deviation of y at $\bar{x}$ = 0.0433. For lactate accumulation, $y = -0.0466x + 1.7426$; slope ±95% C.I. = −0.0466 ±0.0053; standard deviation of y at $\bar{x}$ = 0.0749.

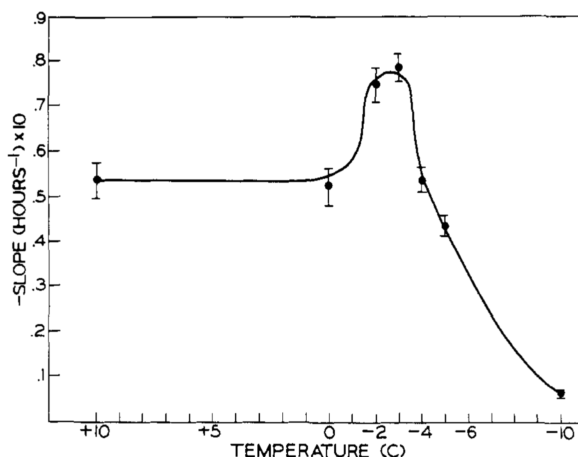

Figure 7. Rates of ATP depletion (slope ±95% C.I.) in *sternomandibularis* (beef neck) muscles at various temperatures.

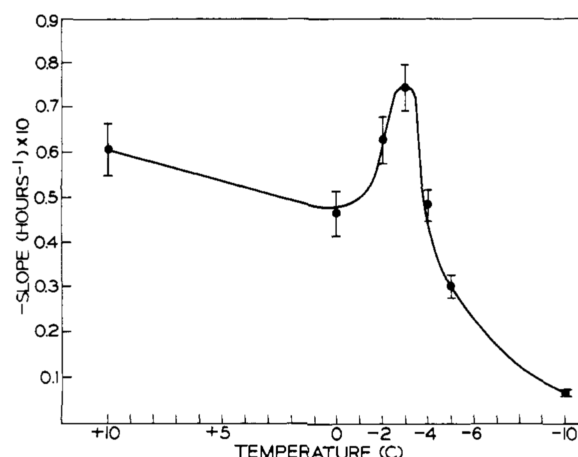

Figure 8. Rates of lactate accumulation (slope ±95% C.I.) in *sternomandibularis* (beef neck) muscles at various temperatures.

freezing in cold air (−10°) than following immersion in liquid nitrogen. Contradictory results were, however, obtained by Khan (1966) and Khan and van den Berg (1967). These investigators studied chicken breast muscle frozen after aging and reported that less denaturation of myofibrillar protein and greater activity of adenosinetriphosphatase (ATPase) was obtained after rapid freezing (immersion in Dry Ice–methanol) than after slow freezing (samples insulated while cooled in air at −18°). The discrepancies in the results of the above investigations may be attributable to differences in the age of the chickens utilized or to differences in freezing treatments. It should be reemphasized that the above investigators used poultry that was aged prior to freezing, whereas the present study involved muscles frozen prerigor. Thus, similar results should not necessarily be expected.

Beef Muscle. Rates of ATP depletion and lactate accumulation in *sternomandibularis* (neck) muscles were determined at +10, 0, −2, −3, −4, −5, and −10°. Presented in Figure 6 are typical plots of ATP depletion and lactate accumulation *vs.* time at chilling temperatures of +10 and 0°. Regression equations for lines of best fit are cited in the legend of Figure 6. Each regression equation for ATP depletion was calculated from at least 18 values representing the six (or more) times which most closely surrounded the value for 50% ATP remaining [the approximate percentage of ATP remaining when *sternomandibularis* muscle undergoes a rapid loss in extensibility (Newbold, 1966)]. Each regression equation for lactate accumulation was calculated from triplicate values representing eight times before the lactate content reached its maximum value.

In addition, several muscles of different lactate content were analyzed for pH. For pH values between 5.7 and 6.8, the linear regression equation is as follows: $y = -57.97x + 433.37$, where y is lactic acid concentration in micromoles per gram and x is the pH value. The slope ±95% confidence interval is −57.97 ± 4.10, and the standard deviation of y at $\bar{x}$ is 3.70.

The rates (slope ±95% confidence interval) of ATP depletion and lactate accumulation in *sternomandibularis* muscle at the various temperatures are summarized in Figures 7 and 8. Figure 7 (rate of ATP depletion in neck muscle *vs.* temperature) indicates that the rate of ATP depletion did not change significantly between +10 and 0°, attained a maximum subfreezing value between −2 and −3°, and decreased sharply as the temperature was lowered from −3 to −10°. Rates of ATP depletion were significantly ($p < 0.05$) greater at −2 and −3° than at +10, 0, −4, −5, or −10°.

Figure 8 (rate of lactate accumulation in neck muscle *vs.* temperature) illustrates that the rate of lactate accumulation decreased significantly ($p < 0.05$) as the temperature was lowered from +10 to 0°, attained a maximum subfreezing value at −3°, and decreased sharply as the temperature decreased from −3 to −10°. The rate of lactate accumulation was significantly ($p < 0.05$) greater at −3° than at +10, 0, −2, −4, −5, or −10°.

As previously mentioned, various investigators have reported that ATP depletion and lactate accumulation in muscles of frogs, haddock, cod, trout, and carp occur at maximum subfreezing rates just below their respective freezing points. However, in beef *(extensor digitalis communis)*, Partmann (1961, 1963) reported that the rate of ATP depletion decreased sharply from 0 to −8°. Part-

mann's results differ greatly from those obtained in this study (Figure 7), wherein a maximum subfreezing rate of ATP depletion in *sternomandibularis* muscle of beef was obtained between −2 and −3°. Although an explanation for this discrepancy is not obvious, it seems possible that utilization of different muscles or different handling procedures could be responsible. Since the rate of lactate accumulation in neck muscle of beef also was found to be maximal at −3° (Figure 8), this lends credibility to the concept of a maximum rate of glycolysis in prerigor beef muscle at temperatures just below its freezing point.

Also investigated was the effect of different initial freezing treatments on rates of ATP depletion and lactate accumulation at −3°. A reaction temperature of −3° was chosen because it is apparent from Figures 7 and 8 that rates of ATP depletion and lactate accumulation were maximal at this temperature. Samples were exposed to the following initial freezing treatments: A, immersed in liquid nitrogen to an internal sample temperature of approximately −30° and then packed in crushed Dry Ice; B, packed in crushed Dry Ice; C, placed in cold (−26°) circulating air to an internal sample temperature of −23° and then packed in crushed Dry Ice; or D, placed in cold (−26°) circulating air to an internal sample temperature of −23° and then transferred to a well-insulated chest to maintain a near constant temperature.

Rates (slope ±95% confidence interval) of ATP depletion and lactate accumulation in beef neck muscle at −3° following different freezing treatments are summarized in Figure 9. The treatments can be ranked in rate of freezing (fastest to slowest) as A > B > C = D. They can also be ranked in depth of freezing as A = B = C, all of which involved a greater depth of freezing than D. Thus, treatments A, B, and C differed only in freezing rate and treatments C and D differed only in freezing depth. The results in Figure 9 indicate that the freezing rates tested had no significant effect on the rate of ATP depletion or lactate accumulation at −3°, and the freezing depths tested had no significant effect on the rates of these same two reactions at −3°, although there was a consistent tendency for those samples which received a freezing treatment of the least depth (treatment D) to exhibit the greatest reaction rates. The authors are not aware of any previous study relating the effects of freezing rate or depth with rates of glycolysis in beef muscle.

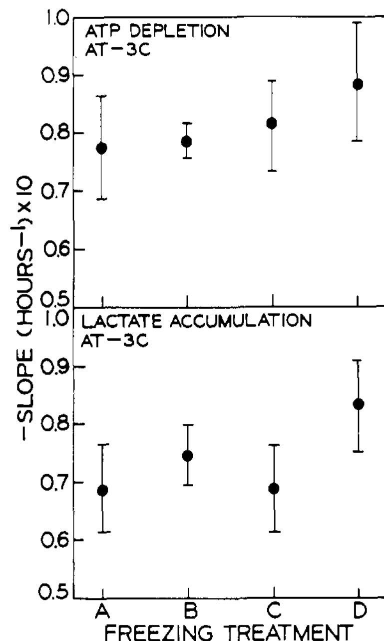

Figure 9. Effect of various freezing treatments on the subsequent rates of ATP depletion and lactate accumulation (slope ±95% C.I.) in *sternomandibularis* (beef neck) muscles at −3°. Freezing treatments: A = immersed in liquid nitrogen until an internal (geometric center) temperature of about −30° was obtained, and then packed in Dry Ice until an internal temperature of about −78° was obtained; B = packed in Dry Ice until an internal temperature of about −78° was obtained; C = placed in cold (−26°) moving air until an internal temperature of −23° was obtained, and then packed in Dry Ice until an internal temperature of about −78° was obtained; D = placed in cold (−26°) moving air until an internal temperature of −23° was obtained.

Table I. Rates of Reactions as Influenced by Temperature and Concentration of Solutes during Freezing

Situation	Change in rate of reaction caused by: Lowering of temperature (effect T)	Change in rate of reaction caused by: Concn of solutes (effect S)	Relative influence of the two effects	Total effect of freezing on reaction rate
1	Decrease	Decrease	Cooperative	Decrease
2	Decrease	Slight increase	T > S	Slight decrease
3	Decrease	Moderate increase	T = S	None
4	Decrease	Great increase	T < S	Increase

Causes of Increased Rates of Reaction during Freezing. A complete explanation as to why some reactions undergo increases in rate during the early stages of freezing cannot be given at this time. However, it is well known that reactants are concentrated in the unfrozen phase during freezing (freeze-concentration effect) and this is undoubtedly a major causative factor in many instances (Fennema, 1971, 1972; McWeeny, 1968; Pincock and Kiovsky, 1966; Thompson and Fennema, 1971). According to this view two factors are primarily responsible for changes in rates of reaction during the course of freezing (Fennema, 1971, 1972) and they are the temperature and concentration of solutes in the unfrozen phase. The relative importance of these two factors can vary, producing the consequence shown in Table I. A decrease in temperature, when considered independently, invariably decreases reaction rates. On the other hand, an increase in the concentration of solutes, when considered independently, can either decrease or increase rates of reactions, depending on the type of reaction and the circumstances. Thus, in Situation 4 of Table I, the rate-accelerating effect of solute concentration overbalances the rate-decreasing effect of temperature lowering, and a net increase in reaction rate occurs. Quantitative treatments of this concept were presented by Pincock and Kiovsky (1966) and by Thompson and Fennema (1971).

Although the freeze-concentration effect can account almost entirely for the extent to which many reactions accelerate during freezing, it has been speculated that one or more of the following factors also may be involved (Alburn and Grant, 1965; Bruice and Butler, 1965; Grant and Alburn, 1965a,b, 1967; Grant *et al.*, 1961): a catalytic effect of ice crystals; the greater mobility of protons in ice than in water; a favorable substrate-catalyst orientation caused by freezing; or an effect resulting from the difference in the dielectric constants of ice and water. Furthermore, freezing can have adverse effects on cellular integrity and functionality of membranes, and this may have a profound effect on reactions rates. For example, the activities of some enzyme systems are greater following a freeze-thaw treatment than they are prior to freezing (Fishbein and Stowell, 1969; Rhodes, 1961; Tappel, 1966). The proposed explanation is that the freeze-thaw treatment damages cellular membranes and thereby facilitates access of enzymes to their substrates. The activity of enzymes in frozen tissues could be enhanced by the same

means, and it is likely that the results reported here can be explained at least partly on this basis.

LITERATURE CITED

Alburn, H. E., Grant, N. H., *J. Amer. Chem. Soc.* **87**, 4174 (1965).
Bate-Smith, E. C., Bendall, J. R., *Brit. Med. Bull.* **12**, 230 (1956).
Bate-Smith, E. C., Bendall, J. R., *J. Physiol.* **106**, 177 (1947).
Bate-Smith, E. C., Bendall, J. R., *J. Physiol.* **110**, 47 (1949).
Bendall, J. R., *J. Physiol.* **114**, 71 (1951).
Bendall, J. R., Hallund, O., Wismer-Pederson, J., *J. Food Sci.* **28**, 156 (1963).
Bergmeyer, H. U., Ed., "Methods of Enzymatic Analysis," Academic Press, New York, N. Y., 1965.
Bito, M., Amano, K., *Proc. Int. Cong. Refrig., 9th* **3**, 85 (1960).
Bruice, T. C., Butler, A. R., *Fed. Proc.* **24**(2), S 45 (1965).
Cassens, R. G., Newbold, R. P., *J. Food Sci.* **32**, 269 (1967a).
Cassens, R. G., Newbold, R. P., *J. Food Sci.* **32**, 13 (1967b).
Cassens, R. G., Newbold, R. P., *J. Sci. Food Agr.* **17**, 254 (1966).
de Fremery, D., *J. Agr. Food Chem.* **14**, 214 (1966).
de Fremery, D., Pool, M. F., *Food Res.* **25**, 73 (1960).
Fennema, O., in "Low-Temperature Preservation of Food and Living Matter," Chapter 4, Fennema, O., Ed., Marcel Dekker, New York, 1972.
Fennema, O., *Proc. Meat Ind. Res. Conf.*, March 25, 35 (1971).
Fishbein, W. N., Stowell, R. E., *Cryobiology* 6(3), 227 (1969).
Fraser, D. I., Punjamapirom, S., Dyer, W. J., *J. Fish Res. Bd. Can.* **18**, 641 (1961).
Grant, N. H., Alburn, H. E., *Biochemistry* **4**, 1913 (1965a).
Grant, N. H., Alburn, H. E., *Science* **150**, 1589 (1965b).
Grant, N. H., Alburn, H. E., *Arch. Biochem. Biophys.* **118**, 292 (1967).
Grant, N. H., Clark, D. E., Alburn, H. E., *J. Amer. Chem. Soc.* **83**, 4476 (1961).
Huber, C. S., Stadelman, W. J., *J. Food Sci.* **35**, 233 (1970).
Huntsberger, D. V., "Elements of Statistical Inference," Allyn and Bacon, Boston, Mass., 1967.
Khan, A. W., *Cryobiology* **3**, 224 (1966).
Khan, A. W., *J. Food Sci.* **36**, 120 (1971).
Khan, A. W., Nakamura, R., *J. Food Sci.* **35**, 266 (1970).
Khan, A. W., van den Berg, L., *J. Food Sci.* **32**, 148 (1967).
Khan, A. W., van den Berg, L., Lentz, C. P., *J. Food Sci.* **28**, 425 (1963).
Lawrie, R. A., *J. Physiol.* **121**, 275 (1953).
Locker, R. H., Hagyard, C. J., *J. Sci. Food Agr.* **14**, 787 (1963).
Marsh, B. B., *Biochim. Biophys. Acta* **9**, 127 (1952).
Marsh, B. B., *J. Sci. Food Agr.* **5**, 70 (1954).
Marsh, B. B., Leet, N. G., *J. Food Sci.* **31**, 450 (1966).
Marsh, B. B., Thompson, J. F., *J. Sci. Food Agr.* **9**, 417 (1958).
McWeeny, D. J., *J. Food Technol.* **3**, 15 (1968).
Newbold, R. P., *Physiol. Biochem. Muscle Food, Proc. Int. Symp.* **1**, 213 (1966).
Nowland, S. S., Dyer, W. J., *J. Fish. Res. Bd. Can.* **26**, 2621 (1969).
Partmann, W., *J. Food Sci.* **28**, 15 (1963).
Partmann, W., Z., *Ernaehrungswiss.* **2**, 70 (1961).
Pincock, R. E., Kiovsky, T. E., *J. Chem. Educ.* **43**, 358 (1966).
Rhodes, D. N., *J. Sci. Food Agr.* **12**, 224 (1961).
Sharp, J. G., *Biochem. J.* **29**, 850 (1935).
Sharp, J. G., *Proc. Roy. Soc., Ser. B* **114**, 506 (1934).
Smith, E. C., *Proc. Roy. Soc., Ser. B* **105**, 198 (1929).
Smith, E. C., Moran, T., *Proc. Roy. Soc., Ser. B* **106**, 122 (1930).
Tappel, A. L., in "Cryobiology," Meryman, H. T., Ed., Academic, New York, N. Y. 1966, p 163.
Thompson, L. U., Fennema, O., *J. Agr. Food Chem.* **19**, 121 (1971).
Tomlinson, N., Jonas, R. E. E., Geiger, S. E., *J. Fish. Res. Bd. Can.* **20**, 1145 (1963).

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Aggregate Formation in Frozen Aqueous Solutions of Nucleic Acid Derivatives and Aromatic Amino Acids. Energy Transfer and Complex Formation

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The formation of aggregates of nucleic acid derivatives and aromatic amino acids is induced by freezing aqueous solutions of these molecules. Luminescence and absorption studies provide information about molecular interactions in these aggregates. Extensive migration of the excitation energy at the triplet level is demonstrated by phosphorescence quenching studies, indicating that molecules are stacked in the aggregates. Complex formation between two molecular species can be induced by freezing aqueous mixtures. Thus, tryptophan forms intermolecular complexes with nucleic acid bases which involve charge-transfer interactions. It has also been shown that neutral cytidine is able to form a charge-transfer complex with protonated cytidine. These results demonstrate the usefulness of aggregate formation in frozen aqueous solutions to study molecular interactions between biologically-important molecules.

Weak interactions between identical or different organic molecules in dilute aqueous solutions may be greatly enhanced by freezing these solutions. Rapid cooling of an aqueous solution induces the formation of a microcrystalline structure. Solute molecules are excluded from the growing ice crystals and accumulate in the interstices of solvent (ice) crystallites (Szent-Györgyi, 1960; Wang, 1960, 1961, 1965). This phenomenon has been previously reported by a number of workers. The formation of aggregates has been suggested to be responsible for such different phenomena as the photodimerization of thymine by uv radiation (Wang, 1961, 1965), which occurs to a large extent in frozen aqueous solution although it is completely inefficient in the fluid state (Beukers and Berends, 1960a,b), dipolar broadening of electron spin resonance spectra of paramagnetic cations (Mn^{2+}/Gd^{3+}) in frozen aqueous solutions (Ross, 1965), and enhancement of bimolecular reaction rate constants upon freezing or changes in kinetic order of reactions in the same medium (Bruice and Butler, 1965). Some colorless mixtures in a fluid medium, *e.g.*, quinone and indole derivatives (Szent-Györgyi, 1960; Stom, 1967), exhibit characteristic colors in frozen solutions.

All the foregoing results support the proposed concept that in the aggregates obtained in frozen aqueous solu-

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