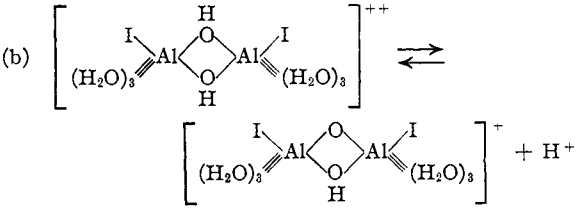
which is similar to the reaction

$$(Cr(H_2O)Cl)^{++} \underset{\text{Green}}{\overset{H_2O}{\rightleftharpoons}} \underset{\text{Violet}}{(Cr(H_2O)_6)^{+++}} + Cl^-$$

Inasmuch as heating these sols greatly favors oxolation the following type of reaction must be considered

(b) 

Allowing the heated sol to stand at room temperature should cause a reversal in (b) which was actually observed (Table IV) and has been reported before from this Laboratory. It is seen also that heating caused reaction (a) to go to the right, with reversal on subsequent aging at room temperature.

When the results recorded in this paper are reviewed in the light of the nature of the sols described in the previous paper,[1] it seems that increased concentration and elevation of temperature of preparation of aluminum oxyiodide sol favors the formation of bound iodide.[5] Excessive heating, however, tends to make the hydrous alumina less reactive toward the peptizing agent, hydriodic acid, thus causing less iodide to be bound.

Thus one can account for the fact that Sol 5 made at room temperature had the smallest promotion factor while Sol 4 made at 90° had the largest promotion factor. The cases of Sols 1 and 2 are of interest in this respect. Sol 1 had a smaller catalytic effect than Sol 3 although the latter sol had less iodide in the system. Sol 1 was prepared by boiling for twelve hours whereas Sol 4 was boiled for seven hours.

(5) In this connection one may cite the case[6] of $Cr(H_2O)_6Cl_3$ or $(Cr(H_2O)_5)_2(SO_4)_3$ where boiling causes the anions to enter the complex. High concentration also favors this reaction.

(6) Stiasny, "Gerbereichemie (Chromgerbung)," Verlag Theodor Steinkopff, Dresden, Germany, 1931.

Summary

It has been shown by means of the catalytic decomposition of hydrogen peroxide by aluminum oxyiodide sol that: 1, water molecules can displace bound iodide; 2, addition of potassium iodide to a sol or the preparation of a sol in the cold causes relatively small amounts of iodide to become bound; 3, aging of a sol at room temperature causes part of the bound iodide to become unbound. This last effect can be made very noticeable upon heating the sol and using silver–silver iodide electrodes to measure the iodide ion activity.

An explanation of these results has been given based on the assumption that the micelles are polyolated and polyoxolated structures.

NEW YORK, N. Y. RECEIVED OCTOBER 31, 1938

[FOOD RESEARCH DIVISION CONTRIBUTION NO. 395, BUREAU OF CHEMISTRY AND SOILS, U. S. DEPARTMENT OF AGRICULTURE]

The Energy of Activation of Enzyme Reactions, and their Velocity below 0°

BY HANS LINEWEAVER

Chemical changes suffered by biological material in cold storage are due chiefly to the intervention of microörganisms, and to the reactions catalyzed by inherent tissue enzymes. The former effects may be minimized, perhaps eliminated, by proper handling or treatment; the latter cannot be avoided without considerable injury to the tissues. This paper outlines certain conclusions reached by a study of the effects of low temperatures on these changes which are due to the ever-present enzymic factor.

Comparison of Enzymic and Non-Enzymic Catalysis.—The empirical Arrhenius equation,[1] $\ln k = -\mu/RT + C$, relating temperature, T, and reaction velocity, k, is suitable for the calculation and comparison of the changes in the velocities of many enzymic and non-enzymic reactions as the temperature is lowered. Consideration of the justifications and limitations of this pro-

(1) Stearn [*Ergeb. Enzymforsch.*, **7**, 1 (1938)] has used a different equation in connection with mechanism considerations, but extrapolations with either equation over the short temperature ranges to be used in this paper would lead to practically the same results.

TABLE I

CALCULATED RELATIVE CHANGE OF REACTION VELOCITIES WITH TEMPERATURE AS FUNCTION OF CATALYST

Reaction	Catalyst	μ	Relative velocities (vel. at 30° = 100) 0°	−30°	Ratio of enzymic to non-enzymic velocity +30°	0°	−30°	Reference
	None (dust)	18,000	3.75	0.063	1.0	9.8	167	(2), page 225, and (3)
Hydrogen peroxide decomp.	$Fe(OH)_3$, MnO_2, I^-	13,500	8.5	.40	1.0	4.3	26	
	Colloidal Pt	11,700	11.9	.83	1.0	3.1	12.6	
	Liver catalase	5,500	36.7	10.5				
	H^+ ion	26,000	0.87	0.0024	1.0	10.7–14.1	204–375	(4) and (5)
Sucrose inversion	Yeast invertase	11,500	12.3	.90				
	Malt invertase	13,000	9.3	.49				
	HCl	20,600	2.45	.022	1.0	3.1–4.7	12.5–33	(2), page 225
	Trypsin-kinase	14,400	7.5	.28				
Casein hydrolysis	Trypsin, Crystalline trypsin, Crystalline chymotrypsin	12,000	11.5	.73				(6)
Ethyl butyrate	H^+ ion	13,200	9.1	.46	1.0	5.1	39	(7)
Ethyl butyrate	Pancrease lipase	4,200	46.5	17.9				(8)

cedure will be taken up in the discussion. Throughout this paper μ will be used to indicate the Arrhenius energy of activation as well as the temperature characteristic of growth of bacteria, etc.

Table I illustrates that the reaction velocity decreases with temperature far less in the case of enzymic catalysis than in the case of non-enzymic catalysis. Column three gives the observed energy of activation, always lower for the enzyme catalyzed reactions than for the non-enzymic reaction. Columns four and five give the velocities calculated by the Arrhenius equation at 0 and −30° on a scale where the velocity at 30° is arbitrarily set equal to one hundred. Columns six, seven and eight show the ratio of the enzymic to the non-enzymic rates of decomposition for each substrate and each temperature. The non-enzymic rate is thus seen to decrease relatively more as the temperature is lowered than does the enzymic rate. The greatest difference between the behavior of the enzymic and non-enzymic velocities is seen in the case of the inversion of sucrose by hydrogen ion and yeast invertase—a 14.1-fold difference at 0° and a 375-fold difference at −30°.

The table also shows that the energy of activation differs with the type of enzyme reaction. Hence, if a system capable of carrying out several reactions, whose μ values are different,[9] were maintained at different temperatures there would not only be a difference in the amount of change but a qualitative difference in the composition of the products formed. That is, the amounts of different products formed and of reactants removed per unit time would vary with the temperature. Such a case is exemplified by the well-known fact that the quality of fruits ripening in storage depends on the storage temperature.[10] It therefore becomes evident that reactions responsible for the deterioration of a product at room temperature may be of negligible importance in deterioration in cold storage and vice versa.

In order to facilitate the examination of any system of individual choice with regard to this qualitative effect of temperature, Fig. 1 has been prepared. It may be used to estimate the ratio of the rates at 0, −15 or −30°, of any two reactions that proceed with the same speed at +30°, from the μ values of the respective reac-

(2) Moelwyn-Hughes, "Kinetics of Reactions in Solutions," Oxford University Press, 1933.

(3) Rotini, *et al.*, *Ann. Lab. Ricerche Ferment.*, 2 (1931).

(4) Sizer, *J. Cellular Comp. Physiol.*, **10**, 61 (1937).

(5) Sizer, *Enzymologia*, **4**, 215 (1938).

(6) Balls and Lineweaver, *Food Research*, **3**, 57 (1938).

(7) "International Critical Tables," Vol. VII, p. 132.

(8) Kastle and Loevenhart, *Am. Chem. J.*, **24**, 491 (1900).

(9) Blagoveschenski [*Enzymologia*, **2**, 203 (1938)] showed that at least polypeptidase and catalase have the same μ for any one plant. However, it hardly seems likely to the author that other enzymes, particularly oxidative enzymes, would fall in line in this respect. Tang [*J. Gen. Physiol.*, **14**, 631 (1931), and **15**, 87 (1931)], on the other hand, has shown that a difference of the order of 5000 cal. exists between the μ for oxygen consumption and carbon dioxide production by both *Lupinus albus* and *Zea mays*. Crozier and Stier [*ibid.*, **10**, 516 (1927)] show a difference of 16,000 cal. between μ for heart beat and gill movements measured simultaneously in *Asellus*.

(10) R. Plank [*Food Research*, **3**, 175 (1938)] has presented a scheme based on the different quantitative effect of temperature on the rates at which toxic bodies are formed and are used up by living fruit in storage, but in the storage of dead material such as meat, similar considerations are not to be neglected.

TABLE II

RELATIVE RATES OF PROTEOLYSIS AT CONSTANT TEMPERATURE IN THE FROZEN AND SUPERCOOLED LIQUID STATES

The method of determination was that of Anson [*J. Gen. Physiol.*, **20**, 565 (1937)], or Anson and Mirsky, *ibid.*, **17**, 151 (1933)]. In this method the split products not precipitated by trichloroacetic acid are estimated colorimetrically with the phenol reagent which gives a blue color with tyrosine, tryptophane, and cysteine. In column 5 the relative times required to give an increase in color equivalent to 0.047 mg. of tyrosine in 5 ml. of filtrate is given. In the solid state experiments the enzyme-substrate mixture was frozen quickly at −20 or −30°, and then placed at the desired temperature. For analysis a tube was thawed quickly (<60″) under the hot water tap while shaking with trichloroacetic acid.

Reaction mixture	Condition	Initial velocity	Ratio to velocity at 30°	Time req. for Δ of 0.047	Ratio of time to time req. at 30°
	− 5° liquid	20	1/12[11]	1	1/12
Chymotrypsin and 2% casein	− 5° frozen	1	1/240	35	1/420
	−17° frozen	0.1	1/2400	500	1/6000
Cathepsin and 2% hemoglobin	− 2.8° liquid	3	1/10[12]	1	1/10
	− 2.8° frozen	1	1/30	3.8	1/38

tions. For example, if we desire to compare catalase and trypsin we find the difference in μ to be 12,000 − 5500 = 6500 cal., and the ratio of velocities at 0 and −15° to be about three and six, respectively. The trypsin activity at 0° will therefore be $^1/_3$ of the catalase activity for concentrations of enzyme such that the two activities are equal at 30°. If the velocities differ at 30° a proportionate correction applies, that is, if the trypsin activity were already $^1/_{10}$ the catalase activity at 30°, then at 0° it would be $^1/_{10} \times {}^1/_3 = {}^1/_{30}$ the catalase activity, or if it were 9 times the catalase activity it would be $9 \times {}^1/_3 = 3$ times the catalase activity, etc.

The energy of activation data of Blagoveschenski[9] may be used for deductions similar to that just described. He reported μ values that vary from source to source for a single enzyme (for catalase μ = 5000 to 20,000 cal.), and also with the stage of development of the organism, *e. g.*, the μ value for guinea pig catalase was reported to increase from 1400 cal. at ten days of age to 6300 cal. at adult age. These two observations suggest the possibility of predicting from temperature–enzyme studies made before storage, what products will be most stable and at what stage of development they will be most stable, when placed at low temperatures. For such a prediction one should know the enzymes responsible for deterioration and their μ values for each material.

Discontinuity in the Velocity–Temperature Relation.—The extrapolations and comparisons which have been made in the foregoing are valid when the enzyme system remains essentially the same from temperature to temperature. When the system freezes a sharp discontinuity in the velocity–temperature curve generally occurs. Little work is available on this phenomenon and no predictions seem warranted at present. Table II shows summary data comparing the velocity of chymotrypsin and muscle cathepsin action in supercooled liquid and frozen at the same temperature. Chymotrypsin shows a 20-fold difference in rate between the liquid and frozen system and cathepsin only a 3-fold difference.

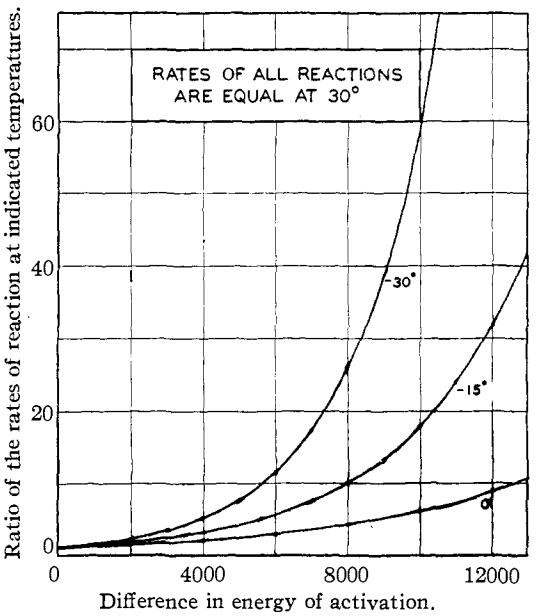


Fig. 1.—The effect of temperature on the ratio of the rates of two reactions as a function of their difference in energy of activation. The rates at 30° are placed equal.

Balls and Lineweaver[6] reported data of Balls, Matlack and Tucker that showed less than a two-fold difference between the rate of hydrolysis of olive oil by pancreas lipase frozen and unfrozen at −12°. A different series of determinations by Balls and Tucker[13] has been used to deduce a

(11) μ = 11,450 cal.

(12) μ = 11,350 cal.

(13) Balls and Tucker, *Ind. Eng. Chem.*, **30**, 415 (1938).

liquid to frozen state ratio of 5 to 6 for the same hydrolysis at −18°. This ratio was arrived at as follows. The initial rates of hydrolysis yield values of μ within the limits 9500 to 10,000 cal. for the temperatures 30, 20, and −3°. Extrapolation of the liquid state velocity to −18°, using a μ value of 9800, seems justified and yields a velocity 5 to 6 times that of the observed velocity in the frozen state. No adequate explanation of these variations in ratio can be given at this time, although the lipase emulsion system is obviously physically different from the proteinase system. Nevertheless the observations emphasize the fact that variable behavior is to be expected when different systems are frozen. Determination of the limits of variation for various types of material should be of both practical and theoretical importance.

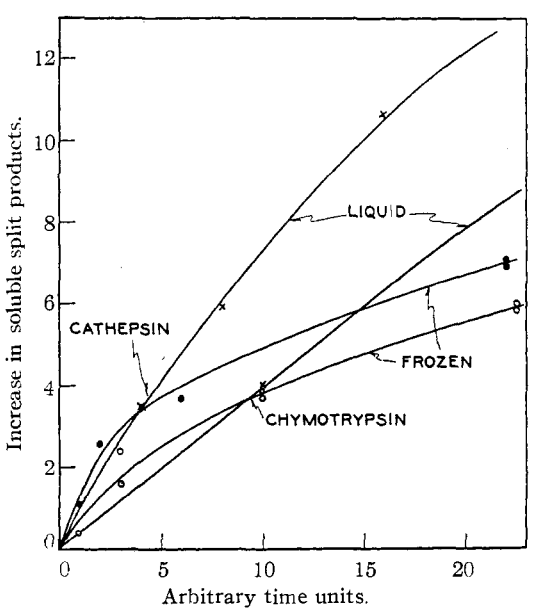


Fig. 2.—The time course of proteolysis in the frozen state compared with supercooled liquid. The time scales are adjusted so that the activities in the frozen and liquid states superimpose at a relative activity of about 3.5. Cathepsin and chymotrypsin digestions were carried out at −2.8 and −5°, respectively. See Table II for method and substrates.

Figure 2 shows that in the frozen state, the time course of hydrolysis by cathepsin and chymotrypsin is altered in such a manner that the extent of hydrolysis is decreased more than the initial velocity (also compare columns 4 and 6 in Table II). Thus freezing is doubly effective against extended hydrolysis (secondary changes).

The effect of freezing on cell structure and the rapid chemical changes that occur after thawing cannot be discussed at this time. The homogeneous proteinase systems that we have used exhibit the same rate of hydrolysis whether previously frozen and thawed, or never frozen.

Discussion

Discussion of the merits of the preceding comparison of enzymic and non-enzymic reactions on the basis of the Arrhenius equation will be taken up under three heads.

Constancy of Energy of Activation Values in Simple Systems.—Older data from which it appeared that μ for enzyme reactions did vary with temperature have been criticized by Bodansky[14] on the grounds that the velocity values were incorrectly estimated. Sizer[4,5] studied invertase in considerable detail and concluded that μ was practically constant from 0 to 40°. Rotini[3] and Pratalongo[15] report data between 0 and 30° that show practically constant μ values for various amylases, trypsin, glycerophosphatase and catalase.[16] Hence it has been assumed that extrapolations of the type used in Table I generally can be made without great error.

Apparent Inconstancy of Energy of Activation Values in Organized Systems.—Where several reactions are concurrent one would expect μ to vary with temperature unless particular caution is taken to see that the system is in exactly the same state at different temperatures. *E. g.*, if the disappearance of protein is followed in a system that is simultaneously producing or removing acid (*i. e.*, formation of lactic acid or decarboxylation) the rate of change of *p*H would vary with temperature and, since the activity of the proteinase should vary with *p*H, an apparently different time course of proteolysis would be observed. For similar reasons the measurement of any single un-isolated biological activity as a function of

(14) Bodansky, *J. Biol. Chem.*, **120**, 555 (1937).

(15) Pratalongo, *Riv. freddo.*, **23**, 45 (1937).

(16) Although the extrapolations of interest here are essentially empirical, the form of the equation used is similar to that in mechanism considerations of chemical reactions [Stearn[1] and Moelwyn-Hughes, *Ergeb. Enzymforsch.*, **2**, 1 (1933); **6**, 23 (1937)]. However, in view of the uncertainty of much of the older enzyme data, from the standpoint of activators, inhibitors, etc., and because of its incomplete nature, mechanism considerations without additional information would seem to be of dubious value. Thus, in this regard definite molecular concentrations of enzymes may now be employed for a number of reactions so that the use of these systems together with information obtained from careful temperature, substrate concentration, and inhibition studies should be sufficient to supply more trustworthy conclusions about such points as the heat of activation, the entropy of activation, the absolute reaction velocity of the activated complex, etc., that is, the reaction mechanism.

TABLE III

ILLUSTRATIVE μ VALUES FOR MICROÖRGANISMS

Source of data	Microörganism	Method of velocity meas.	Temp. range, °C.	
Burk[22]	Azotobactor	Velocity constant	17–29	19,300
	B. coli		20–42	14,000
Lane-Claypon[24]	*B. typhosus*	Velocity constant	20–42	14,000
	B. enteritidis		20–42	10,000
Barber[25]	*B. coli*	Hanging drop generation time	20–37	17,000
			At 12	55,000
Sherwood and Fulmer[26]	Yeast	Velocity constant	At 10	12,500[28]
Berry[27]	Yeast	Generation time	−2.2–+21	26,000

temperature might lead to variable temperature characteristics. Crozier, Stier and their collaborators have shown repeatedly that the temperature characteristic of various activities such as frequency of heartbeat, locomotor activity, breathing rhythm, carbon dioxide production, and oxygen consumption, is different in different temperature ranges. Such activities, however, are undoubtedly the result of very complicated reactions. There seems to be less tendency for μ to vary or change with temperature in simpler cases. Sizer[17] recently showed that sucrose inversion exhibited a μ value of about 11,000 cal. at certain temperatures whether live yeast, toluene-killed yeast, or yeast invertase preparations were used. As Sizer pointed out, the behavior of invertase in the cell appears to be the same as in cell-free preparations. If this is true for enzymes in general, more confidence could be placed in the practical inferences drawn from isolated enzyme studies. Although these considerations at present place limits on the precise quantitative conclusions that can be drawn with regard to organized or mixed systems, it is nevertheless possible to draw important semi-quantitative inferences concerning low temperature enzyme activity from existing data. A further limitation, the intervention of microbial contamination, will be treated briefly in the following section.

Limitation of the Growth of Microörganisms by Lowered Temperature.—Numerous authors have shown that microörganisms not only survive but multiply at temperatures below zero degrees centigrade. Smart[18] worked at −8.9°, Berry and Magoon[19] at −4°, while Wallace and Tanner[20] give an excellent summary of the literature in connection with frozen food research and point out that growth may occur as low as −10 to −20°. See also Prescott and Tanner.[21]

It appears to be characteristic of the growth of microörganisms that a constant value of μ is not obtained. However, the variation in μ, particularly at lower temperatures, need not be due to incorrectly measured velocity constants. The characteristics of the organism (water, nitrogen, carbohydrate content, morphology, etc.) vary with temperature; therefore the reaction being measured is changing qualitatively as well as quantitatively. This of course is not the case with simple enzymic reactions such as the hydrolysis of sucrose. Burk[22] for growth of *Azotobacter vinelandii* found a constant value of 19,300 cal. between 17 and 29°, but unreported data obtained by the author in Dr. Burk's laboratory indicated that below 17° the value of μ increased, as has generally been found to be the case (Buchanan and Fulmer).[23]

Table III gives illustrative data for the μ values of microörganism growth and indicates also how the values may be found to increase on lowering the temperature. Without going extensively into the literature, Tables III and I may be compared to find support, in spite of exceptions, of the view that the enzymic μ values are generally considerably lower than those for microörganisms, particularly at low temperatures. The amount of chemical change produced by microorganisms in biological material must therefore decrease with temperature to a far greater extent than the amount produced by the partially disorganized inherent enzyme activity.

(17) Sizer, *J. Gen. Physiol.*, **21**, 695 (1938).
(18) Smart, *Science*, **29**, 525 (1934).
(19) Berry and Magoon, *Phytopathology*, **24**, 780 (1934).
(20) Wallace and Tanner, *Fruit Prod. J. Am. Vinegar Ind.*, **13**, 52, 109, 275, 366 (1933 and 1934); **14**, 145 (1935).
(21) Prescott and Tanner, *Food Research*, **3**, 189 (1938).
(22) Burk, *Ergeb. Enzymforsch.*, **3**, 23 (1934).
(23) Buchanan and Fulmer, "Physiology and Biochemistry of Bacteria," Baltimore, 1930, pp. 68 and 89.
(24) Lane-Claypon, *J. Hygiene*, **9**, 239 (1909).
(25) Barber, *J. Infectious Diseases*, **5**, 379 (1908).
(26) Sherwood and Fulmer, *J. Phys. Chem.*, **30**, 738 (1926).
(27) Berry, *Science*, **80**, 341 (1934).
(28) Medium without ammonium chloride.

Acknowledgment.—The writer is greatly indebted to Dr. A. K. Balls for his suggestions and assistance in the preparation of this manuscript.

Summary

The temperature characteristics of growth of microörganisms and other organized biological processes bear a formal relation to the energies of activation of enzymically and non-enzymically catalyzed reactions. Although certain variations, observational or real, occur in these energy values with temperature, velocity extrapolations justified within reasonable limits lead to some valuable considerations. 1. Extrapolations to cold storage temperatures show the relatively enhanced importance of unorganized enzyme reactions compared to microbial action or non-enzymically catalyzed reactions. 2. Materials whose constituent ferments have high energies of activation will be aided most by storage at low temperatures. 3. The magnitude of the various changes that occur in a single product will not all be quantitatively altered in the same degree by lowering the temperature, as evidenced by the differences in fruits ripened at low and at ordinary temperatures. 4. From the activity and temperature characteristic data of reactions in any particular product at ordinary temperatures it should be possible to predict the nature of the major changes that would occur in storage. In the critical region of temperature where liquid systems become frozen, generally 0 to −5°, the extrapolation of reaction velocities is, as might be expected, no longer readily feasible. Determinations with cathepsin, chymotrypsin and pancreas lipase show that in some, but not all, cases the velocity of simple enzyme reactions is greatly decreased when the change of state occurs.

WASHINGTON, D. C. RECEIVED OCTOBER 21, 1938

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, VANDERBILT UNIVERSITY SCHOOL OF MEDICINE]

Diacylureas. II. Preparation and Properties of Diacylureas Derived from Branched Aliphatic Acids

BY ROGER W. STOUGHTON, H. L. DICKISON AND O. GARTH FITZHUGH

In the first paper of this series[1] a survey of the available data on diacylureas was presented and the preparation and properties of diacylureas derived from normal aliphatic acids were described. Because of the interesting hypnotic properties exhibited by these substances, this work has now been extended to derivatives of branched chain aliphatic acids. These N,N′-diacylureas were prepared by the action of an acid chloride on the appropriate monoureide. They could not be prepared by the condensation of an ester with urea in the presence of sodium ethylate, and all attempts to condense esters of both secondary and tertiary acids gave only sodium cyanate and the corresponding amide. The properties of these compounds are very similar to those reported for the straight chain analogs. It had been hoped that derivatives of secondary or tertiary acids would be more resistant to hydrolysis, but they were found to be only slightly more stable to alkali than the primary derivatives.

(1) Stoughton, *J. Org. Chem.*, **2**, 514 (1938).

A preliminary evaluation of the hypnotic activity of these compounds on white mice was made, using the procedure described in the previous paper. The data obtained are summarized in Table I.

As in the case of derivatives of normal acids, the anesthesia produced by intravenous administration lasted only a very short time, about two to four minutes. Intraperitoneally the minimum effective dose was much greater, but anesthesia lasted from fifteen minutes to one hour. The introduction of a tertiary alkyl group did not increase the activity but did increase the length of time the animals remained anesthetized. The anesthesia produced was again characterized by marked analgesia and absence of excitement.

Experimental

Monoacylureas.—All monoacylureas were prepared by the action of the proper acid chloride on urea according to the general procedure previously described for *n*-butyrylurea.[1] The isobutyryl-, 2-methylbutyryl-, and 2,2-dimethylpropionyl-ureas were recrystallized from water; the others, from dilute ethanol. Yields of 75 to