Reversible Cold Inactivation of a 17β-Hydroxysteroid Dehydrogenase of Human Placenta: Protective Effect of Glycerol*

Joseph Jarabak, A. Elmore Seeds, Jr.,† and Paul Talalay

ABSTRACT: Purified preparations of the pyridine nucleotide linked 17β-hydroxysteroid dehydrogenase of human placenta lose activity rapidly in buffered aqueous solutions at temperatures below 10–15°. The rate of the cold inactivation is reduced by the addition of low concentrations of 17β-estradiol and of certain pyridine nucleotides, and by high concentrations of some polyvalent anions. The addition of 20% or more of glycerol totally protects the enzyme against cold inactivation. The loss of enzyme activity which occurs at low temperatures may be largely restored by warming the enzyme solutions at 30° under carefully controlled conditions but is no longer reversible after prolonged cooling. Cold treatment of the enzyme preparations is accompanied by the formation of enzymatically inactive high molecular weight components which may be separated from the active enzyme by gel filtration on Sephadex or by electrophoresis on polyacrylamide gels.

The process of cold inactivation appears to involve complex structural changes, including probable alterations in the configuration of the native protein as well as a series of aggregations resulting in the formation of multiple polymeric species. The properties of other cold-inactivated enzymes and the protective effect of glycerol are discussed.

A method for obtaining highly purified preparations of the pyridine nucleotide linked 17β-hydroxysteroid dehydrogenase of human placenta has been described (Jarabak *et al.*, 1962). This enzyme, which may function as either a dehydrogenase or a pyridine nucleotide transhydrogenase (Talalay and Williams-Ashman, 1958, 1960; Jarabak *et al.*, 1962), catalyzes the following freely reversible dehydrogenation reaction[1]

$$17\beta\text{-estradiol} + NAD^+ (NADP^+) \rightleftharpoons \text{estrone} + NADH (NADPH) + H^+$$

Difficulties were encountered during the development of satisfactory methods for the purification of this enzyme because of its profound instability under conditions commonly employed in enzyme fractionation. Addition of pyridine nucleotides, 17β-estradiol, or glycerol stabilized the enzyme (Langer and Engel, 1958; Talalay *et al.*, 1958; Jarabak *et al.*, 1962; Talalay, 1962). The effect of glycerol was especially striking in this respect. Whereas dilute solutions of partially purified 17β-hydroxysteroid dehydrogenase lost more than 90% of their activity upon storage for 24 hr near 0°, the same solutions in 50% glycerol could be stored for many months at this temperature and could be heated at 67° for several hours without the slightest loss in enzymatic activity. Success in obtaining highly purified preparations of this enzyme depended upon carrying out all the steps of the procedure in mixtures of glycerol and water of varying composition (Jarabak *et al.*, 1962). In these media, the enzyme was isolated in good yield by a method involving salt fractionation, heat treatment, and ion exchange chromatography on DEAE- and Ecteola-celluloses. The final product was substantially homogeneous by chromatographic and electrophoretic criteria. Other glycols and their ethers, sucrose, and appropriate concentrations of ethanol were also found to stabilize the enzyme (Jarabak, 1962; Jarabak *et al.*, 1962; Talalay, 1962). Closer examination of the behavior of the enzyme led to the realization that it had the interesting property of losing activity rapidly upon exposure to temperatures below about 10–15°, but that the addition of glycerol protected against this cold inactivation. Upon warming the enzyme under carefully controlled conditions, a substantial fraction of the lost activity could be restored. This paper describes some characteristics of the reversible cold inactivation of the placental 17β-hydroxysteroid dehydrogenase and the protective effect of glycerol and other agents.

Experimental Section

The 17β-hydroxysteroid dehydrogenase was purified

* From the Department of Pharmacology and Experimental Therapeutics and the Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland. *Received December 6, 1965.* These studies were supported in part by Grant AM 07422 from the U. S. Public Health Service.

† Present address: Department of Gynecology and Obstetrics, The Johns Hopkins University School of Medicine, Baltimore, Md. Graduate Training Grant NIH 2 T1 HD-23-04.

[1] Abbreviations used: NAD^+, nicotinamide-adenine dinucleotide; $NADP^+$, nictotinamide-adenine dinucleotide phosphate; NADH, reduced nicotinamide-adenine dinucleotide; NADPH, reduced nicotinamide-adenine dinucleotide phosphate.

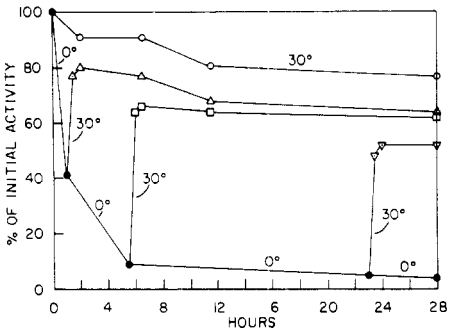


FIGURE 1: Inactivation of placental 17β-hydroxysteroid dehydrogenase at 0° and reactivation at 30°. The enzyme (120,000 units/ml; 1.28 mg of protein/ml) was diluted to a protein concentration of 25.6 μg/ml by the addition of 0.005 M potassium phosphate at pH 7.0. The final glycerol concentration was 1%. One portion of the diluted enzyme was maintained at 30°, while another portion was rapidly cooled and stored at 0°. At the indicated times (1, 5.5, and 23 hr) aliquots were removed from the cold solution, rapidly warmed to 30°, and maintained at this temperature. Periodic measurements of activity were carried out, and the results are expressed in terms of the original activity of the solution. No apparent reactivation occurs during the assay as judged by linearity with time.

according to the procedure of Jarabak *et al.* (1962). Details of chromatographic and electrophoretic purity were given at that time. With the exception of the experiment described in Figure 4, all other studies were carried out on a single purified preparation which was stored for many months at 4° in a medium containing 50% glycerol, 0.005 M potassium phosphate, 0.001 M EDTA, and 0.007 M β-mercaptoethanol, at pH 7.0. The protein concentration of this preparation was 1.28 mg/ml. The enzymatic activity varied somewhat during the course of this work, and is given with the appropriate protocols. Unless otherwise stated, activity measurements were carried out with 3-acetylpyridine–adenine dinucleotide as hydrogen acceptor according to Jarabak *et al.* (1962). The assay cuvets contained in a final volume of 3.0 ml: 440 μmoles of sodium pyrophosphate buffer at pH 10.2, 25 mg of crystalline bovine serum albumin (usually added as 0.5 ml of a 5% solution), 0.3 μmole of 17β-estradiol in 0.04 ml of 95% ethanol, 1.9 μmoles of 3-acetylpyridine–adenine dinucleotide, and sufficient enzyme to produce a change in absorbance at 363 mμ of between 0.001 and 0.040/min in a cuvet of 1.0-cm light path. This normally required about 0.5 μg of protein. The final pH was between 9.3 and 9.4. All activity measurements were carried out at 25°, irrespective of the earlier temperature history of the enzyme. The enzymatic reactions were initiated by the addition of enzyme (in a small volume) to the otherwise complete reaction system which had been equilibrated at 25 ± 0.5°. The reaction rate was determined from changes in absorbance at 363 mμ against a blank cuvet from which nucleotide was omitted. The initial reaction rates were strictly constant over the period examined (the initial 3–5 min). When different aliquots of a single enzyme preparation were assayed in this system, the observed initial reaction rates were strictly proportional to enzyme concentration over a wide range. One unit of enzyme activity is defined as the quantity producing an absorbance change of 0.001/min at 363 mμ under the stated conditions.

Reagent grade urea was recrystallized from ethanol. Spectroscopic quality glycerol was used without further purification. The concentrations of glycerol are expressed by volume. Sephadex G-100 and G-200 beads (Pharmacia) were thoroughly equilibrated with appropriate buffer systems, and the fine particles were removed by decantation before packing of the columns. The void volumes of the Sephadex columns were determined with Blue Dextran 2000 (Pharmacia). Disk electrophoresis on polyacrylamide gels was carried out according to the general directions of Ornstein (1964) and Davis (1964). More detailed information on the electrophoresis is given in the legend of Figure 7.

Results

Cold Inactivation and Its Reversal. The time course of inactivation of 17β-hydroxysteroid dehydrogenase upon storage at 0 and 30° in 1% glycerol is shown in Figure 1. The activities of all samples were assayed at 25°. The enzyme stored at 0° undergoes a rapid initial loss of activity which is followed by a prolonged and much more gradual inactivation. The enzyme preparation stored at 30° lost activity at the same rate as the slow phase of the enzyme stored in the cold. At 0°, more than 50% of the initial activity was lost within the first hour of storage, and only 3–4% of the activity remained after 28 hr. When aliquots of the cold-inactivated enzyme were warmed to 30°, they rapidly regained a substantial fraction of their initial activity. Upon continuing storage at 30°, the reactivated enzyme lost activity at the same slow rate as the control enzyme which had been stored at 30° from the beginning of the experiment. After prolonged storage in the cold the activity of the enzyme cannot be restored on warming. Although the addition of glycerol arrests the cold-inactivation process, the enzyme may be readily reactivated by warming at 30° in the presence of 20% glycerol. All the measurements shown in Figure 1 were made in the assay system containing 3-acetylpyridine–adenine dinucleotide, but entirely parallel changes in activity were observed with NAD or NADP as hydrogen acceptors.

Effect of Temperature on Rate of Inactivation and Reactivation. Examination of the effect of temperature on the inactivation of the enzyme reveals that little loss in activity is observed in 1 hr until the temperature is lowered to below about 11–12° (Figure 2A and 2B). The initial rapid phase of inactivation is highly dependent on temperature whereas the rate of the subsequent slower inactivation appears to be little affected by temperature changes within the range examined (Figure 2A). The biphasic time course of the inactivation is more clearly

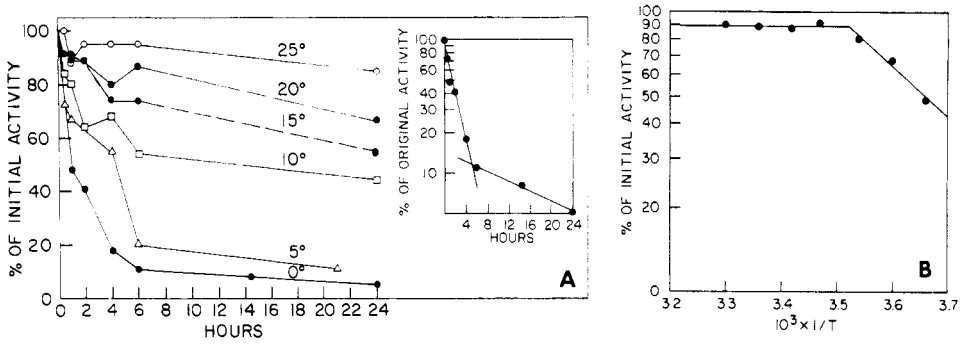


FIGURE 2: Effect of temperature on the rate of inactivation of the 17β-hydroxysteroid dehydrogenase. (A) The enzyme preparation described in Figure 1 was diluted with 0.005 M potassium phosphate buffer at pH 7.0 to a final protein concentration of 25.6 μg/ml. Aliquots were assayed immediately after dilution and after various times of storage at the indicated temperatures. The insert is a semilogarithmic plot of the activity as a function of time for the sample stored at 0°. (B) The fraction of the initial enzyme activity remaining after 1 hr of storage at various temperatures is plotted *vs.* the reciprocal of the absolute temperature (°K). The results obtained from Figure 2A at 1 hr have been used for this plot.

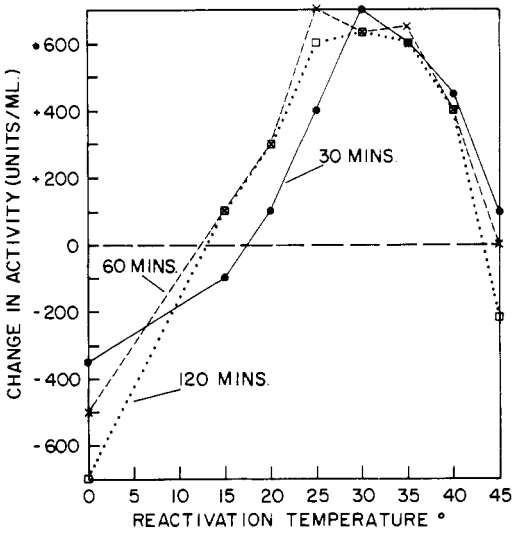


FIGURE 3: Reactivation of cold-inactivated 17β-hydroxysteroid dehydrogenase as a function of temperature and time. The enzyme solution was diluted to a protein concentration of 25.6 μg/ml as described in Figure 1. After storage at 0° for 1 hr, the activity of the diluted enzyme had fallen from 2200 units/ml to 1000 units/ml. The graph shows the change in activity from the latter level after incubation of the enzyme for 30, 60, and 120 min at the indicated temperatures.

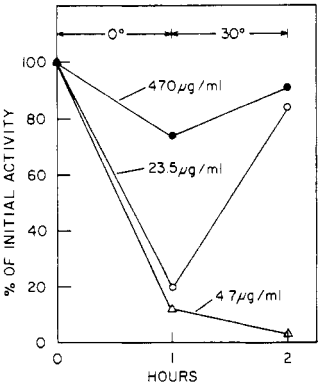


FIGURE 4: Effect of enzyme concentration on cold inactivation and subsequent reactivation on warming. The preparation of 17β-hydroxysteroid dehydrogenase used in these experiments had an initial protein concentration of 18.8 mg/ml (1.06×10^6 units/ml). Dilutions were made so that the enzyme contained 0.005 M potassium phosphate and 1% glycerol at pH 7.0. The initial assays were performed immediately upon dilution. The solutions were rapidly cooled at 0° and appropriate aliquots were assayed after 1 hr, warmed to 30°, and reassayed after standing for 1 hr at the latter temperature. Samples which were not cooled retained more than 90% of their original activity after 2 hr.

shown on a semilogarithmic plot (see insert in Figure 2A).

The optimum temperature of reactivation of the cold-inactivated enzyme is 25 to 35° (Figure 3). Above about 11–12° the cold-inactivated enzyme gains activity under the conditions described. Figure 3 also shows that the rate and ultimate extent of reactivation are temperature dependent.

Effect of Protein Concentration. The rate and extent of cold inactivation of the enzyme and its reversal are also profoundly affected by the protein concentration. Figure 4 shows that only 25% of the activity was lost in 1 hr at 0° when the protein concentration was 470 μg/ml, whereas nearly a 90% loss of activity occurred at a concentration of 4.7 μg/ml. The activity of the very dilute cold-inactivated enzyme could not be restored on

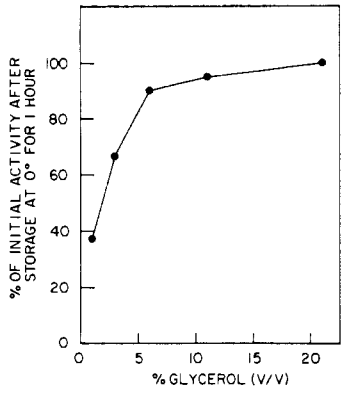


FIGURE 5: Protective effect of varying concentrations of glycerol on the cold inactivation of the placental 17β-hydroxysteroid dehydrogenase. The enzyme preparation described in Figure 1 was diluted to a protein concentration of 25.6 μg/ml with a medium containing 0.005 M potassium phosphate at pH 7.0 and various concentrations of glycerol. The enzyme was assayed immediately after dilution, cooled rapidly to 0°, and assayed for a second time after storage for 1 hr at this temperature. All assays were conducted at 25°.

warming, whereas at the higher concentration the process was almost fully reversible.

Effect of Composition of Medium on Cold Inactivation. The composition of the medium influences not only the rate but also the extent of the cold inactivation of the enzyme. In contrast to the rapid loss of activity which occurs at 0° in 1% glycerol, the presence of 50% glycerol stabilized the enzyme for many months. The efficiency of various concentrations of glycerol in protecting the enzyme against cold inactivation is shown in Figure 5. The purified enzyme is very stable in 50% glycerol at room temperature and at higher temperatures (Talalay, 1962; Jarabak *et al.*, 1962). Although the enzyme appears to be quite soluble in almost pure glycerol, it loses activity in this medium. For example, after 24 hr at 23° in 99.5% glycerol, the enzyme had lost 27% of its activity while after 144 hr under these conditions, the loss was 85%.

Table I summarizes the effect of 17β-estradiol, various pyridine nucleotides, and potassium phosphate on the stability of the enzyme after storage for 1 and 24 hr at 0°. The protective effects of the pyridine nucleotides appear to be related to their Michaelis constants (Talalay *et al.*, 1958; Talalay and Williams-Ashman, 1960). High concentrations of potassium phosphate efficiently protected the enzyme against cold inactivation. In other experiments, it was found that high concentrations of potassium sulfate (1.0 M) also exercised a protective effect, but similar concentrations of potassium chloride or potassium nitrate greatly accelerated the loss of enzyme activity in the cold. 17β-Estradiol (Table I), and equivalent concentrations of testosterone, offered some degree of protection against cold inactivation. It is of interest that diethylstilbestrol (1–10 μM) also protected the enzyme against cold inactivation although it is not oxidized by the enzyme and actually competitively inhibits the oxidation of 17β-estradiol (Adams *et al.*, 1962).

The addition of urea to dilute solutions of the enzyme accelerates the loss of activity at 23 and 0°, but the cold inactivation is stimulated to a much greater extent by this reagent. Table II shows that the inactivating effect of urea increases with concentration and can be counteracted by the incorporation of glycerol into the solu-

TABLE I: Protection of 17β-Hydroxysteroid Dehydrogenase against Cold Inactivation by Various Substances.[a]

Additions	Concentration	Residual Activity after Storage at 0°: 1 hr (%)	24 hr (%)
No additions		41	3
Pyridine nucleotides			
NADP	1 μM	89	71
NADP	10 μM	91	84
NAD	1 μM	63	5
NAD	100 μM	96	60
3-Acetylpyridine-AD	1 μM	50	3
3-Acetylpyridine-AD	100 μM	82	19
3-Pyridine aldehyde-AD	100 μM	82	21
3-Thionicotinamide-AD	100 μM	90	80
Steroids			
17β Estradiol	1 μM	88	42
17β-Estradiol	10 μM	93	83
Potassium phosphate, pH 7.0	100 mM	54	7
Potassium phosphate, pH 7.0	500 mM	87	41
Potassium phosphate, pH 7.0	1 M	87	87

[a] The enzyme preparation described in Figure 1 was diluted to 25.6 μg of protein/ml in the appropriate medium. The activity was assayed immediately after dilution and the enzyme cooled rapidly to 0°. The activity was measured again after 1 and 24 hr. All media contained a final concentration of 1% glycerol and 5 mM potassium phosphate (except when phosphate was added). The 17β-estradiol was added in ethanol to give a final concentration of 2%. All assays were carried out with 3-acetylpyridine–adenine dinucleotide (3-Acetylpyridine-AD) as acceptor. The final concentrations of protective nucleotides were too low to affect the assay system in the cuvets.

TABLE II: Effect of Temperature on the Stability of the Placental 17β-Hydroxysteroid Dehydrogenase in Urea and in Glycerol.[a]

	Residual Activity after Storage (%)							
	1% Glycerol		21% Glycerol		51% Glycerol			
Additions	1 hr at 0°	1 hr at 23°	1 hr at 0°	1 hr at 23°	1 hr at 0°	24 hr at 0°	1 hr at 23°	24 hr at 23°
None	40	95	100	100	100	100	100	100
Urea, 1 M	5	74	44	95				
Urea, 4 M	0	0	0	28	87	66	101	99
Urea, 8 M							0	

[a] The enzyme preparation described in Figure 1 was diluted 50-fold with 0.005 M potassium phosphate buffer at pH 7.0 and urea in the concentrations indicated. The activity was assayed immediately, the solutions were rapidly cooled at 0°, and activity measurements were made after 1 and 24 hr of storage at this temperature.

tions. This is exemplified by the finding that in 1 hr at 23° the entire activity was lost in 4 M urea and 1% glycerol, but the entire activity was retained in a solution containing 4 M urea and 51% glycerol.

Sephadex Gel Filtration. The nature of the structural changes which accompany the cold inactivation of the 17β-hydroxysteroid dehydrogenase was examined by filtration of the enzyme on Sephadex gels. When a purified preparation of the enzyme which had been stored in 50% glycerol was applied to a Sephadex G-100 column (operated at 23°, previously equilibrated with 0.01 M potassium phosphate buffer at pH 7.0), the protein was eluted in two distinct peaks. The first was eluted in the void volume and was enzymatically inactive, whereas the second peak was eluted at 1.25–1.35 void volumes and contained the entire enzymatic activity (Figure 6A). It is not clear whether the material eluted in the void volume represents an impurity present in the enzyme, an inactive transformation product of the enzyme formed during filtration, or a combination of both such materials. If the same enzyme preparation was first dialyzed for 18 hr at 0° against 0.01 M potassium phosphate at pH 7.0, and then applied to the column again operated at 23°, the elution pattern shown in Figure 6B was obtained. The enzymatically inactive high molecular weight fraction appearing with the void volume was greatly increased in quantity whereas both the total activity and the amount of protein in the peak appearing at about 1.3 void volumes was considerably diminished. These findings suggest that the enzyme is capable of existing in at least two forms of which only the low molecular weight species is catalytically active.

When the lower molecular weight fractions obtained from the column operated at 23° (Figure 6A) were combined and reapplied to the same column, the protein once again became distributed between the two peaks, indicating that there may be a tendency for the enzyme to aggregate in dilute solution, or upon passage through the Sephadex column.

The experimental design was then modified to permit study of both cold inactivation and reactivation on warming. The purified enzyme was directly applied to a Sephadex G-100 column of similar dimensions, but maintained at 4° and developed with 0.2 M potassium phosphate buffer at pH 7.0 (Figure 6C). After determination of the protein concentration and enzymatic activity of the cold fractions, these were incubated at 30° for 1 hr and their activity again assayed. Figure 6C shows that reactivation of the enzyme on warming occurs primarily in the fractions containing the low molecular weight species, although some increases in enzyme activity resulting from reactivation of higher molecular weight forms cannot be excluded.

The interpretation of the experimental findings with the Sephadex columns is somewhat complicated because equilibrium conditions may not always exist during the gel filtration. If during the operation of the Sephadex columns the environmental conditions existing in the columns induce changes in the distribution of molecular species, the resolution of these species may be affected. It should also be pointed out that a part of the reactivation that occurs upon warming of the fractions shown in Figure 6C may reflect activity lost by the lower molecular weight material during storage in the cold after elution from the column.

The total recovery of enzyme activity in Figure 6B is considerably higher than that in the experiment illustrated in Figure 6C (before warming). This difference may be related to the fact that the column described in Figure 6B was operated at 23°, and some recovery of activity may have occurred during passage of the enzyme through the Sephadex bed at this temperature. It may be concluded from these experiments with Sephadex filtration that cold inactivation is associated with the formation of material of high molecular weight, but that other complicating factors preclude a quantitative analysis of this phenomenon.

Gel Electrophoresis. When gel filtration was performed on Sephadex G-200, the high molecular weight material produced on cooling was found to contain several components, which were not sharply resolved. However, electrophoresis of the cold-inactivated enzyme on polyacrylamide gel separated these components and permitted a more detailed analysis of the

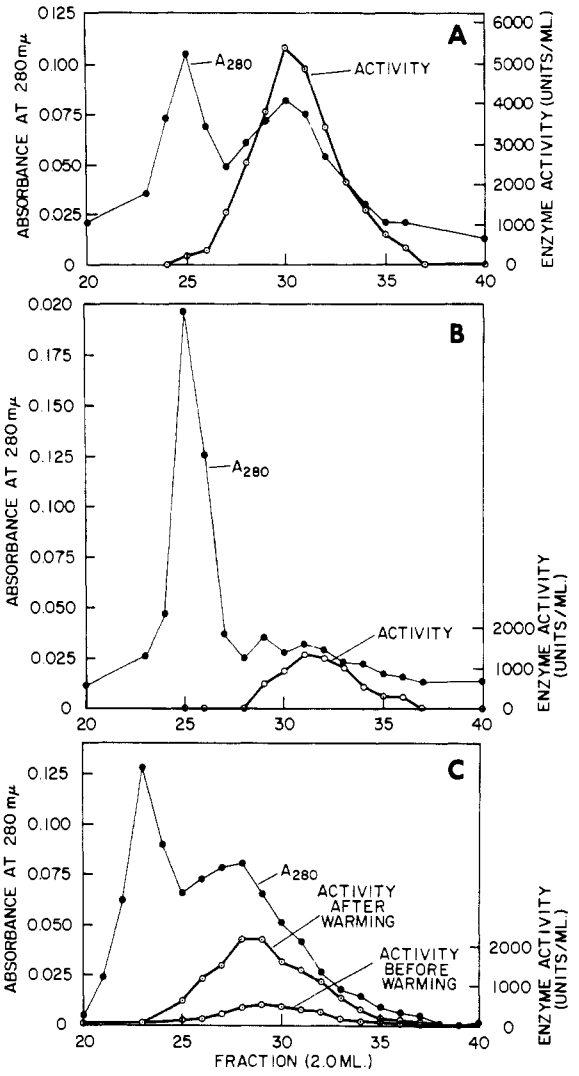


FIGURE 6: Filtration of 17β-hydroxysteroid dehydrogenase on a Sephadex G-100 column. The distribution of enzyme activity and absorbance at 280 mμ is shown. (A) A column of Sephadex G-100 (470 × 20 mm) was equilibrated with 0.01 M potassium phosphate buffer at pH 7.0. The column was operated at 23°. Enzyme (1 ml; 1.28 mg of protein and 80,000 units of activity) in 50% glycerol, 0.005 M potassium phosphate, 0.001 M EDTA, and 0.007 M β-mercaptoethanol at pH 7.0 was applied directly to the column. The column was developed by the addition of 0.01 M potassium phosphate buffer at pH 7.0. Fractions of 2.0-ml volume were collected at a flow rate of 0.8 ml/min. The exclusion volume of the gel was 50 ml. (B) Approximately the same quantity of protein as in A was applied to the same column after the enzyme had been dialyzed for 18 hr at 0° *vs.* 0.01 M potassium phosphate buffer at pH 7.0. The elution was carried out at 23° in the same manner as described in part A. (C) Another column of Sephadex G-100 (470 × 20 mm) was equilibrated with 0.2 M potassium phosphate buffer at pH 7.0. The column was operated at 4°. The enzyme (1 ml) described in part A was applied and the column was developed with 0.2 M potassium phosphate buffer at pH 7.0. Fractions of 2.0-ml volume were collected at a flow rate of 0.7 ml/min. The exclusion volume of the gel was 46 ml. All of the enzymatically active fractions had appeared from the column within 110 min after application of the enzyme. The fractions were stored at 4° until after their protein concentration and enzymatic activity had been determined. All of the initial assays had been completed within 270 min from the time of the application of the enzyme to the column. The fractions were then warmed for 1 hr at 30° and their activity again measured.

changes occurring during the cold-inactivation process and subsequent warming. An additional advantage of this method over Sephadex gel filtration was that small amounts of very dilute protein solutions could be analyzed since these solutions are initially concentrated during the electrophoresis upon passage through the stacking gel. Consequently, the entire process of cold inactivation and reactivation by warming could be examined at enzyme levels similar to those used in Figure 1.

The enzymatic activity is associated with a single band which migrates relatively rapidly upon electrophoresis on polyacrylamide gel (indicated by the arrow in Figure 7). As the enzyme is cooled for increasing periods, and becomes progressively inactivated, the band of active enzyme diminishes in intensity. After 14 days of storage at 0°, the enzyme is totally inactive and the band corresponding to active enzyme is no longer detectable. During the early stages of cold inactivation, a diffuse zone of less rapidly migrating protein appears just behind the band of active enzyme. As cooling is continued, this diffuse band also becomes less intense and is replaced by a series (at least 12) of sharply defined components which migrate even more slowly than the diffuse zone. On warming the enzyme stored in the cold for 4–24 hr, activity is restored and two changes in the electrophoretic pattern are noted. The band associated with enzymatic activity becomes more intense, and the multiple, sharply defined, more slowly moving components are also intensified.

When the distance of migration of these individual slow moving components is plotted against the logarithm of their order, beginning with the band representing the active enzyme, a strictly linear relation is obtained. Since the migration of components is probably a function of both size and charge, no unequivocal interpretation of these findings can be drawn, but it seems reasonable to suggest that the slow moving sharp bands represent simple multiples of molecular weight of the active enzyme. The formation of similar multiple protein bands was observed also after treatment of the enzyme with urea, sodium dodecyl sulfate, or *p*-(hydroxy)-mercuribenzoate.

When bovine serum albumin was polymerized by treatment with ethanol (Hartley *et al.*, 1962), multiple

bands were observed on gel electrophoresis, and once again the distance of migration of these bands was a linear function of the logarithm of their order.

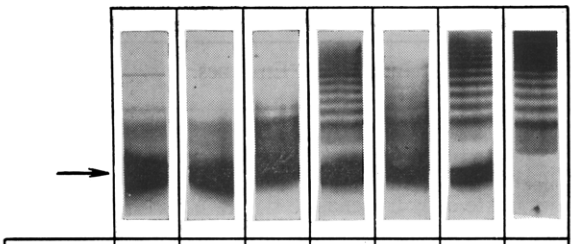
One simple interpretation of the electrophoretic patterns shown in Figure 7 is the following. On cooling the enzyme undergoes a series of conformational changes. This altered enzyme now migrates in a diffuse band just behind the active enzyme. On further and prolonged cooling the molecules of altered conformation aggregate and form a series of species of increasing molecular weight represented by the sharp slowly migrating bands. These high molecular forms are no longer converted to active enzyme on warming. If the enzyme is warmed during the early stages of cold inactivation, some of the molecules of altered conformation may revert to the native conformation and add to the intensity of the band of active enzyme, while others undergo a series of aggregations with the formation of the slowly moving bands. In the absence of quantitative information on the distribution of protein in the electrophoretic gels, this interpretation is necessarily tentative.

Sephadex filtrations of the cold-inactivated enzyme followed by polyacrylamide electrophoresis has confirmed the interpretation that the materials which migrate more slowly than the active enzyme on electrophoresis are those eluted in the exclusion volume on Sephadex G-100.

Discussion

Cold-Inactivated Enzymes. Numerous reports dating from the very beginning of quantitative studies on enzymes have dealt with the influence of temperature on these catalysts and on the rates of the reactions which they promote (Dixon and Webb, 1964). It is commonly accepted that, with a few exceptions, elevated temperatures result in protein denaturation and loss of enzymatic activity, and that the temperature coefficient of this process is very high. This observation led to the widespread and often correct belief that strict maintenance of low temperatures (0–5°) is an essential prerequisite for success in enzyme isolation and fractionation. Uncritical adherence to this dogma can no longer be recommended since the past few years have brought to light some 12 examples of cold-sensitive enzymes (Table III) which display far greater stability near room temperature than in the icebox, and this property may be far more common than has been hitherto suspected. It is to be recommended that enzymes displaying unusual instability during the ordinary isolation procedures should be examined for cold sensitivity.

The temperature at which an enzyme is assayed and the temperature at which it is stored prior to assay are frequently different and may be varied at will. It is possible therefore to distinguish experimentally the effects of these two parameters on the activity of an enzyme. The experiments recorded in this paper deal exclusively with the influence of storage temperature on enzymatic activity, all assays being performed at a fixed temperature. We feel it has been possible to maintain the



Time at 0°	0	30 MIN	4 HR	4 HR	24 HR	24 HR	14 D
Time at 30°	0	0	0	1 HR	0	1 HR	0
Activity %	100	65	22	70	8	40	0

FIGURE 7: The effect of cold inactivation and subsequent warming on the polyacrylamide gel electrophoresis patterns of the 17β-hydroxysteroid dehydrogenase. Disk electrophoresis was performed in a cold room at 4° by a modification of the procedure described by Davis (1964). Both the 3.5% (stacking) and the 7.5% cross-linked gels were polymerized in solutions containing 20% glycerol. The Tris–glycine buffer of pH 8.3 used for the electrophoresis also contained 20% glycerol. The enzyme described in Figure 1 was diluted to a protein concentration of 64 μg/ml by the addition of 0.005 M potassium phosphate buffer of pH 7.0. The enzyme was assayed immediately and stored at 0°. The assays were repeated after 30 min, 4 hr, 24 hr, and 14 days (designated 14 D) at 0°. Portions (25.6 μg of protein or 0.4 ml) were brought to a glycerol concentration of 30%, and were placed on top of the polyacrylamide stacking gel. The Tris–glycine buffer containing brom phenol blue was then added and the electrophoresis started. The initial current was 0.5 ma for each gel, and was increased to 1.0 ma as soon as the dye had entered the 7.5% cross-linked gel. The total time of electrophoresis was about 3.5 hr. The temperature of the buffers never rose above 10°. The gels were fixed and stained with Amido–Schwartz dye in acetic acid. Aliquots of the cold-inactivated enzyme were removed after 4 and 24 hr at 0°; they were warmed at 30° for 1 hr, assayed, and then subjected to electrophoresis as described. The direction of migration is from top to bottom. The electrophoretic patterns are shown above together with the activities of the solutions just prior to electrophoresis. An aliquot of the sample cooled for 14 days remained completely inactive upon warming for 1 hr at 30°, and the electrophoretic pattern did not change after warming. The conditions of the electrophoresis were selected in order to minimize changes in enzyme activity resulting from cold inactivation or heat reactivation. This was achieved by using a glycerol concentration of 20% and maintaining temperatures below 10° during electrophoresis. In separate experiments, the gels were sectioned prior to staining, the enzyme was eluted, and the activity was assayed spectrophotometrically with 3-acetylpyridine–adenine dinucleotide. Only the band indicated with the arrow contained 17β-hydroxysteroid dehydrogenase activity. Histochemical tests (Goldberg, 1963) with intact gels also located the enzymatic activity in this single band, when NAD, NADP, or 3-acetylpyridine–adenine dinucleotide was used as hydrogen acceptor.

TABLE III: Cold-Inactivated Enzymes.

Enzyme	Source	Reference
Urease	Jack bean	Hofstee (1949)
Glutamate dehydrogenase	*Neurospora crassa* (mutant)	Fincham (1957)
Adenosine triphosphatase	Beef heart mitochondria	Pullman *et al.* (1960)
Adenosine triphosphatase	Yeast	Racker *et al.* (1963)
Carbamyl phosphate synthetase	Frog liver	Raijman and Grisolia (1961)
D(−)β-Hydroxybutyrate dehydrogenase (DPN)	*Rhodospirillum rubrum*	Shuster and Doudoroff (1962)
Glutamate decarboxylase	*Escherichia coli*	Shukuya and Schwert (1960)
Nitrogen-fixing enzyme	*Clostridium pasteurianum*	Dua and Burris (1963)
Arginosuccinase	Steer liver	Havir *et al.* (1965)
Glycogen phosphorylase	Rabbit muscle	Graves *et al.* (1965)
Pyruvate carboxylase	Chicken liver mitochondria	Scrutton and Utter (1965)
Glucose 6-phosphate dehydrogenase	Human erythrocyte	Kirkman and Hendrickson (1962)

separation of the effects of assay temperature and storage temperature because the enzyme undergoes no significant changes in activity during the assay procedure, irrespective of its earlier storage temperature. Enolase (Rosenberg and Lumry, 1964) and pyruvic kinase (Kayne and Suelter, 1965), which undergo inactivation at lowered assay temperatures, were not included in Table III because of insufficient information concerning the effect of storage temperature on their activities.

The concept of cold inactivation is naturally relative to a specific reference temperature. From an operational viewpoint we are here concerned with enzymes which maintain their activity much better on storage between about 20 and 30° than between 0 and 20°. The more complex changes which occur during freezing and thawing of enzyme solutions will not be considered.

The cold inactivation of the 17β-hydroxysteroid dehydrogenase has certain notable characteristics: it is partially reversed by warming, it may be reduced by certain anions as well as cofactors and substrates, it is accompanied by changes in both molecular weight and configuration, and it is prevented by glycerol. A comparison of the cold inactivation of the 17β-hydroxysteroid dehydrogenase with that of the enzymes which appear in Table III reveals many similarities, but particularly striking are the changes in molecular weight and configuration and the protection by glycerol or related compounds.

Changes in Molecular Weight and Configuration. In every case which has been examined, cold inactivation of susceptible enzymes was accompanied by changes in molecular weight. Dissociation into subunits on cooling has been observed in the case of pyruvate carboxylase (Scrutton and Utter, 1965), carbamyl phosphate synthetase (Grisolia and Raijman, 1964), arginosuccinase (Havir *et al.*, 1965), erythrocyte glucose 6-phosphate dehydrogenase (Kirkman and Hendrickson, 1962), and in the adenosine triphosphatase of beef heart mitochondria (Penefsky and Warner, 1965). In contrast to these enzymes, Graves *et al.* (1965) have clearly shown by ultracentrifugation that glycogen phosphorylase *b* undergoes cold inactivation accompanied by the appearance of a component or components which migrate with a sedimentation constant of about 20 S, whereas the active enzyme has a sedimentation constant of approximately 8 S. On rewarming the enzyme, a single component with a sedimentation constant of about 8 S is again obtained, and the high molecular weight material is almost totally obliterated. Hofstee (1949) suggested, on the basis of indirect evidence, that urease also aggregates in the cold. The experiments described in this paper indicate that the placental 17β-hydroxysteroid dehydrogenase undergoes a series of aggregation reactions on cooling. This conclusion is based on the behavior of the enzyme on Sephadex molecular sieves and on polyacrylamide gel electrophoresis, and is confirmed by preliminary experiments in the analytical ultracentrifuge. The placental enzyme displays the hitherto unique property of forming a large number of molecular aggregates on cooling.

Although cooling can induce either aggregation or dissociation into subunits, the extent of these physical changes does not necessarily correlate well with the enzyme activity. The kinetics of cold inactivation frequently do not obey simple rate laws, thus indicating that the physical changes may be quite complex. Havir *et al.* (1965) and Scrutton and Utter (1965) have intimated that other changes, probably of a conformational nature, precede physical changes in the size of the protein. Several findings also indicate that aggregation may not be the earliest cold-induced change in the placental enzyme. If aggregation were the sole cause for loss of enzyme activity, this might be expected to occur more rapidly at high protein concentrations, as is the case with phosphorylase *b* (Graves *et al.* (1965), whereas the opposite is observed for the placental enzyme. It has been shown that preparations of cold-inactivated 17β-hydroxysteroid dehydrogenase can be separated into high and low molecular weight components by Sephadex gel filtration. On warming these fractions, reactivation was observed largely, and perhaps exclusively, in

the low molecular weight species (Figure 6C). This observation suggests that conformational changes occur upon cooling, and that these changes precede and are possibly a necessary prerequisite for the formation of molecular aggregates. Furthermore, the finding that inactivation of the 17β-hydroxysteroid dehydrogenase by such chemically dissimilar agents as sodium dodecyl sulfate, *p*-(hydroxy)mercuribenzoate, and urea is accompanied by the formation of molecular aggregates, suggests that there is no direct relation between aggregation and cold inactivation, but that cold and the chemical agents all bring about a disruption of the native configuration of the enzyme and that this favors the subsequent aggregation.

Stabilization by Glycerol and Other Substances. Varying concentrations of glycerol exert a profound stabilizing effect on the placental 17β-hydroxysteroid dehydrogenase. Glycerol protects the enzyme not only against the deleterious effects of cooling and urea, as shown in the present studies, but also against heat inactivation (Langer and Engel, 1958; Jarabak *et al.*, 1962). This protective influence is not confined to glycerol, but is shared by other polyhydroxylic compounds such as glycols, glycol ethers, and sucrose, as well as certain inorganic salts in high concentration (Table II; Jarabak, 1962; Talalay, 1962). The addition of glycerol has stabilized enzymes against cold inactivation in each instance where this question has been adequately examined. Racker *et al.* (1963) have shown that the dinitrophenol-stimulated adenosine triphosphatases of beef heart mitochondria and of yeast are both cold-sensitive enzymes which could be stabilized by the addition of 20–50% glycerol. Meyerhof and Ohlmeyer (1952) partially purified yeast adenosine triphosphatase in the presence of glycerol, long before its cold sensitivity was recognized. Similarly, the 17β-hydroxysteroid dehydrogenase of placenta was also purified in glycerol–water mixtures, before we became aware of its cold inactivation. Glycerol also stabilized the cold-sensitive carbamyl phosphate synthetase (Novoa and Grisolia, 1964). Complete protection against cold inactivation of pyruvate carboxylase is provided by 1.5 M sucrose (Utter *et al.*, 1964). The addition of 10% by volume of methanol, propylene glycol, or dimethyl sulfoxide completely protected phosphorylase *b* against cold inactivation (Graves *et al.*, 1965). Dua and Burris (1963) could find no stabilizing effect of glycerol and sucrose on the cold-inactivated nitrogen-fixing enzyme in extracts of *Clostridium pasteurianum*, but the concentrations used (0.1–0.001 M) were much lower than those required for significant stabilization of other enzymes.

Although the stabilizing effect of steroids and pyridine nucleotides can be attributed to the maintenance of the configuration at the active site, the mechanism of the stabilizing effect of glycerol and related compounds against cold, heat, and urea inactivation is at present obscure. The question also arises whether the stabilizing effect of high concentrations of certain anions is in any way related to the protective effect of glycerol. One suggestion which has been advanced to account for the protective effect of various organic solvents is that they may have the common property of stabilizing networks of "structured" water molecules which are essential to the maintenance of the proper spatial configuration of the protein in the native state (Talalay, 1962). The protective effect of anions might also be considered within this framework in view of their capability of inducing changes in the structure of the solvent medium. In a recent study, Fridovich (1963) has given a detailed analysis of the inhibition of acetoacetic decarboxylase by anions, and has analyzed the effect of replacing the aqueous medium by 50% glycerol or 50% ethylene glycol. His explanation for the specificity of anion inhibition is based on the effects on the structure of water produced by a combination of the ions with a specific binding site on the protein. He also states that the effects of replacing water partially by glycols is in accord with this proposal. Recent measurements of the anomalous dielectric constants of glycerol–water mixtures have been interpreted as indicating the existence of "packages" of glycerol and water of different composition in these solutions (McDuffie *et al.*, 1962). Thus the formation of water–glycerol structures around the protein molecules is not unreasonable, and may account for the stabilizing forces protecting the protein against inactivation. Evidence for the structured nature of water and the importance of this structure in the maintenance of protein configuration has been reviewed in a recent symposium (Frank, 1965; Klotz, 1965). The validity of such suggestions based on the stabilization of the hydrated structure of proteins must await a far better understanding of the structure of water surrounding biological macromolecules at different temperatures than is presently available (Jencks, 1965).

Of the bonds believed to play an important part in maintaining the three-dimensional structure of proteins, only hydrophobic bonds become significantly weaker as the temperature is lowered (Kauzmann, 1959; Scheraga *et al.*, 1962; Tanford, 1962). Therefore, it is perhaps only to be expected that some proteins become less stable when the temperature is lowered. Brandts (1964) has found that the temperature of maximum stability of chymotrypsinogen is 10°. Extensive studies have led him to conclude that the temperature dependence of hydrophobic bonding is an important factor in accounting for this. He has suggested that ". . .those proteins having a large number of hydrophobic interactions which are solvated during the unfolding process would be expected to have a maximum stability at a higher temperature than those which exhibit less hydrophobic character in their transition." In the light of this, it would be desirable to obtain information on the influence of glycerol on the strength of hydrophobic bonds of proteins in solution.

The behavior of proteins in nonaqueous solvents is attracting increasing attention. The thoughtful and interesting review of this subject by Singer (1962) created order out of many isolated observations. Numerous studies attest to the fact that the internal configuration of some protein molecules may undergo profound changes in nonaqueous systems, whereas no evidence

for such changes can be obtained in other systems or with other proteins. For instance, Kay and Brahms (1963) showed that in 30–60% ethylene glycol, myosin A undergoes profound changes in internal configuration, a reduction in helicity, and an increase of about 2.5 times in adenosine triphosphatase activity. However, other fibrous muscle proteins do not change the proportion of their helical content in ethylene glycol. It would, therefore, be of great interest to study the conformational changes which cold-sensitive enzymes undergo during inactivation, and the influence of glycerol and related substances on these processes.

Although the use of glycerol in enzymology was widespread in earlier days, this agent was only rarely employed following the introduction of refrigeration. However, a number of recent reports have stressed the advantage of purifying enzymes in glycerol–water mixtures. Many of these enzymes are probably not cold inactivated, but they appear to enjoy considerably enhanced stability in this medium. In this connection, mention might be made of the purification of glucose 6-phosphate dehydrogenase from mammary gland (Levy, 1963), RNA polymerase from *M. lysodeikticus* (Nakamoto *et al.*, 1964), RNA polymerase from testis (Ballard and Williams-Ashman, 1966), 3α-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* (Boyer *et al.*, 1965), 20α-hydroxysteroid dehydrogenase of corpus luteum (Wiest and Wilcox, 1961), steroid Δ^4-5β-dehydrogenase (Davidson and Talalay, 1966), and glycerokinase (Bublitz and Kennedy, 1954). In the last case, the protection by glycerol occurs at quite low concentrations, probably because glycerol is one of the substrates for this enzyme.

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Properties and Metabolism of 2-Alkylalkanoates. Cholesteryl 2-Methylalkanoates*

Edward A. Napier, Jr.

ABSTRACT: Substitution of a methyl group at the α-methylene carbon of methyl esters of laurate, myristate, and palmitate results in characteristic infrared spectra that readily distinguishes them from the straight-chain homologs, and facilitates their unequivocal identification when separated from complex lipid mixtures by gas–liquid partition chromatography (glpc). Spectral changes observed with the methyl esters are also evident in the cholesterol esters of 2-methylalkanoates and also serve in their identification. No significant absorption of either cholesterol-4-C^{14}, as determined by radioassay, or of 2-methylalkanoate, as determined by glpc, could be demonstrated when given to the rat as cholesteryl-4-^{14}C 2-methyl alkanoate, either incorporated in normal rat chow, fed by peroral intubation, or perfused *in situ* through isolated intestinal loops. These studies thus support the thesis that neither cholesterol nor long-chain fatty acids is readily transported across the intestinal mucosa as the cholesterol ester, as such, and would minimize the role of pinocytosis as a mechanism for the absorption of fatty acid esters, at least in the case of cholesterol esters.

Substitution of alkyl or aryl groups at the methylene carbon adjacent to the carbonyl group of fatty acids is known to alter the metabolic pathways of fatty acids and their ester derivatives. Metabolic anomalies associated with α- or 2-alkyl fatty acids apparently arise because of steric hindrance to β-oxidation, with resultant compensatory ω-oxidation of such acids, and in the case of esters of 2-alkyl substituted acids, to inhibition of hydrolysis by esterases (*cf.* Carter, 1941; Weitzel, 1951; Tryding, 1957a). Glycerides of 2-methylstearic, *e.g.*, have been shown to be resistant to hydrolysis by pancreatic lipase (Tryding, 1957b), and after feeding 2-methylstearic acid-1-^{14}C, 1–2% of the radioactivity was recovered in the urine of rats as 2-methyladipic and 2-methylsuccinic acid (Tryding and Westoo, 1957). The long-chain 2-methylalkanoic acids are readily separated in complex lipid mixtures by gas–liquid partition chromatography (glpc)[1] (Napier, 1963), and with verification of structure by infrared spectra, metabolic studies of these nonnaturally occurring acids are facilitated. Furthermore, when these novel acids are esterified with ^{14}C-labeled alcohols such as sterols, methanol, and glycerides, the fate of both moieties of the effectively double-labeled compound may be unequivocally followed, particularly as related to the transport of lipids and the stereospecificity of hydrolases and esterases.

The present communication summarizes the properties and characteristic infrared spectra of both the methyl and cholesterol esters of 2-methyllauric, 2-methylmyristic, and 2-methylpalmitic acids, as well as studies of the absorption of cholesteryl-4-^{14}C 2-methylalkanoates. Evidence is presented which supports the thesis that neither long-chain fatty acids nor cholesterol are transported as cholesterol esters, as such, across the intestinal mucosa.

Experimental Section

Syntheses. The 2-methylalkanoic acids were synthesized by the diethyl methylmalonate procedure described by Cason *et al.* (1953).[2] Samples collected from

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[1] Abbreviations used: glpc, gas–liquid partition chromatography; tlc, thin layer chromatography; BSP, bromsulfonphthalein.

[2] The novel alkyl migration in dialkyl ketones, to produce 2-alkyl substituted acids in the presence of SeO_2 (Sonoda and Tsutsumi, 1959), was initially employed at the suggestion of Dr. Hugh S. Wiggins. Although the reaction was indeed found applicable to the synthesis of long-chain 2-methyl fatty acids, the yields of the 2-methyl fatty acids were found to be in approximately a 1:1 ratio with the straight-chain isomer as determined by glpc (*e.g.*, 2-tridecanone → 2-methyllauric + tridecanoic).