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INTRODUCTION

Low temperatures are frequently the limiting factor in the distribution of those native or introduced insects which must undergo a period of hibernation during the winter. In temperate climates all insects with the exception of those taking advantage of the heat and shelter provided by man, or of the body heat of warm-blooded animals, undergo a more or less rigorous period of exposure to unfavorable conditions. It is desirable in the case of an established infestation or an expected outbreak of an insect pest to know how well it can resist winter temperatures. By means of this advance knowledge preparations for control, which are often expensive, may be made more intelligently.

Low temperatures are used to advantage by entomologists in the "cold-sterilization" of infested stored products, mills, and dwellings. In temperate regions it is often convenient during the winter to expose infested goods or buildings to the cold atmosphere. A knowledge of the conditions required to make effective such an exposure is necessary.

The present study deals with some of the more fundamental problems of insect freezing and seeks particularly to clarify in so far as possible at the present time the physical concepts involved in the freezing of insects.

The insects used in the experimental work are representative of several types. Some insects, such as the wood borers and bark beetles, are exposed to extremes of winter temperature; others, as the box-elder bug, are exposed to moderately low temperatures in hibernacula, but often are insufficiently protected; and, lastly, insects such as most stored-product pests are normally never exposed to low temperatures. As great a variety of insects as available was used in this work.

DISCUSSION OF LITERATURE

The work on the effects of low temperature on insects, as in the development of winter-hardiness, has followed the lead of the older and more complete work on plants. Altho individual problems vary greatly in the two fields, fundamentally they are similar and are approached usually from the same standpoint. A comparison of the literature in these fields has been given by Payne (1926c). Uvarov (1931) discussed the literature on the effects of low temperatures on insects in his review, "Insects and Climate." More recently Bělehrádek (1935) in his monograph, "Temperature and Living Matter," has devoted one

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chapter to freezing and frost resistance, and another to chilling, chill-coma, and death by chilling. The literature with reference to both plants and animals is discussed very thoroly. In the present discussion of low temperature studies, therefore, only that directly pertinent to the experimental work will be considered.

Réaumur (1736) was one of the earliest to observe that some insects can withstand freezing while others cannot. Using his newly improved thermometer, he recorded the temperatures to which his experimental insects were subjected and thus procured definite information concerning the effects of the exposures. Göppert (1839) found that ice formation took place in both cells and intercellular spaces of freezing plants. Sachs (1860) discovered that in the process of freezing water was withdrawn from the cells of plants and froze in the intercellular spaces. Similarly, Molisch (1897) observed that when a cell (such as *Spirogyra* or *Amoeba*) is cooled below the freezing point of water, ice crystals are formed around its surface as water is withdrawn from the interior. Considerable dehydration of the cells thus occurs and is considered by most workers to be the actual cause of death from freezing. However, it seems certain that this is not the sole cause, since many organisms can withstand a considerable amount of freezing without ill effects. The extent of the withdrawal of water from the cells is shown by reports of various workers that, after being frozen and thawed, the bodies of certain insects are quite moist on the surface. It is possible that moisture was condensed on the cooled insect exposed in warm air, altho cases described by Payne (1928) seem to be authentic examples of the withdrawal of water. This author observed that a liquid apparently passed through the body wall of larvae of the Japanese beetle (*Popillia japonica* Newm.) where the chitin was thinnest. If this liquid gave tests for amino acids or proteins, the larvae invariably died. If, however, the liquid were water alone, the larvae generally lived and the water was frequently reabsorbed. Sectioned larvae showed no gross abnormalities.

One of the earliest theories of the cause of death from freezing, namely that ice forming in the cells expanded sufficiently to burst them, was definitely abandoned when a number of workers failed to find any histological evidence in favor of it.

Sachs (1860) advanced the opinion that the cell was killed by thawing. It has been suggested that the injury is produced by the sudden flood of distilled water. The corollary to this, that the unfrozen tissues are more concentrated and thus toxic, has received considerable support. Such a situation is possible regardless of whether the ice formation occurs around or within the cells.

The idea that freezing causes an irreversible change in the permeability of the cell wall has been advanced by Maximov (1912). He considered the plasma membrane as the least resistant part of the cell.

In 1905 Mez advanced the theory that death occurs by the direct action of cold and that ice serves rather as a protector due to its low thermal conductivity.

Concerning the hypothetical causes of death from the "quantity factor of cold" as defined by Payne (1927a), or "chilling" as Bělehrádek termed it, the latter author has given a very good discussion (1935).

The earliest workers thought that this type of death was due to a disproportion among the velocities of several vital functions. Some modern workers, however, think that it is caused by the accumulation of toxic products which at normal temperatures are burned or eliminated. This explanation is far from satisfactory in many cases, as, for instance, those in which death from chilling occurs at exposures of as low as ten minutes.

The monographic work of Bachmetjew (1901) on the freezing of insects has been discussed in most papers on this subject, but it is necessary again to review his work and some of the criticisms of it that have been made. Concerning his method of recording temperatures he says, "The thermoelectric needle was in these experiments usually placed in the thorax of the insect, it being considered that since the juice flowing out of the wound was removed at once with blotting paper, the body temperature of the insect was not lowered by the evaporation of the blood. One can by the use of finer wires produce a thermoelectric needle which has a rounded point and can be very easily inserted up the anus of an insect without injuring it."

Bachmetjew formulated the rule, based on 153 determinations on various species of insects, that death occurs when the temperature drops as low as the undercooling point after the rebound has taken place. He found that 16 per cent of the observations did not fit the rule, pointing out himself that it could not be taken too literally.

The thermocouple when used to pierce the insect becomes a good center of ice nuclei formation. Piercing or injuring the insect therefore prevents accurate recording of the undercooling points. Hence it is evident that Bachmetjew formulated his rule from inaccurate data, altho the rule is a very useful and exact one if properly understood and applied. If that relatively small group of insects be excluded which can survive freezing, namely those which pass the winter exposed to the low temperature extremes of a cold climate, then the remaining group of insects follow Bachmetjew's rule. All these insects are killed by freezing. Since freezing does not occur until after the undercooling point is reached, then this point is the minimum lethal temperature. The temperature of an insect in hibernation follows closely the temperature of its environment. If the undercooling point is reached freezing occurs, and altho the temperature of the insect is raised by the liberation of the heat of fusion, yet this change is momentary, and the temperature of the insect returns shortly to that of the environment.

Robinson (1928d) observed that piercing the insect resulted in undercooling and rebound points considerably higher than when the insects were not pierced. He attributed this to the freeing of bound water when the insect was subjected to a surgical shock. He reported that the bound-water content of pierced pupae of *Telea polyphemus* Cram. was lower than of unpierced individuals. It should be mentioned here that the method developed by Robinson (1928c) to calculate the internal temperature from the surface temperature is merely a means of calculating the lag of the inside temperature behind that of the surface, and not a means of calculating the correct undercooling and rebound points.

Bachmetjew measured temperature directly from the deflections of his galvanometer, but unfortunately he used a factor averaged from

several readings, to convert deflection into temperature. Altho he recognized that the deflections varied as the distance from the null point changed, he considered the difference to be negligible, and used an average deflection for each degree of temperature.

In determining the rebound point of an insect, one must reduce undercooling as much as possible to reduce in turn the error in the observed rebound point. In Bachmetjew's data there occur a few cases of several determinations made with the same species where the rebound points are approximately the same. These are the highest rebound points obtained and may be taken as the most precise. If several are together at or slightly lower than this high point, then the latter is approximately correct. In such cases the undercooling is less than in the other cases.

Bachmetjew was criticized by Maximov (1913) for stating that pupae of *Celerio euphorbiae* L. were completely frozen at —4.5° C. This is the result of an error that many subsequent writers have also made, namely, of assuming that physical rigidity or hardness is a sign of complete freezing.

Knight (1922) attempted to find the undercooling point of the pentatomid, *Perillus bioculatus* Fabr., by piercing with the thermocouple. In one case it would seem that the piercing failed to make any difference, for freezing did not take place as low as —26° C. (the other undercooling points approximated —7° to —9°). These bugs when in hibernation have very little liquid in them, certainly not enough to run or drip, and it is possible that the thermocouple was not in contact with the body fluids. Knight concluded that these bugs showed a periodicity by reducing their undercooling points in the fall when they eliminated much of their body moisture. The writer has failed to find this to be true in the similar species, *Chlorochroa sayi* Stål.

Pirsch (1923) carried on some very limited work on the freezing of honey bees. Body temperatures were determined by piercing with a thermocouple. Three bees undercooled to —2.3° and rebounded to —0.8° C. Another bee undercooled to —4.3° and rebounded to —2° C. The latter point is evidently too low, judging from the previous cases, which in all probability are the true rebound points.

Carter (1925) worked on the effects of low temperatures on *Bruchus obtectus* Say. Even by piercing these insects, he obtained fairly low undercooling points of around —8° to —10° C. When he recorded the points without piercing, they lay at lower temperatures of about —15° to —20° C. In the latter cases the rebound was very slight, while with piercing the rebound was about three or four degrees. The rebound points were so low because of the large degree of undercooling and the small size of the insects. Actually the freezing points should be very little below zero, while the normal undercooling points apparently should be about —15° to —20° C. Whether the insects were pierced or not, they died when frozen. Carter concluded that an uninjured insect could withstand more cold than a pierced one altho he did not point out the real reason, namely, that altho freezing caused death in each case, it took place at a higher temperature in injured material. Carter gave some valuable data on the time and temperature required to kill the various stages of *B. obtectus*. The normal undercooling points as evi-

denced by the minimum lethal temperatures lie around —20° C., and not at the points recorded for pierced insects.

Robinson (1926) studied the effect of the "quantity factor" of cold on the granary weevil, *Sitophilus granarius* L., and the rice weevil, *S. oryzae* L., and found that these insects cannot become dormant. This is a rather general characteristic of insects infesting stored products. Both abrupt and gradual exposures were studied as well as the relation of the results to certain moisture relationships of the two species.

Later work by the same author is mostly concerned with the determination of bound water in insects. Kistler (in an unpublished manuscript) points out that Robinson (1928a) determined bound water in groups of granary weevils for which he gave the freezing points of at least half of the individuals as below —20° C. The method used to determine bound water was to cool the insects to —20° C. on the assumption that at this temperature all of the free water and none of the bound would be frozen. Yet the data indicated that at least half of the individuals were not frozen at this temperature. The "freezing" points listed by Robinson for these weevils, however, were many degrees too low, but the undercooling points were at least somewhat lower than those listed as the "freezing points." Many other examples could be cited of insects which have undercooling points far below —20° C., and which, if this method of determining bound water be used, would show all their water in the bound condition even tho no freezing had taken place.

Payne (1926a) pointed out that a periodicity occurs in the cold-hardiness of some insects, as shown by the relative positions of their undercooling points. These points are high during the summer, drop during the fall as lower temperatures arrive, reach a minimum during the winter, and rise again on the approach of higher temperatures in the spring. This hardening was observed with insects that inhabited the bark of trees and were therefore subjected to temperature extremes. The same effect was secured artificially by exposure to temperatures of about 0° C. in the laboratory, and subsequent exposure to higher temperatures. Dehydration also produced a decrease in the undercooling points of *Synchroa punctata* Newm.

Payne (1926b) carried on a comparison of three types of insects: the oak borers, normally exposed to temperature extremes; the aquatic insects, never exposed to a temperature lower than 0° C.; and the stored-product pests, representing, supposedly, a tropical or sub-tropical group. In the first group the undercooling points were periodic; the second and third groups showed no such phenomenon. It was found also that the first group could survive freezing when in a winter-hardy condition, but not in a non-hardy condition. Neither of the other groups could withstand freezing.

Payne (1927a) introduced the terms "quantity factor of cold" and "intensity factor of cold," referring respectively to exposure to moderately low temperatures for fairly long periods of time, and to exposure to temperatures so low that time has little if any influence. The term "chilling" is used by some authors to refer to the "quantity factor" of cold. The lethal action in chilling is directly proportional to the length

of exposure as well as the degree of cold. The "intensity factor" is determined by the location of the undercooling point, and whether or not the insect is killed by freezing.

Further studies by the same author (1927b) led to the conclusion that hardening is effected by the dehydration that the insect undergoes in the fall. She found that hardiness could be measured by determining blood conductivity.

Working with the Japanese beetle, *Popillia japonica* Newm., Payne (1928) found that the larvae exhibit a small degree of periodicity and that cold-hardiness is affected by disease, nutritional state, and degree of dehydration. Working then on the effects of humidity on hardiness, she found (1929) that cold-hardiness bore a direct relationship to absolute humidity which could be expressed in a simple equation. In the eggs and larvae of the tussock-moth, *Hemerocampa leucostigma* S. and A., the equation is

$$H_a T_1 = K$$

where H_a is the absolute humidity in millimeters of vapor pressure, T_1 is the survival temperature in degrees Centigrade, and K is a constant.

Payne (1929) determined the temperature lag between the inside and outside of a tussock-moth egg mass and found an extremely slight lag as the temperature fell from 25° to —20° C. Robinson (1928a), on the other hand, found that as the cabinet temperature dropped from 7° to —14° C. over a period of three weeks, the internal temperature of pupae of *Callosamia promethea* Drury dropped only to —6.5° C. The author explains this as due to the heat of imbibition as water was being bound in the pupae. How he determined the internal temperature he does not say.

Mail (1930), working on the effects of low temperature on certain wireworms, followed the technique of Robinson. His work on soil temperatures (1930, 1932) forms a very important contribution to the subject, while papers by Mail and Salt (1933) and Patton and Mail (1935) are practical applications of the study of low temperature effects on two economically important species.

Saccharov (1930) was interested in the fat and water contents of hibernating insects and the interrelationships of these two factors. He found that the fat content was higher in cold-resistant insects than in non-resistant ones, and that an insect prepared for hibernation in one way by storing fat. He determined also the percentage of freezable water in the insects by the dilatometer method of Bouyoucos (1917) and concluded that this was lowered as the species became more resistant to cold. Thus hardiness to cold was due to an increase in fat content and a decrease in the amount of freezable water. This freezable water has been likened to "free" water as contrasted to "bound" water, but the relation is doubtful. A fairly large sample was needed for a dilatometer determination so it is probable that Saccharov used a few grams of insects ground up into a liquid mass, altho he does not mention the state of his material. Obtaining constant temperatures by the use of criohydrate solutions, he determined the percentage of the water freezing at such temperatures.

In this connection the recent unpublished work of Kistler (1935)

and Kistler *et al.* (1935) is of great importance. The authors of these manuscripts consider that what has been measured as bound water by the freezing method is merely undercooled water. They showed that water in a finely divided state has a strong tendency to remain in an undercooled condition, and freezes completely only after a long time. At low temperatures the chances of an ice crystal forming and of its growing when once formed are both reduced considerably. The rate of crystallization in an undercooled solution depends on the temperature in such a way that there is a maximum velocity at a definite temperature. This temperature has been given by Tamann as —9.1° C. for water, altho from the experiments of Zandbergen it would seem to be about —20° C. Kistler points out also that "as a crystal of ice grows in a hydrophilic colloid it dehydrates the solution in its immediate vicinity and becomes coated with a protective layer of dehydrated colloid. This crystal can serve to seed or inoculate the solution surrounding it only in case a crack forms through the sheath. Unlimited growth of a single crystal is also prevented by the slow rate of diffusion of water through the dehydrated colloid, the diffusion rate probably being greatly reduced by low temperatures." He suggests also that an ice crystal in one cell cannot seed the adjoining cells.

There are in the literature several excellent papers dealing with the practical aspects of the action of low temperatures on some economic insect pests. Those of Fox (1935), Beal (1933), Miller (1931), Batchelder and Questel (1933), and Nagel and Shepard (1934) are selected references. The papers of Beal (1933) on the southern pine beetle, *Dendroctonus frontalis* Zimm., and of Miller (1931) on the Western pine beetle, *D. brevicomis* Lec., are of special interest since the authors found higher winter mortalities under wet hibernation conditions than under dry ones. This is a practical demonstration of the role of contact moisture in freezing. The work of Hodson (1936) is also in agreement.

Shibata (1935 a,b) published two papers on the effects of low temperatures on the fruit fly, *Chaetodacus cucurbitae* Coqu. His first paper deals with the "supercooling" death of insects, which is another way of expressing death from chilling. In his second paper he described a method of limiting the amount of undercooling by mechanical shocks. Freezing was allowed to progress to varying degrees, when the survival was recorded. It is possible this method may be used to advantage under certain conditions.

APPARATUS

Low-temperature work with insects generally is carried on in two different ways: First, by exposure of the insects, usually in fairly large numbers, to a constant low temperature, the time and possibly other factors being varied; and second, by exposure of individuals in contact with a thermocouple which records the body temperature. For either type of work, a refrigerator which will maintain constant temperatures as low as —50° C. is almost essential. The refrigerator used in this work consists of a one-half horse-power compressor using Freon gas in 250 feet of one-half–inch copper coil. This cooling coil is immersed in 30 gallons of alcohol in an insulated metal tank. A stirrer, a knife-

heater, and two sensitive mercury-toluene thermoregulators (one to control the compressor, and the other the heater) keep the temperature of the alcohol constant at any desired temperature. A pint jar is immersed almost to its top in the alcohol and covered with a rubber stopper. When insects are frozen individually, each one in a thermocouple holder is inserted through the stopper to a depth of about four inches. If the insects are exposed in bulk, a number of test tubes are immersed to about three-quarters of their depth in the alcohol and the insects placed in these.

Body temperatures of individual insects are recorded by the thermoelectric method. Most previous workers have used a potentiometer reading in millivolts, convertible to degrees Centigrade, connected to a galvanometer used as a null instrument. Then two thermocouples are connected in series, one being placed in an ice and water bath, giving a constant temperature of 0° C., the other against the body of the insect. Using this method it is possible to obtain readings of the body temperature only at intervals, a reading of the potentiometer requiring about half a minute. In the present work the galvanometer deflections were read directly on a 12-foot scale calibrated in tenths of degrees for the range of temperatures desired, as a continuous record of body temperature. This apparatus takes some time to set up, the calibration being effected by placing the couple in contact with the bulb of a standard thermometer reading in tenths of degrees, both then being placed in a large container filled with cold alcohol from the refrigerator tank, and used together as a stirrer. As the temperature rises, the deflections of the galvanometer are marked on the large scale at each half-degree interval. These are checked several times as the temperature is raised and lowered. The tenths of degrees are then marked in at even intervals between the halves. The larger intervals cannot be marked off evenly, because the deflections of the galvanometer vary as the distance from the null point changes. An appropriate resistance is placed in series with the galvanometer to limit the deflections so the scale will include all of the temperature range desired. While it is admitted that the scale calibration may be slightly inaccurate, the error remains constant and thus is not very serious.

The method by which the thermocouple is arranged is of extreme importance in recording efficiently the body temperature of the insect. In the present work a type of thermocouple holder was used which ensures a good contact between the thermojunction and the insect, and at the same time eliminates the danger of water of condensation forming on the insect. The holder consists of a six- or seven-inch length of three- to ten-millimeter glass tubing into one end of which is inserted a piece of rubber cut from a large rubber stopper. The projecting rubber is cut to a size about 1½ inches long, ¼ to ½ inch wide, and a depth tapering from ¼ to ½ inch at the base to ⅛ inch at the tip. The rubber is then slit open evenly from the top two-thirds of the way back to the base, to form two tapering flaps which can easily be bent back with the fingers. On the inside surface of each flap, in the center, the rubber is cut out to form a cavity of approximately the same shape and size as the insect to be used in it. Each thermocouple wire is led through the

glass tube separately, over the back of one of the flaps, and through the flap into the depression. The tips of the wires are then fused and filed down to form a small flat junction which lies against the rubber in the center of one of the excavated depressions. When using the holder the free flap is bent back, the insect placed in the depression against the junction, and the flap released and fastened to its mate by means of a small wire clamp. When an insect is being frozen in such a holder, it is lowered into the pint of cold air in the refrigerator to a depth of about four inches. Since the holder is warmed and dried by a blast of compressed air each time before using, the rate of cooling is constant for a given holder and a given refrigerator temperature. The fact that the thermojunction is in contact with the rubber as well as the insect is of no importance when recording the undercooling points, since both the rubber and insect surface are cooling together and are at the same temperature. In recording the rebound points, an error in measurement is introduced which will be dealt with later.

METHODS AND CONCEPTS

In the freezing of a cold-blooded organism, the body temperature falls to a point below zero from which it suddenly rises. This point is known as the undercooling point, and the rise in temperature, which is caused by the heat of fusion of ice forming in the body, is called the rebound. The temperature to which the rebound proceeds has been called the freezing point or the rebound point. The organism may be regarded as a solution containing electrolytes. In such a solution, when the first crystals of ice form, the remaining unfrozen solution is consequently more concentrated and has a lower freezing point. Thus if a gram of pure water is undercooled to —1° C. before ice crystallization begins, one-eightieth of the water will separate as ice (Gortner, 1929). Since the latent heat of fusion of water is 80 calories, the one-eightieth gram of ice formed liberates one calorie of heat. Since the specific heat of water is 1.0° C., the temperature of the gram of water is raised one degree from —1° to 0° C. Had the water undercooled to —3° C. before crystallization began, three-eightieths of the gram of water would have frozen, liberating three calories of heat which would raise the temperature of the water three degrees, or from —3° to 0° C. Using these values, one can easily correct the observed depression of the freezing point by means of the formula,

$$\Delta = \frac{\Delta'(V - \frac{uV}{80})}{V}$$

or

$$\Delta = \Delta' - 0.0125u\,\Delta',$$

where V = weight of the water (solvent),
Δ = corrected depression of the freezing point,
Δ' = observed depression of the freezing point, and
u = degrees of undercooling.

The true freezing point, therefore, cannot be recorded directly, but must be calculated from the observed "depression" of the freezing point

or the "observed rebound point." However, it is obvious that in order that the true freezing point may be calculated the "observed rebound point" must be determined with precision. Unfortunately, it appears at present to be impossible to record the rebound point very accurately in insects, except possibly in the case of very large insects with a high fluid content. The reason for this is that a portion of the heat of fusion is extracted by the surroundings, such as air, rubber, thermocouple wires, and glass. Some of this heat is absorbed by parts of the insect which do not freeze until lower temperatures are reached. In the type of thermocouple holder described above as used by the writer this loss of heat to the holder must be considerable, but under the old system of freezing the insect in cold air the loss would be much greater, since air currents aid the dissipation of heat.

It follows that the error of the observed rebound point increases as the amount of water first freezing in the organism decreases. The surroundings absorb a greater proportion of the heat of fusion of a small amount of water than of a large amount, and a condition is approached where the rebound is recorded merely as a hesitation in the falling temperature and finally is obliterated entirely.

The extraction of some of the heat of fusion from the organism by the surroundings is of course directly dependent on the differential in temperature between the two as well as the heat conductivity of each. Unless heat is injected into the environment to accompany the organism in its rebound, there must be a differential of at least the same number of degrees as the rebound. However, since the environmental temperature was falling when the rebound occurred, it would fall further even tho the insect's temperature rose, and therefore the differential would increase. This increase is dependent on the rate of cooling used in the experimental work, so it follows that a slow rate of cooling lessens the error of the rebound and a fast rate of cooling increases it. Since a certain amount of differential must be present in any case, the error cannot be entirely eliminated.

In order to obtain the freezing point of a salt solution, for instance, undercooling is avoided as much as possible by agitating the liquid. If the rate of cooling is fairly constant, the temperature will drop to the freezing point, remain there until most or all of the liquid that can freeze at that temperature is frozen, and again drop. However, in the case of an insect, especially a small one, it is very difficult if not impossible to eliminate undercooling by means of mechanical shocks, altho they may lessen the extent of it. If an insect is allowed to undercool and rebound normally, the temperature theoretically should remain at the rebound point until all the water that can freeze at that temperature has done so, and then drop again. This may possibly be the case with very large insects, but the writer has never seen it occur in any insect that he has frozen normally, including those ranging from the size of a mature *Phyllophaga* larva down to the saw-toothed grain beetle. If and when it does occur, this flat portion of the time-temperature curve may represent the true rebound point. It is conceivable, however, that, after the first burst of heat of fusion has raised the temperature of the insect and thermocouple somewhat, the heat liberated by subsequent

freezing becomes balanced at some point by the heat extracted by the system. The heat liberated is first greater than, then equal to, and finally less than that extracted by the system. The length of time that the two remain balanced, as shown by the flat part of the time-temperature curve, is directly proportional to the amount of water freezing at that temperature, provided that the rate of extraction of heat, or rate of cooling, remains constant. Actually, in all of the thousands of insects of many species and stages that the writer has frozen, the rebound begins with a fairly rapid rise in temperature, slows down to a stop, and within a few seconds at the most, the body temperature starts down the scale again. This indicates that the system represented by a normal insect of average size liberates, when frozen, insufficient heat to carry its temperature to its true rebound point. Moreover, this unfortunate state cannot be overcome merely by lessening the rate of cooling, since even with a very slow rate the rebound itself causes a differential in temperature which must be restored to an equilibrium at the undercooling point.

A sample of the approximate rate of cooling used by the writer in most of this work may be of interest here. In cooling a series of pupae of *Ephestia kühniella* Zell., the time was recorded in one case with a stop watch. The pupae ranged in weight from 13.1 to 22.8 milligrams and had a moisture content of 8.6 to 15.4 milligrams, as determined by desiccation at 105° C. for 24 hours. The rate of cooling, however, depended on the temperature of the cabinet and the size, surface, and heat conductivity of the rubber thermocouple holder, and not on the size of the pupae. The individual under consideration cooled as recorded in Table 1.

Table 1. The Rate of Cooling of a Pupa of Ephestia kühniella

Room temperature to −15° C.	not recorded
−15 to −16	38 seconds
−16 to −17	39 "
−17 to −18	40 "
−18 to −19	52 "
−19 to −20	54 "
−20 to −21	59 "
−21 to −22	62 "
−22 to −23	74 "
−23 to −24	79 "
−24 to −24.3	25 "

The rebound occurred at −24.3° C. and went to −18.6°, a rebound of 5.7° C. Actually, however, the true freezing point of these pupae lies approximately at −2°, requiring a rebound of about 22° C. Yet the rate of cooling was sufficiently slow so that the unavoidable differential in temperature caused by the rebound was increased but slightly by the existing rate of cooling. The rebound, halting, and falling of the temperature back to the undercooling point in this case required less than a minute, during which time the differential in temperature could have increased not more than one degree, and probably much less. Such experimental evidence leads to the conclusion that the observed rebound points are not the true freezing points.

The fact is often overlooked that living organisms are far from homogeneous in their physical and chemical structure. It is obvious that different parts of an organism will have different undercooling and freezing points. However, the critical undercooling and freezing points are usually those of that tissue or organ freezing at the highest temperature. Inoculation of those tissues having lower freezing points by the ice formed in the first crystallization prevents further undercooling and consequent rebound in most cases. Exceptions are found when different tissues are sufficiently separated physically to prevent inoculation of one by ice in the other, as for example when the Colorado potato beetle has prepared for hibernation by emptying its alimentary tract.

An insect being heterogeneous in structure, it follows that at any given temperature below the highest freezing point, the insect will freeze only to a certain extent, and that, as the temperature is lowered, freezing becomes more complete. It is possible that water under certain conditions, notably in a state of fine division, may be undercooled tremendously. Such fine particles of water and perhaps even individual cells may be isolated from surrounding tissues by colloidal material dehydrated during the process of freezing. This material may form a coating around the water or cell, but may eventually be cracked and permeated. Due to such phenomena, a considerable period of time is necessary to establish an equilibrium of ice and unfrozen solution in an organism at a given temperature.

Another important aspect of the technique of low temperature work is an appreciation of the effect that contact moisture may have when an insect is cooled below freezing. This is obviously of importance also in nature, since hibernacula of insects are frequently wet or icy, and the contact of such moisture with the insect body produces a center of ice formation which may inoculate or seed the body fluids. In such a case, undercooling is eliminated or greatly lessened and freezing begins at or slightly below the highest freezing point of the insect tissues. If the insect were dry, or if inoculation failed to occur, freezing would not take place until the temperature dropped to the undercooling point, thus providing a margin of safety equal to the difference between the undercooling and freezing points. This difference ranges from a very few to 30 or 40 degrees Centigrade, depending on the species, stage, and physiological condition. In experimental work, then, care must be taken to eliminate all possibility of the occurrence of moisture in contact with the body of the insect, unless such a condition is expressly desired. The principal way in which such moisture would occur is as condensation moisture, produced when a cooled insect or any of its immediate surroundings comes in contact with warm air. It has been necessary to warm and dry the insect holder by a blast of compressed air each time that it was withdrawn from the refrigerator, since a considerable amount of ice formed on it immediately on removal. During the cooling process, using such a holder, there was no chance of acquiring moisture on the insect as long as the holder was not withdrawn from the cabinet, each run being started with the holder dry and at room temperature.

A similar source of error is not so easily prevented. Many insects, when irritated in some way, as by handling, eject liquids from the mouth,

anus, or other opening. Adults of the confused flour beetle, *Tribolium confusum* Jacq.-Duv., secrete a volatile liquid; larvae of *Lucilia sericata* Meig. secrete a slimy liquid from the body surface and the salivary glands; other insects pass fecal matter when so disturbed. In such cases, as well as those in which the insect is wounded, inoculation may frequently take place, and undercooling will be lessened or eliminated.

The adults of both *Sitotroga cerealella* Oliv. and *Tribolium confusum* Jacq.-Duv. are so active that considerable difficulty was experienced in fitting them properly into the cavity of a thermocouple holder. Inactivating them temporarily by exposing to low temperatures cannot be resorted to, since condensation moisture on the cooled insects may seriously affect the results. In the case of the adults of *S. cerealella,* chloroform was used in a concentration just sufficient to keep them inactive for a few seconds. Ten adults frozen after this treatment had undercooling points averaging —14.81°, while adults frozen without this treatment had under-cooling points averaging —14.83° C. Adults of *T. confusum* are not only very active, but when disturbed they secrete a liquid which inoculates them when frozen. Adults that were anesthetized with ether, both in heavy and light dosages, showed no difference either in the percentage of inoculated individuals or in the location of the undercooling points. This treatment was used only in the two cases mentioned, where it was necessary to freeze the insects individually in a thermocouple holder.

THE RELATION OF WATER CONTENT TO THE UNDERCOOLING POINT

Since the freezing of an insect involves the crystallization of a fraction of the water content as ice, it is natural to expect that water relationships play an important part in the phenomena of freezing. Payne (1926a) found that larvae of *Synchroa punctata* Newm. could be "hardened" by dehydration. Exposure for 24 hours over calcium chloride reduced the undercooling points approximately 18 degrees. An increase in hardiness with a reduction of absolute humidity was also found by Payne in Japanese beetle larvae (1928, 1929).

Laboratory conditions are usually warm and dry. For example, the temperature of the laboratory used by the writer varied but little from 25° C. and the relative humidity was about 15 to 20 per cent. Under such conditions, some insects would in a few minutes undergo sufficient desiccation to affect the location of their undercooling points. While working with larvae and puparia of *Musca domestica* L. and *Lucilia sericata* Meig., weighing them on a balance correct to .0001 gram was complicated by a continuous loss of weight during the weighing process. In a short test experiment, larvae of *Musca domestica* exposed in the laboratory lost 2.2 per cent of their weight in 34 minutes, while puparia lost 2.2 per cent in two hours. It is thus obvious that active insects like house-fly maggots are very sensitive to drying, and that exposure to dry conditions for a short period of time elapsing between their removal from the culture medium and actual use in the experiment may create a very significant error.

An experiment to bring out the correlation between drying and under-cooling points in puparia of *Musca domestica* was designed and executed. Thirty puparia were used in three groups of ten. In one series, the puparia were removed from the culture medium one at a time, dried carefully on the surface with a blast of compressed air, weighed, and immediately frozen. The individuals of the other two series were weighed one after another, the time of weighing being recorded. One series was then frozen after being exposed to 25° C. and 18 per cent relative humidity for 7 to 11 hours, and the other for 23 to 27 hours. Results for each series are averaged and presented in the following table.

Table 2. The Effect of Drying on the Undercooling Points of Musca domestica Puparia

	Per cent loss in weight	Undercooling point
Initial series,—no desiccation	.....	— 12.12° ± 3.18° C.
25° C., 18% R.H. for 7-11 hours	4.0	— 22.25 ± 0.71
25° C., 18% R.H. for 23-27 hours	9.8	— 21.65 ± 1.85

In the puparia of *Musca domestica,* therefore, exposure to dry laboratory conditions was accompanied by a considerable lowering of the under-cooling points. It is evident, however, that this lowering took place chiefly during the early stages of the exposure, and did not correspond to the further loss in weight.

An interesting point in this connection is brought out by experiments with larvae of *Lucilia sericata.* A series of 10 prepupal larvae were weighed both before and after freezing, reasonable haste being observed. It was found that the maggots lost an average of 5.3 per cent of their weight by being frozen. This, it is believed, is due to a peculiar habit of these larvae. When handled they secrete a liquid from the mouth, probably from the salivary glands, and lash out from side to side with the fore part of the body. They also secrete a slimy liquid from the body surface in general, according to observations by A. C. Hodson. These liquids serve to inoculate the body fluids when the maggots are frozen, and thus draw water to the surface of the body, which is the center of ice formation. The loss in weight consists therefore of secretions of the insect and the liquids withdrawn from the body when these secretions freeze.

Another test was carried on with adults of *Leptocoris trivittatus* Say. These bugs were collected during the winter from hibernacula consisting of niches and holes in a cliff of soft sandstone at Battle Creek Park, near St. Paul, Minnesota. There was a high percentage of survival of those collected at this time, so that the ones used do not represent especially hardy individuals. On the same day that they were collected, 30 active individuals were divided into three equal series. Ten were placed in a desiccator over calcium chloride at a temperature of 20° C., 10 were placed in a desiccator over calcium chloride at 2° C., and the remaining 10 were frozen individually in an inclosed rubber thermocouple holder. By using the two temperatures for desiccation,

one below the threshold of development where very little metabolism takes place, and the other high enough to ensure plenty of activity (no food was available to either series), two different factors are operating. At 2° C. the loss in weight should be almost entirely water; at 20° C. the loss would be both water and the material used during metabolism. The series at 2° C. was continued for 18 days, the individual weights being recorded five times during this period, after which they were frozen under conditions identical with the preliminary check series. The series at 20° C. lost weight very rapidly and at the end of three days, two of the ten were dead, so the remaining eight were frozen. The results for each series are averaged and listed in Table 3.

Table 3. The Effects of Desiccation on the Undercooling Points of Adult Leptocoris trivittatus

	Per cent loss in weight	Undercooling point
No desiccation		— 16.65° ± 1.93° C.
Desiccated at 2°, 18 days	20.8	— 16.35 ± 2.35
Desiccated at 20°, 3 days	21.3	— 16.50 ± 2.24

It is concluded that in the case of the adults of *Leptocoris trivittatus*, desiccation, either slowly at a low temperature or quickly at a high one, to the extent of 20 per cent of the total weight, has no effect on the undercooling point.

A species which demonstrates a doubtful relationship between desiccation and undercooling points is the sugar beet root maggot, *Eurycephalomyia myopaeformis* Roeder. Since the larvae of this species can survive freezing if removed shortly after the rebound point has been reached, they present excellent material for such an experiment. There were too few of these larvae, however, for extensive experiments.

In one experiment five larvae were weighed and frozen individually, the cabinet temperature being —30° C. The undercooling points averaged —6.4° C. They were then placed in a desiccator over calcium chloride at a temperature of 25° C. and after two days were weighed and re-frozen. One had died, but the average loss of weight of the remaining four was 27 per cent; the average undercooling point was —6.2° C. These survivors died after desiccation. A more gradual drying at 2° C. was therefore substituted.

Five larvae were frozen and then placed over calcium chloride at 2° C. Of the three that survived the exposure of 24 days, one lost 22.5 per cent of its weight and its undercooling point dropped from —9.3° to —13.2° C; another lost 20.3 per cent and its point dropped from —7.8° to —9.6° C; the third lost 27.6 per cent and the point dropped from —6.2° to —8.2° C.

In another experiment with the same species 10 larvae were weighed and frozen, the cabinet temperature being —30° C. All 10 survived and were re-frozen the next day. Again all 10 survived and were weighed and re-frozen the next day. Two died from this treatment, but the eight survivors were weighed and frozen for the fourth time

on the following day. Between treatments, they were kept in ½-ounce tins in the laboratory without soil or food. Immediately after the last freezing they were placed in an oven at 105° C. for 24 hours to determine their water content. The averaged results are given in Table 4.

Table 4. The Effects of Desiccation on the Undercooling Points of Larvae of Eurycephalomyia myopaeformis

	Per cent loss in weight	Undercooling point
First freezing	.....	— 6.2° C.
Second freezing	.....	— 8.1
Third freezing	10.4	— 7.1
Fourth freezing	15.8	— 6.9

It is therefore very doubtful if desiccation had any effect on the undercooling points of larvae of this species.

It has been seen that drying of an insect may or may not lower the undercooling point, depending on the species. To an insect that cannot withstand freezing and to which the undercooling point is the minimum lethal temperature, the ability to harden by a lowering of this point becomes a valuable asset. Why this ability should vary so greatly in insects exhibiting apparently the same need for it cannot be explained, nor is the mechanism of the process known. One would expect, however, that a loss of water would be accompanied by a decrease in the true freezing point. Whether or not the facts fit this supposition is not as yet known, since recording of freezing points has hitherto been inaccurate. When a convenient method of obtaining the true freezing point of an insect is developed, this question can easily be settled.

It is shown on page 28 that toward the end of the larval development of the Mediterranean flour moth, the undercooling points drop considerably as the feeding larvae enter the prepupal stage. The water content, expressed both in milligrams and as per cent of total weight, was recorded for these stages, and is shown in Table 5.

Table 5. The Relation of the Water Content of Larvae of Ephestia kühniella To Their Undercooling Points

	Undercooling	Water content	
		Absolute	Per cent
A	— 5.79° ± .835° C.	16.17 mg. ± 3.15	66.12 ± 2.80
B	— 8.07 ± 1.46	14.97 ± 2.81	65.37 ± 2.35
C	— 21.30 ± 1.73	12.73 ± 1.95	65.30 ± 2.27

A — Last instar, feeding larvae; B — prepupal larvae, not feeding; C — prepupal larvae from cocoons.

It will be seen that while the water content remained practically constant, the undercooling points dropped over 15 degrees.

A case in which the change in water content of a hibernating insect which had just resumed feeding had no apparent effect on the under-

cooling point is exemplified by adults of Say's grain bug, *Chlorochroa sayi* Stål. Two series of these bugs were individually frozen, one series being taken directly from hibernacula and storage for a few days at 2° C., and the other series differing only in that they were fed on sprouted wheat for five days before being frozen. The results are given in Table 6.

Table 6. The Effect of Feeding on the Undercooling Point and Water Content of Adult Chlorochroa sayi

Condition	No. used	Undercooling point	Per cent water content
Dormant at 2° C.	9	— 18.5° C.	53.3
Fed for five days	5	— 20.0	67.0

Thus, while the water content increased 14 per cent upon feeding, the undercooling points remained approximately the same, the difference having no apparent significance because of the small size of the sample.

Another series was removed to room temperature from 2° C., fed for five days, and then exposed to decreasing temperatures of 5°, 0°, and —5° over a period of three weeks, to observe if any "hardening" was demonstrated by a decrease in undercooling points. After the treatment the points averaged —18.7°, thus exhibiting no effect of the artificial hardening process.

THE EFFECT OF CONTACT MOISTURE ON THE UNDERCOOLING POINT

The first indications of the importance of contact moisture were obtained when a series of 10 *Lucilia sericata* larvae were frozen individually in an enclosed rubber thermocouple holder, one after the other in fairly rapid succession. In a somewhat irregular manner the larvae undercooled less and less from the first to the last one frozen, as follows: —20.5, —12.2, —13.2, —21.3, —15.5, —11.2, —7.0, —6.1, —6.0, —8.9° C. It was finally decided that condensation moisture was the cause of these results, since moisture in contact with a leaf surface has long been known by plant physiologists to inoculate the plant fluids. The thermocouple holder, upon being taken out of the refrigerator after an insect is frozen, and being at a temperature below zero, immediately becomes covered with a thin layer of ice formed by condensation. The ice soon melts and leaves the surfaces of the holder and thermocouple moist. That such moisture, frozen again, was responsible for the descending series of undercooling points listed was soon proved by freezing a second series which differed only in that the larvae and thermocouple holder were thoroly warmed and dried on the surface before being cooled. When each insect thus received the same start as the preceding one, the undercooling points were more uniform, as follows: —21.3, —17.8, —15.7, —21.4, —22.2, —20.1, —18.2, —19.6, —18.8, —20.9° C. There is no semblance of a descending series here, nor does there seem to be any possibility of inoculation having taken

place except possibly to a slight degree in the third one. As already mentioned, these larvae when irritated are prone to secrete a liquid that may inoculate them. Thus considerable care had to be exercised in freezing that latter series.

In all further work, unless contact moisture was desired, the thermocouple holder was warmed and dried with a blast of compressed air, and the insect surface-dried. The possibility of the insect inoculating itself by some secretion was also taken into consideration. It must be pointed out here that contact moisture, when effecting inoculation, is in the form of ice.

It will be noted that in the first series of larvae undercooling was not eliminated but only reduced. More of the same species were artificially inoculated. Larvae dipped in water and then frozen gave rebounds at very high temperatures, the undercooling points being around —2° to —4° C. No further rebounds were recorded in any case, even if cooling was carried down to —30° C. This was at first taken to mean that the single rebound observed just below zero was that of the water and that undercooling of the insect was entirely eliminated. In view of later investigations another explanation may be presented, namely, that the undercooling was limited by the free water in contact with the insect, but that the rebound point recorded was a combination of the freezing of both the water and the insect. The freezing point of these larvae is not much below 0° C. even tho the undercooling points may be around —20° C. Larvae inoculated by a small droplet of water at one end and removed when the temperature had dropped to only —2° or —2.5° C. were hard when pressed with a blunt object. This discovery led to an investigation of the true freezing points of insects.

When larvae of *Lucilia sericata* were frozen in a holder with a small piece of moist blotting paper on the side opposite the thermojunction, so that the latter would remain dry, the reaction was similar. Altho the rebounds were always very slightly below zero, perhaps two or three degrees, as many as three distinct rebounds were recorded for the same insect. One of these, at least, was that of the water in the filter paper; one other may have been that of the insect itself, in which undercooling may not have been entirely eliminated; a third seems to be from water accidentally in the holder or on the insect and removed from physical contact with the water in the filter paper.

When full-grown *Musca domestica* larvae were exposed to the same treatment, inoculation took place when the filter paper was wet, but not when it was just moist. Puparia of the same species, frozen in contact with a droplet of water, were inoculated in only two out of ten instances.

It might be expected that the type and extent of cuticular covering would affect the susceptibility of an insect to inoculation by contact moisture. Strangely, adult wasps of the genus *Polistes,* having a rather hard exoskeleton, showed a considerable susceptibility when dipped in water and vigorously shaken once or twice before cooling. Pupae of the Mediterranean flour moth, *Ephestia kühniella,* showed individual differences. When they were exposed to —22.0° C. in a vial, 53 per cent were killed when the pupae were dry, 73 per cent when they were placed in the vial by means of a wet camel's hair brush, and 97 per cent when exposed in contact with a drop or two of water. Death at this tempera-

ture was due only to freezing, the time factor being negligible. A similar experiment with pupae of *Tribolium confusum* exposed at —16.0° gave mortalities of 22, 66, and 86 per cent, respectively.

Ten Mediterranean flour moth pupae were frozen one after another without warming the thermocouple holder. In two cases no rebound at all could be observed even tho the temperature was closely watched from 0° to —28°. The undercooling points of the 10 pupae, given in order, were: —22.7, —23.0, none, —8.1, —23.6, —23.6, —21.6, —20.2, —23.5, none. Inoculation was of course not expected with the first one, since the holder was dry. It occurred, however, in one-third of the remaining cases merely from condensation moisture. In the two cases where no rebound occurred, and in the single case where the rebound was slight, at —8.1°, the insects were frozen solid when removed at —28.0° C.

Prepupal larvae of the Indian-meal moth, *Plodia interpunctella* Hbn., exhibited a considerable susceptibility to inoculation. Out of 25 larvae exposed on moist filter paper to —4° for one hour, 24 were inoculated and frozen hard long before the hour had elapsed. Prepupal larvae of *Ephestia kühniella* were affected in a similar manner.

The indications are that the individual susceptibilities exhibited within a group of insects of the same species are due to the element of chance in securing a good physical contact. Whether the contact is a direct one through the cuticula, or with the cuticula itself, or of some other type is not known. There certainly does exist, however, a variation in the susceptibility of different species and stages to contact moisture inoculation. An extensive experiment with eggs of the Angoumois grain moth, *Sitotroga cerealella,* failed to show any significant difference in the temperature-mortality curves of eggs exposed on dry filter paper and eggs exposed on moist filter paper. The data are given in the discussion of lethal temperatures of this species.

Eggs of the grasshopper, *Camnula pellucida* Scudder, were similarly resistant to inoculation. When frozen in contact with water, the rebound of the water was recorded but was always followed by the normal undercooling point of the egg at —23° to —27° C. Soaking of the eggs before freezing had no effect, even tho soaked for periods varying from half an hour to one week. Unhatched larvae of *Malacosoma disstria* Hbn., overwintering in the eggs, could not be inoculated by soaking in water.

An insect that exhibits a doubtful or low susceptibility to inoculation is *Eurycephalomyia myopaeformis.* Larvae were in no case inoculated when the thermocouple holder was not warmed and dried for each one, nor by dipping them into water before freezing. Under the latter treatment, however, the undercooling points were slightly higher than otherwise, tho not very much so.

THE OBSERVED REBOUND POINTS AS DETERMINED BY TWO DIFFERENT METHODS

The value of the observed rebound points has been shown to be open to question. If these points are too low because of the extraction of heat by the system, as seems to be the case, then the error should be reduced by a better method of recording the actual internal temperature of the insect. A good way to secure better contact without piercing

the insect is to use adult beetles and place the thermocouple under the closed elytra. This was done with a series of 15 adults of *Attagenus piceus* Oliv. At the same time an equal number were frozen in the usual manner. Both series were cooled in an enclosed rubber thermocouple holder. The results are given in Table 7.

Table 7. Comparative Results Obtained by Changing the Location of the Thermojunction on Adults of Attagenus piceus

	Undercooling point	Rebound point	Size of rebound
Contact through elytra	— 20.50° ± 1.59° C.	— 19.20 ± 1.44° C.	— 1.35 ± 0.78° C.
Contact under elytra	— 19.23 ± 1.84	— 14.13 ± 2.69	— 5.20 ± 1.51

The difference shown in the undercooling points is not conclusive evidence of a lag in the cooling of the beetle when frozen in the usual manner. However, the location of the observed rebound points and the size of the rebound is definitely changed. The actual internal temperature was more closely recorded by the couple under the elytra than by the one above them, altho without much doubt the actual rebound temperature still lay considerably above either set of recorded points.

RELATION OF THE SIZE OF THE REBOUND TO THE WATER CONTENT

As a rule, insects were desiccated at 105° C. after being frozen individually for 24 hours, and the water content calculated. The undercooling and observed rebound points were always recorded. Thus the data on most of the insects used could be analyzed for relationship between water content and the magnitude of the rebound.

Early in this work, while pupae of *Sitotroga cerealella* were being frozen, it was noticed that the larger individuals gave larger rebounds than did the small ones. To make certain of this point 30 pupae were weighed and frozen individually. The rebounds were plotted against the water content in milligrams as a correlation chart, which on analysis gave a correlation coefficient of 0.965 with a probable error of ±0.032. Such a relationship is very nearly a perfectly linear one, and indicates among other things that the experimental error was very low. The moisture content varied from 2.3 to 12.5 milligrams with a mean of 5.37 milligrams, while the rebounds ranged from 0.5° to 5.5° C. with a mean of 2.05° C.

The rebound is the difference between two recorded temperatures, and therefore a change in it may be due, first, to a change in either temperature while the other remains constant; second, to a change in both points in opposite directions; or third, to an unequal change of both points in the same direction. It has already been pointed out that the undercooling points recorded in this work are not subject to much error. It has also been shown that the rebound points as observed are probably too low.

The correlation of the rebound with the water content of the insect is excellent evidence of the inaccuracy of the observed rebound points.

Each of the 30 pupae was frozen by the same technique, the chief source of variation lying in the mass of the insect which in turn is closely related to the water content. The surrounding cooling system (thermocouple holder, wires, etc.) all extract part of the heat of fusion, which if the true rebound point is to be reached must all be retained by the liquid that is freezing. Even those parts of the insect which do not freeze, or which freeze only at very low temperatures, must extract some of this heat of fusion.

The error, however, will be less when the water content is high than when it is low. While none of the observed rebound points was the true one, those of individuals with the higher water contents most closely approached the true points. The relationship is a physical one. The error in measuring it is dependent on the method but it seems to be impossible to avoid. The rebound points become more precise as the amount of water available for freezing increases. Since in most species of insects the percentage water content is practically constant for a series in the same physiological condition, total mass may be substituted for mass of water, altho the latter is more accurate.

It has previously been pointed out that an insect is heterogeneous in structure, and that it freezes not as a unit, but as a mixture of solutions with different physical properties and different freezing points. Yet the pupae of *Sitotroga cerealella* were treated as homogeneous units since the water content of the whole insect was used, and not the individual water contents of different cells, tissues, or organs. No other species tested exhibited the same degree of relationship between water content and rebound, nor did other stages of this same species. The pupae of *Ephestia kühniella* showed only a fair correlation. All other species and stages used showed still less correlation. This is taken to mean that the body fluids of the pupae of *Sitotroga cerealella* used in the experiment possessed a homogeneity that did not occur in any other species or stage tested. The fluids must have acted almost entirely as one unit or solution; otherwise the water content as calculated from oven desiccation would have given only a partial relationship. During pupal metamorphosis, tissues are being broken down and built up into new ones. The pupa at such a time represents a more homogeneous mass than any other stage with the possible exception of the egg.

THE TRUE FREEZING POINT

Robinson (1928b,c) realized that contact measurement of internal body temperatures is inadequate and devised a method by which was measured the difference in temperature recorded by two thermocouples, one piercing the insect, the other in contact with the surface while the insect was cooled and frozen. A correlation chart was constructed from the data for a certain stage of a given species. In further work the experimenter could freeze the same stage with the thermocouple in contact with the body surface, and read from the chart the corresponding internal temperature. The lag in temperature is inversely proportional to the heat conductivity of the insect tissues between the interior and the surface of the insect body, and also inversely proportional to the temperature difference. The latter is determined by the rate of cooling.

The charts, therefore, show the lag in internal temperature in a specific insect and at a specific rate of cooling.

The inaccuracy of the rebound points, as pointed out, is the result of another factor than the mere lag in the recording of the temperature. It is the result of the extraction of heat by the environmental apparatus to such an extent that the freezing liquids cannot attain their true freezing point. Prepupal larvae of *Lucilia sericata* which were cooled in contact with wet filter paper and inoculated by it were hard when removed at temperatures no lower than —2.0°. Their observed rebound points when frozen dry were much lower. The significance of the fact that these larvae were frozen hard at such a high temperature was not appreciated at the time. The next experiments along this line were performed much later, using prepupal larvae of the Mediterranean flour moth. The normal undercooling points of the latter averaged —21.3°, and the observed rebound points —12.5° C. Seven larvae inoculated by a slight film of water on the dorsal surface gave the results shown in Table 8.

Table 8. The Freezing Points of Larvae of the Mediterranean Flour Moth Inoculated with Water

Larva No.	Undercooling point	Observed rebound point	Temperature held for 2 minutes	Results
1	— 4.4°	— 4.2°	— 4.5°	Frozen hard
2	— 3.1	— 3.0	— 3.5	" "
3	— 3.8	— 3.3	— 3.5	" "
4	— 2.9	— 2.8	— 3.3	" "
5	— 1.9	— 1.9	— 2.4	" "
6	— 2.3	— 2.3	— 1.9	Only slightly hardened
7	— 2.2	— 2.1	— 2.2	" " "

From the above data it would appear that the freezing points lie not lower than about —2° C.

Sixteen larvae were then pierced by the thermocouple, which in this case was fairly large and blunt (approximately 0.5 millimeter in diameter). The data are presented in full in Table 9.

These data indicate that the freezing range lies around —1.25 to —1.4° C., as represented by the figures in bold-faced type. In all of the other cases undercooling was too great to allow the true rebound point to be attained. It will be noticed that the greater the undercooling, the more the observed rebound point tended to be lowered. When the true freezing point was reached, or nearly reached, the time that the rebound point was maintained was greater than otherwise. Even when the insects are pierced, dissipation of the heat of fusion is too great to be accurately recorded. It is hoped that a better method of determining the freezing points of insects will soon be found.

At this stage of the experiments, the objection was raised that physical "hardness" was not proof that the body fluids were frozen, it being thought possible that merely the fats were congealed. Opposing this idea was the fact that in no case was a normally cooled insect found to be at all hard upon its removal from the refrigerator, before reach-

ing its undercooling point. At such temperatures, then, the fats were either not congealed or were insufficiently so to make the insect hard. This was determined definitely with larvae of the Mediterranean flour moth and the Indian-meal moth.

Table 9. The Freezing Points of Injured Larvae of the Mediterranean Flour Moth

Larva No.	Treatment	Undercooling point	Observed rebound point	Time at observed rebound point
1	Pierced, badly mashed	— 1.3°	— 1.25°	2 seconds
2	Pierced with needle first	— 4.0	— 1.5	negligible
3	"	— 11.0	— 4.5	"
4	"	— 7.6	— 3.0	"
5	"	— 9.1	— 4.5	"
6	"	— 8.2	— 2.5	"
7	"	— 2.3	— 1.4	2 seconds
8	"	— 3.7	— 1.7	negligible
9	Anal segment removed, threaded on couple	— 4.9	— 1.65	"
10	"	— 3.3	— 1.4	3 seconds
11	"	— 6.2	— 2.6	negligible
12	"	— 5.7	— 1.6	"
13	"	— 6.8	— 1.9	"
14	"	— 2.9	— 1.4	4 seconds
15	"	— 2.6	— 1.3	4 seconds
16	"	— 4.0	— 1.3	3 seconds

Conclusive proof was needed, however, to settle this point. A prepupal larva of *Plodia interpunctella* was cooled normally to —15°. No rebound took place, so the undercooling point therefore lay below —15°. The holder was carefully removed from the refrigerator and warmed almost to room temperature. It was then opened and a very small droplet of water was placed on the caudal tip of the insect, away from the thermojunction. The insect was then cooled very slowly to —3.0° C. and held at this temperature for 5 minutes. The holder was shaken from 0° to —3° to insure that the water was frozen. When removed quickly and tested, the larva was found to be hard all over. Several more specimens treated in the same way gave exactly the same results, as did also larvae of the Mediterranean flour moth. These results are offered as proof that the water of the insect was frozen, and not merely the fats. Thus an average observed rebound point of —12.5° was recorded for the larvae of *E. kühniella* when the actual freezing points lay somewhere between —0.5° and —2.0° C.

Further tests were made with *Plodia interpunctella.* Twenty-five prepupal larvae were exposed on moist filter paper to —4.0° C. for one hour. Before the hour had elapsed, 24 were frozen hard. On removal, the one that had not been inoculated and frozen was normal, as were all of the 25 control larvae which were exposed on dry filter paper. Of 20 larvae of *Galleria mellonella* L. exposed at the same time, 8 out of 10 froze on the moist filter paper, and the other two, as well as the 10 dry control larvae, were normal.

It is interesting to observe in this connection that none of the larvae of *P. interpunctella* which froze at —4° died immediately after freez-

ing. They reacted the same as adults of *Tribolium confusum* discussed in a later section of this bulletin which are designated as "severely affected" by the quantity factor of cold. These larvae could only roll over and over and feebly move their legs and antennae. No spinning occurred, altho the dry larvae exposed to the same temperature resumed spinning shortly after exposure. None of the frozen larvae died until the fifth day, when three died and two pupated abnormally. At the end of 13 days, eight were dead and three had pupated abnormally. When last observed three days later, three more had died. The effect was not from the quantity factor, since the control insects exposed dry showed no ill effects. Yet the similarity of results was striking and opens up an intriguing problem.

It being proved that larvae of the Mediterranean flour moth undercool as much as 20 degrees, it was then natural to test insects having much lower undercooling points. The insects chosen for this work were the oak borers, *Chrysobothris* sp., *Romaleum rufulum* Hald., and *Synchroa punctata* Newm. Much difficulty was experienced with these insects because of the fact that very little freezing took place at the higher sub-zero temperatures, so that their bodies became hard only after fairly low temperatures were reached. For example, the normal undercooling points of *S. punctata* lie below —35°C., and at temperatures of —10° and —7° the insects are still quite hard. However, from —7° upward, melting takes places very gradually and it is impossible under these circumstances to say just where the melting was completed. At —5° they were still slightly frozen, so that the freezing points of these insects lie above —5° C. This was as accurate a determination as the technique would allow.

Larvae of *Romaleum rufulum* with normal undercooling points lower than —32° gave the first indications of melting at about —11 to —13°, but were not completely melted until the temperature was raised considerably higher than this. No end point of the melting could be determined. *Chrysobothris* larvae acted in the same way, except in one respect. The thorax of these larvae remained hard even as high as —2.0° C., regardless of the condition of the abdomen. This appears to demonstrate a variation in freezing points of different parts of the body.

Using the methods of piercing the insect with the thermocouple and of using contact moisture to initiate freezing, the results were no better. At the highest freezing point insufficient heat was liberated to be accurately recorded, while by using contact moisture the same difficulty arose as in the determination of the melting range.

The conclusion drawn from these data on three species of oak borers is that at the initial, or highest freezing point, very little liquid is frozen, certainly insufficient to cause the body to become hard, with the exception of the thorax of *Chrysobothris*. Considerably more cooling is necessary before the bulk of the liquids is frozen, and the body becomes definitely hard. The technique as yet is inadequate to record the initial freezing point accurately.

All of the insects used in the above experiments were soft-bodied larvae which represented the simplest material to work with. The

difficulties accompanying the recording of the true freezing points are increased when using insects with a hard cuticular covering, or insects containing a very low absolute water content. Attempts of the writer to record the freezing temperatures of the hibernating bugs *Chlorochroa sayi, Perillus bioculatus,* and *Leptocoris trivittatus* produced only uncertain results. The difficulties attending this work may in general be said to vary inversely as the amount of the liquid content involved in any determination. It will probably be necessary in future work to express the body fluids from enough insects to give a sample of at least one cubic centimeter. Piercing the insect with the thermocouple may be used where the insect is large and contains much fluid, provided that the junction is located in or close to that tissue which has the highest freezing point.

LETHAL LOW TEMPERATURES OF SOME STORED-PRODUCT INSECTS

The Mediterranean flour moth, *Ephestia kühniella* Zell., is a very common and troublesome pest in flour mills. The larvae spin an enormous amount of silk which mats together the flour and meal, clogging the bolters, purifiers, conveyor spouts, and other machinery. The Angoumois grain moth, *Sitotroga cerealella* Oliv., is a pest of unmilled grain and is especially troublesome in seed wheat and corn. In temperate regions this insect is most destructive in seed-houses and granaries. The black carpet beetle, *Attagenus piceus* Oliv., is more of a pest of sacks, bolting cloth, clothes, and carpets than of cereals or flour, altho it also works extensively in mixed feeds high in protein and oil.

These three pests are usually controlled by fumigation, superheating, or cleaning practices. There are occasions, however, when it is more convenient and economical to make use of natural or artificial low temperatures, and for this reason a study was made of the effects of subzero temperatures on them. The two moths were studied in every stage of their life cycle, but the beetle only in the larval, pupal, and adult stages.

The methods used in these particular studies were of two types. The eggs and newly hatched larvae were exposed in small gelatin capsules, about 4 millimeters in diameter and 15 millimeters in length, suspended inside a test tube at room temperature and lowered into the cold air of the constant-temperature refrigerator. The capsule was suspended by the thermocouple wires which pierced its lid, the thermojunction being in contact with the bottom of the capsule where rested the larvae or eggs. The cabinet temperature was adjusted so that the final temperature of exposure was approached very slowly. When this point was reached, the capsule was withdrawn. Using this method, moderately large numbers of eggs and larvae were exposed at each temperature used. Check lots were used to determine the normal viability of the eggs.

In the case of feeding larvae, prepupal larvae, pupae, and adults, the method was different. Each specimen was exposed individually in the standard type of thermocouple holder, the cabinet temperature being adjusted so that the rate of cooling at the lower temperatures was suffi-

ciently slow for accurate determination of the undercooling points. A sample of the rate of cooling of one specimen has already been given (page 13). The undercooling and observed rebound points were recorded and the insect was removed and warmed soon after the rebound point had been reached. In all cases the insect was removed at or above the undercooling temperature as this was approached the second time, and in all cases death resulted from the freezing that had taken place during these few seconds. Thus the undercooling point of any individual of these three species represents its minimum lethal temperature.

In Table 10 these data are presented in condensed form. In the case of the eggs and newly hatched larvae, the figures are estimated from a temperature-mortality curve constructed from the data obtained in the experiments with these two stages. For all the other stages the figures are exact calculations from the actual undercooling points recorded, which, as stated above, represent the minimum lethal temperatures. The percentage water content, as determined by desiccation at 105° C. for 24 hours, is also given in some cases to show its uniformity during a major change in the undercooling temperatures.

Table 10. Lethal Low Temperature of Ephestia kühniella, Sitotroga cerealella, and Attagenus piceus

Stage	No. used	Lethal temperature					Per cent water content	
		Maximum	Minimum	Median	Mean		Mean	
Ephestia kühniella:								
Eggs	1196	— 22.0°	— 27.0°	— 24.8°	.....	.....	.....	.....
Newly hatched larvae	958	— 21.0	— 27.0	— 24.3	.....	.....	.....	.....
Feeding larvae	12	— 4.7	— 7.3	.....	— 5.77°	± 0.835	66.12	± 2.80
Prepupal larvae before spinning	15	— 5.3	— 11.2	.....	— 8.07	± 1.46	65.37	± 2.35
Prepupal larvae after spinning	10	— 17.3	— 23.2	.....	— 21.30	± 1.73	65.30	± 2.27
Pupae	50	— 21.1	— 24.6	.....	— 23.13	± .895	65.88	± 2.97
Adults	10	— 20.0	— 22.0	. .	— 20.90	± .502	.....	.....
Sitotroga cerealella:								
Eggs	6579	— 19.0	— 25.0	— 23.5	.....	.....	.....	.....
Larvae, in eggs and newly hatched	821	— 21.0	— 27.5	— 24.6	.....	.....	.....	.....
Feeding larvae	15	— 6.0	— 12.4	.....	— 9.07	± 1.82	65.60	± 3.88
Prepupal larvae	25	— 13.0	— 21.6	.....	— 18.10	± 2.58	65.51	± 2.17
Pupae	50	— 14.0	— 22.6	.....	— 17.74	± 2.41	66.57	± 4.38
Adults	20	— 11.7	— 17.7	.....	— 14.70	± 1.59	.....	.....
Attagenus piceus:								
Larvae	30	— 11.0	— 22.3	.....	— 16.96	± 2.91	.....	.....
Pupae	20	— 17.5	— 24.0	.....	— 21.25	± 1.73	.....	.....
Adults	30	— 16.1	— 24.0	.	— 19.87	± 1.84	55.31	± 4.22

The minimum lethal temperature of any stage is that temperature just low enough to kill all individuals of that stage by freezing. Similarly the maximum lethal low temperature is that one above which no

individual is killed by freezing. This does not mean that above the maximum lethal temperature an insect will not die of the effects of cold. It merely excludes death from freezing in an environment free of contact moisture. Any one of the three species under consideration may be killed by exposure to a temperature above the maximum lethal temperature, provided that that temperature is maintained for a certain period of time. Freezing does not take place under such circumstances unless the insect is mechanically agitated or exposed to contact moisture while at a temperature below its true freezing point. Death from cold, without any freezing whatever, must be much different from death by freezing.

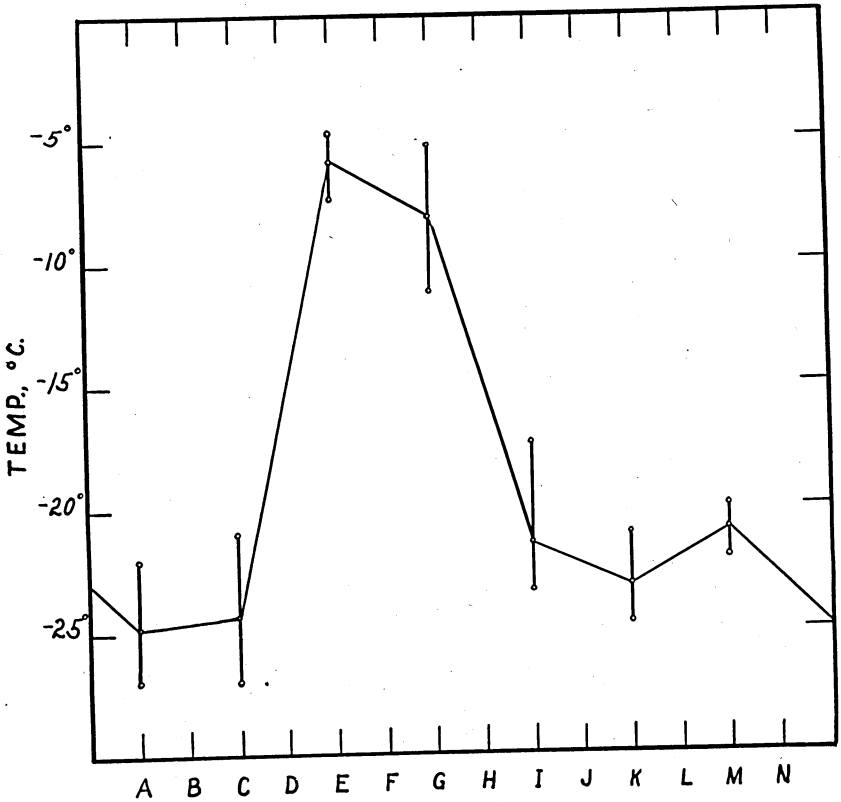

FIG 1. THE MEAN LETHAL LOW TEMPERATURE FOR ALL STAGES OF THE MEDITERRANEAN FLOUR MOTH

A—Eggs
B—Hatching
C—Newly hatched larvae, unfed
D—First feeding
E—Feeding larvae, all stages
F—Emptying of alimentary tract
G—Prepupal larvae, before spinning
H—Spinning of cocoon
I—Larvae from cocoons
J—Pupation
K—Pupae
L—Emergence
M—Adults
N—Oviposition

In Figure 1 are presented graphically the data obtained for *Ephestia kühniella.* The curve is drawn through the median lethal temperature of the eggs and newly hatched larvae, and through the mean lethal temperatures (mean undercooling points) of the remaining stages. The points above and below each median or mean represent the maximum and minimum lethal low temperatures, respectively. The abscissa is divided into equal parts, lettered to indicate either a stage of the life cycle or some process which has an effect on the data presented. For instance, the first few bites of food taken by the larvae send the undercooling points soaring from a mean of —24.3° C. before feeding to —5.77° C. after feeding. Therefore one point on the abscissa is labeled as the first meal, to show that that was the factor causing the change in undercooling points. Similar spaces are used for the processes of hatching, spinning of silk, pupation, emergence, and oviposition. This arrangement helped to explain some of the abrupt changes from one stage to another, altho there may be to some extent a gradual change within the stage. For instance, only a normal distribution of undercooling points was recorded for the feeding larvae of each species. If a change did take place during feeding it was extremely slight. Similarly, the data for the prepupal larvae, pupae, and adults showed no change during the development of each stage. In the case of the eggs of *Sitotroga cerealella,* however, a drop was observed in the undercooling points as the eggs aged. On examination of the data and the conditions under which it was recorded, it became apparent that this drop took place between the time of oviposition and the formation of the fully developed larvae within the chorion. The larvae remain in the shell for a short time before hatching, and at this time have the same undercooling points as after hatching. This fact is not surprising, since there is no visible liquid or other matter left in the shell when the young larva reaches its full growth within the chorion. The shell is almost transparent and is brittle and dry to the touch. In the table, therefore, the eggs are listed in one row and the newly hatched larvae, along with the fully formed larvae in the egg shell, are listed in another row.

LETHAL LOW TEMPERATURE OF OVERWINTERING LARVAE OF FOREST TENTLESS CATERPILLAR

The forest tentless caterpillar, *Malacosoma disstria* Hbn., overwinters as an unhatched larva in the egg. The eggs, which are deposited in uniform rings around small twigs of the host trees, are covered with a coating of a spongy material. They are laid in early summer and develop during the same season, so that the young larvae are fully developed in the fall. They do not hatch from the eggs, however, until the following spring, and thus are exposed to the extremes of winter temperatures. The spongy coating, moreover, absorbs water readily, so that the eggs may be covered with water or ice at various periods of the winter.

One experiment was designed to test the effects of low temperatures on the newly hatched larvae. Larvae were allowed to hatch normally from the eggs in the laboratory and were then subjected to low tempera-

tures, the exposure being gradual over a period of 10 or 15 minutes. The final temperature was maintained for two minutes. A considerable variation in resistance was noted, but since this did not seem to be correlated with age, which was the only variable factor, the results were averaged. The data, which produce a typical S-shaped temperature-mortality curve when plotted, are condensed in Table 11.

Table 11. The Mortality of Newly Hatched Forest Tentless Caterpillars at Different Low Temperatures

Temperature of exposure	Per cent mortality
— 9° C.	0.0
— 10	10.0
— 11	16.7
— 12	21.7
— 13	56.8
— 14	74.2
— 15	97.7
— 16	98.0
— 17	100.0
— 18	100.0

Mortality was thus complete at —17° C. If, however, larvae were exposed before they hatched from the eggs, there was no mortality whatever even at —27° C. These larvae chew their way out of the egg shell and surrounding spongy covering, and altho it was not determined definitely that they ate this material, it seems likely they did. It has been seen that the undercooling points of newly hatched larvae of the Mediterranean flour moth are raised considerably by their first food.

An attempt to determine the minimum fatal temperature of the over-wintering larvae was unsuccessful because they became less cold-hardy when mailed in from northern Minnesota and stored at 2° C. One series stored at 2° C. for several weeks was completely killed by exposure to —36° C., while another series stored for about two weeks at 2° C. suffered only 28 per cent mortality at the same temperature.

THE INFLUENCE OF THE LENGTH OF EXPOSURE IN DEATH AT LOW TEMPERATURES

Many insects are killed by low temperatures above their undercooling points. Excluding contact moisture as a factor, there are certain types of insects which are killed by low temperatures without being frozen, provided the time of exposure is of sufficient length. The lower the temperature, the shorter is the exposure necessary to kill them, and vice versa. Examples of such insects are most of those infesting stored products, and also many insect stages which are normally spent in warm summer weather and are never exposed to temperatures below the threshold of development.

Adults of *Tribolium confusum,* being similar to many other species infesting stored products in that they cannot withstand low temperatures, were chosen for use in experiments designed to show the extent to which the "quantity" factor might enter into a study in which the "intensity"

factor was primarily under consideration. This insect is killed also by the initial freezing represented by the rebound, so that the undercooling point of any individual is also its minimum lethal temperature. It is susceptible, therefore, both to "quantity of cold," and to "intensity of cold," as defined by Payne (1927a).

The range of temperatures necessary to give 0 to 100 per cent mortality was from —12° to —20° C. Exposures were made at regular intervals of one degree for periods of one to 20 minutes at one-minute intervals as well as periods of 22, 24, 27, 30, 45, and 60 minutes. The insects were exposed in groups of 25, at least four such groups being used for each exposure. Exposure was effected by dropping each group of 25 insects into a separate test tube immersed to three-fourths of its depth in the alcohol constant-temperature bath. The time required for the insects themselves to attain the required temperature was thus cut to a minimum. The mortality was recorded after a period of 24 hours, the insects being kept at room temperature on flour in ½-ounce tins without lids. Adequate aeration was necessary to prevent asphyxiation from the toxic volatile liquid that these adults secrete when irritated in any way, or after being exposed to low temperatures.

The problem of separating the dead from the live individuals is often a vexing one in such work. After some preliminary experiments, it was decided to use four groupings which worked very satisfactorily. These were, first, those definitely dead, soon turning a dark brown color; second, those definitely alive and apparently normal; third, those able to move their appendages only very feebly, unable to walk, but not discolored; and fourth, those able to walk feebly, but obviously not normal in their actions. The third class was designated as "severely affected," and the fourth as "moderately affected."

Tests by freezing individual adults in a thermocouple holder proved that individuals which rebounded did not survive the process. They soon discolored and were definitely dead. This happened either when they were frozen normally or when they inoculated themselves by secreting fluid. At temperatures as low as —12° C. the insect was killed by freezing whether the freezing was normal or inoculated. Therefore all those insects in the two "affected" groups must have been affected by the quantity factor of cold alone. When adults in these two groups were kept for a considerable period those "severely affected" never recovered their ability to move or to lay eggs, while those "moderately affected" often gained somewhat in activity and laid eggs. The fertility of these eggs was not determined.

From a practical standpoint, the adults designated as "severely affected" may be relegated to the "dead" group, while those designated as "moderately affected" may probably be included with those alive and apparently normal.

In Figure 2 are shown the time-mortality curves, in solid lines, for temperatures of —12°, —13°, —14°, and —15° C. At —12°, mortality started at two minutes exposure, increased until about four minutes, and leveled off, varying from 11 per cent at 4 minutes to 16 per cent at 60 minutes. Variations between these two exposures were from 12 to 30 per cent mortality.

At —13°, mortality started at one minute exposure, increased until four or five minutes, and leveled off, varying from 14 per cent at 4 minutes to 33 per cent at 60 minutes. Variations between these two exposures were from 12 to 30 per cent mortality.

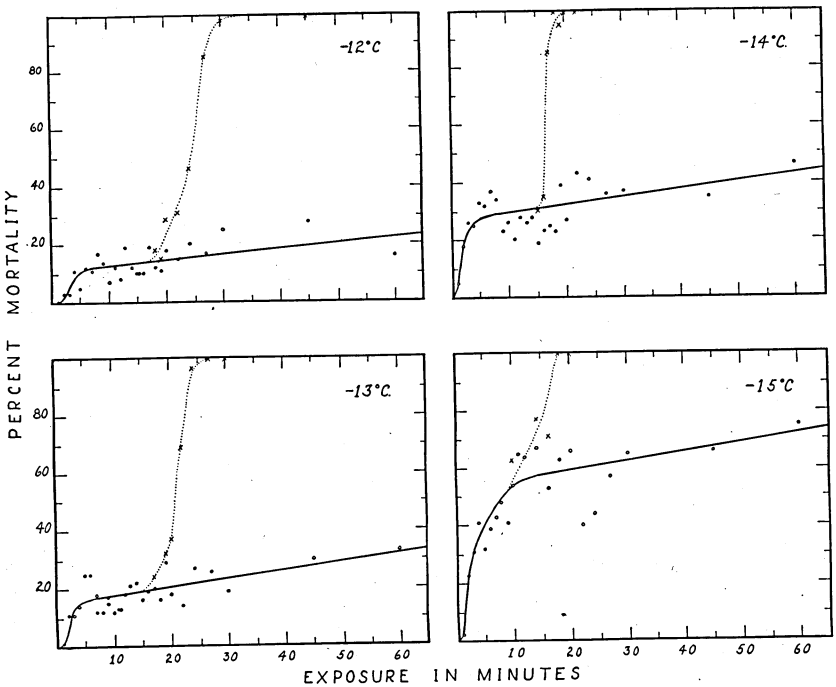

FIG. 2. THE MORTALITY OF ADULT *Tribolium confusum* EXPOSED TO LOW TEMPERATURES FOR DIFFERENT PERIODS

The unbroken line indicates those dead at the end of 24 hours. The dotted line indicates the sum of the dead and the severely affected groups.

At —14°, mortality started at one minute and increased until three or four minutes, when it leveled off, varying from 25 per cent at 4 minutes to 46 per cent at 60 minutes. Variations between these two exposures were from 19 to 43 per cent.

At —15° and down to —20° the curves are disrupted by the dotted line curves, which are obtained by adding to the mortality those which are "severely affected." The insects of this group, altho showing considerable resistance to actual death when kept in the laboratory after exposure, may be considered dead from the practical standpoint, since death to them is only a matter of time, and the powers of movement and egg-laying are lost. If these are included with those dead from freezing, then the curve seems to consist of one S-shaped time-mortality curve representing the effect of the intensity factor of cold or actual freezing at the shorter exposures and another S-shaped time-mortality curve

representing the effect of the quantity factor, at the longer exposures, the two curves being connected by a line of fairly constant mortality. This is better illustrated in the curves for —12°, —13°, and —14° C., where each factor is sufficiently separated from the other to be distinct. As the temperature is lowered, the mortality due to freezing increases and its S-shaped curve lengthens upward. In addition to this, the time required for the quantity factor to operate becomes less, and its S-shaped curve moves to the left. At —15°, finally, the curves interfere with each other, and altho at this temperature it is still possible to distinguish between those killed by freezing and those "severely affected," yet at lower temperatures one cannot be sure that some of the latter group did not actually die as a result of the operation of the quantity factor.

It has been established for some time that the effect of the quantity factor is directly proportional to the length of exposure to a certain temperature. The above experiment indicates the possibility of the interaction of the two factors, and suggests that care be exercised to avoid confusion of the results. It is demonstrated in Figure 2 that at an exposure of about 10 minutes to a temperature of —15° C. the quantity factor is likely to be responsible for deaths attributed to the intensity factor. What, then, is the effect of the quantity factor on an insect that is exposed to a certain rate of cooling designed to be slow enough to allow for accurate data on the intensity factor? If the effect is sufficient to cause death, then perhaps actual freezing is not the cause of death at all in such cases. This would not apply, of course, to insects which are not susceptible to the quantity factor of cold.

A COMPARISON OF MORTALITY CURVES OBTAINED BY INDIVIDUAL EXPOSURE AND BY MASS EXPOSURE

Adults of *Tribolium confusum* do not survive freezing. If a frequency curve of the undercooling points of an adequate number of them is rearranged to read accumulatively, at 1° C. intervals, then this curve should be the same as one plotted from mortalities of insects exposed *en masse* to a series of constant temperatures, arranged in the same 1° C. intervals, provided that the time of exposure is sufficiently long to ensure that the body temperature of the insects reaches the temperature of exposure, and that it is short enough so that the quantity factor of cold does not affect the results. From the work carried on with these adults in a previous experiment, an exposure of 10 minutes was considered as fulfilling these conditions, as completely as possible. At this exposure, the mortality ranged from 0 per cent at —9° C. to 100 per cent at —20° C.

Fifty adults were frozen individually in the usual type of thermocouple holder. Complications soon arose, since 40 out of the 50 were inoculated and gave rebounds of only 0.1° C. and less, altho distinct enough to make possible the accurate recording of the undercooling points. These 40 points all lay between —9.9° C. and —15.8° C. and gave a very good S-shaped mortality curve. The other 10 individuals gave rebounds of 0.3° to 0.7° C. and were obviously not inoculated. Their undercooling points ranged from —14.3° to —18.5° C., a dis-

tinctly lower grouping compared to the inoculated ones, and when plotted also gave a fairly good S-shaped mortality curve. Both curves are plotted in Figure 3. The reason for inoculation lies in the fact that, under certain conditions of excitation, these insects secrete a pungent liquid. Handling and exposure to low temperatures are very effective stimulants for its secretions.

If only 20 per cent undercool normally when frozen individually, what happens when they are cooled *en masse?* That the liquid is secreted in quantity is certain from the strong odor emanating from the insects when they are removed from the refrigerator. After treatment they must be kept well aerated in open containers on flour, or they are asphyxiated and the flour turns a dark pink. The mortality curve of individuals exposed in bulk, representing 2,400 adults, 200 at each temperature, is also shown in Figure 3. That a proportion of the adults were inoculated is shown by the fact that at —14° C. 46 per cent were killed, whereas on the curve for normal undercooling points of individuals there was no mortality at —14° C. That a proportion froze normally is shown by the fact that at —16° C. only 70.5 per cent were killed, whereas on the curve for inoculated undercooling points of individuals there was a mortality of 100 per cent at —16° C.

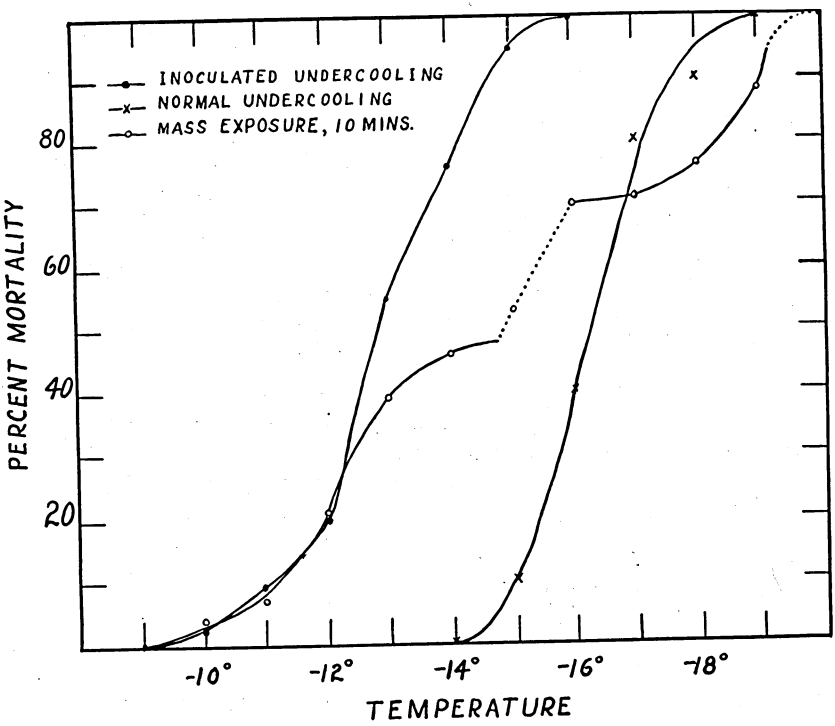

FIG. 3. A COMPARISON OF MORTALITY CURVES OBTAINED BY INDIVIDUAL EXPOSURE AND BY MASS EXPOSURE OF ADULT *Tribolium confusum*

The mortality curve of individuals exposed *en masse* is irregular, but if it is considered somewhat from the same angle as was Figure 2, namely that each end of the curve represents the operation of a different factor, while the middle of the curve is modified by the influence of both factors, then it will be seen to be composed of an S-shaped mortality curve at each end. The curve at the higher temperatures is caused by inoculated undercooling and that at the lower temperatures by normal undercooling. At present some aspects of this curve are unsatisfactory, and it may be that the fitting of the curve to the points as in Figure 3 is incorrect. The upper end should approach 100 per cent gradually, as the dotted line indicates, altho there may be no justification for drawing the curve in this manner.

THE IMPORTANCE OF THE UNDERCOOLING AND FREEZING POINTS IN THE HIBERNATION OF INSECTS

From the standpoint of their reactions to low temperatures, there are two main types of insects. First, there are those insects which can survive freezing and which are killed only by some temperature below their undercooling points. To these insects the freezing and undercooling points are of little if any significance. What finally causes their death at a lower temperature has not yet been determined, but the location of the minimum lethal temperature is a very simple procedure. It appears to bear no simple relation to the undercooling and freezing points, altho it seems probable that death occurs either when a certain vital tissue becomes frozen, or when a certain degree of freezing is reached.

The second group of insects includes all of those which cannot withstand freezing. A sub-group including such insects as many of the stored-product pests cannot withstand even moderately low temperatures for any great length of time, but so far as actual freezing is concerned they react exactly as do the remainder of this group. All of these insects, then, are killed by freezing, but the question arises, "How much freezing is necessary to cause death?" The answer must be qualified depending upon the conditions.

If an insect of this second group is cooled either in the laboratory or in its natural hibernaculum, as in the soil or under trash near the soil surface, then, provided that the insect is not inoculated by contact moisture, and is not mechanically jarred, it will cool to its natural undercooling point. The subsequent rebound, under natural conditions, will be very brief and have little effect, so the insect will soon be at its undercooling point again. If the environmental temperature is falling rapidly, the insect temperature will fall even lower than the undercooling point immediately after the rebound. The amount of freezing that takes place under these circumstances is sufficient to kill the insect. The maximum amount of freezing possible at that temperature does not necessarily take place, since an unknown but probably varying period of time is necessary to establish an equilibrium.

The hibernacula of insects of this group are not always dry, by any means. It has been shown that contact moisture (in the form of ice)

may reduce or even eliminate undercooling in those insects which are susceptible to inoculation by it. In the presence of moisture then, the normal undercooling point is not of much significance. The amount of undercooling that does take place, however, is of great importance and seems to depend on a number of factors such as the extent and quality of the contact and the nature of the integument of the insect. If undercooling is entirely eliminated, freezing will take place at the true freezing point. Thus there are many degrees of freezing, from that occurring at the freezing point, increasing towards that possible at the undercooling point. It has been observed that the lesser degrees of freezing are not always fatal to the animal, but that death often takes place at some point intermediate between the freezing and undercooling points. While death does not always accompany a slight freezing, yet the insect is seriously affected by it.

SUMMARY

The natural and artificial control of insects by low temperatures is forming an increasingly important aspect of economic entomology. The practical application has been and still is hampered by a lack of knowledge concerning the fundamental problems involved. The development of precise and adequate methods of laboratory and field studies is essential. A knowledge of the process of freezing and the factors affecting it is equally important.

Improved apparatus for studying the undercooling and rebound points is described. It provides a continuous and accurate record of body-surface temperatures, eliminates the possibility of interference by contact moisture, and provides a uniform rate of cooling that can be replicated exactly. Many of the complications which may arise in laboratory and field work are discussed.

The use of anesthetics to make extremely active insects quiet while being prepared for freezing was tested. Slight chloroforming had no effect on the undercooling points of adults of the Angoumois grain moth, and neither light nor heavy dosages of ether had any effect on those of adult confused flour beetles.

Insects exhibit a considerable variation in the effect that desiccation or other change in water content has on their undercooling points. Puparia of the housefly lost four per cent of their weight when exposed to warm, dry laboratory conditions for several hours, while their undercooling points dropped from −12° to −22° C. Adults of *Leptocoris trivittatus,* desiccated over calcium chloride at 2° and 20° C., lost over 20 per cent of their weight while the undercooling points remained stationary. Larvae of *Ephestia kühniella* had almost the same percentage water content whether they were last instar feeding larvae, early prepupal larvae, or prepupal larvae from cocoons, yet the undercooling points averaged −5.8°, −8.0°, and −21.3° C., respectively. Hibernating bugs, *Chlorochroa sayi,* increased in water content from 53 to 67 per cent of their total weight when fed for five days, but the undercooling points remained unchanged.

The effects of moisture in contact with the body surface, usually in the form of ice, and the need for careful technique to eliminate this factor

when not desired are pointed out. Contact moisture reduces and sometimes completely eliminates undercooling in many insects, while others are not affected by even a heavy coating of water or ice. The factors governing the action of contact moisture appear to be the extent and "quality" of the contact, the type of integument of the insect, and probably others. Soft-bodied insects such as dipterous and lepidopterous larvae are usually susceptible, altho the heavily sclerotized wasps, *Polistes,* are also very susceptible to ice crystal inoculation. Larvae of *Lucilia sericata, Musca domestica, Ephestia kühniella, Plodia interpunctella,* and *Phyllophaga* are all readily inoculated by contact moisture. The pupae are slightly harder to inoculate, exhibiting an apparent individual variation which probably depends more on the quality of the contact than on the insect. Eggs seem to be very resistant; those of *Cannula pellucida* and *Sitotroga cerealella* were entirely so in spite of all efforts to inoculate them. Fully developed larvae of *Malacosoma disstria* in the egg-shell were also proof against inoculation. Larvae of *Eurycephalomyia myopaeformis,* on the other hand, exhibited a doubtful or very low susceptibility.

The undercooling points of *Attagenus piceus* adults remained practically the same, whether the thermojunction was placed under the elytra or on top of them. The observed rebound points were five degrees higher when recorded from beneath the elytra, showing that this was a more precise method. The true rebound points were still higher than those recorded by either method. The undercooling points are shown to be recorded correctly.

Pupae of *Sitotroga cerealella* exhibited a correlation of 0.965 ± 0.032 between water content in milligrams and the size of the rebound. Analysis showed that the observed rebound points were many degrees lower than the true freezing point, but that the latter was more closely approached as the water content of the pupae increased. Thus the relationship was merely physical and indicated only a very uniform technique. That other stages of the same or different species did not react similarly is due to a more homogeneous liquid composition during the pupal period.

The rebound points as observed were proved, by several different methods, to be incorrect. Larvae of *Lucilia sericata* inoculated by contact moisture were found to be frozen hard at $-2°$ C. The melting point range of Mediterranean flour moth larvae, determined by observing the change from physical "hardness" to "softness" was found to lie above $-1.9°$ C. When the larvae were pierced by the thermocouple, the rebound points lay between -1.25 and $-1.4°$, if the amount of undercooling was not too great. Larvae inoculated by contact moisture were frozen at similar temperatures. Thus larvae whose observed rebound points averaged $-12.5°$ C. had a melting point range near $-1°$, a freezing point (by inoculation) of -1.5 to -2.0, and a rebound point (by piercing) of $-1°$ to $-1.5°$ C. The technique has not yet been perfected sufficiently to bring these slightly different figures together, but they would undoubtedly check closely with more refined methods. It was proved that physical hardness was due to freezing of the body fluids and not to congealing of the fats.

Oak borer larvae with undercooling points of approximately —40° C. also had high freezing points (above —5° C.), but very little freezing took place at these high temperatures. The insects did not become very hard until the temperature dropped a few degrees further. A marked difference in the melting points of the thorax and abdomen was noted in *Chrysobothris* sp. Future work will probably necessitate the use of a method of determining the amount of ice formed at a certain temperature.

Three species of stored-product pests, *Ephestia kühniella, Sitotroga cerealella,* and *Attagenus piceus,* were tested for the location of their minimum lethal temperatures. The variation of these temperatures is given in the form of maximum, minimum, and mean lethal low temperatures. The complete life cycle of *Ephestia,* most of the stages of *Sitotroga,* and the larvae, pupae, and adults of *Attagenus* were studied. A temperature of —27.0° C. kills all stages of *Ephestia,* and —27.5° C. all stages of *Sitotroga.* The most resistant stage in each case is the newly hatched unfed larva. The first feeding of these larvae lessens their cold-resistance so effectively that they are then the least resistant stage. There seems to be evidence that this is a rather general phenomenon in insects, at least of certain types. The forest tentless caterpillar, *Malacosoma disstria,* behaves similarly. The food ingested by the newly hatched larvae of this species as they chew their way out of the egg-shell raises their undercooling points more than 20 degrees Centigrade. It is suggested that this may give important clues in investigations on the factors involved in undercooling, whether such investigations are of a biological or physical nature.

The quantity factor of cold, effective on many insects infesting stored products, was tested with the object of determining whether it could act in a short enough period of time to affect experiments designed for the study of the intensity factor alone. Using adults of *T. confusum,* it was found that at temperatures of —15° C. the quantity factor became lethal in 10 to 15 minutes. From a study of the mortality curves, it is obvious that at lower temperatures and shorter exposures the two factors become hopelessly intermingled so that it seems probable that death results from the interaction of both factors.

A comparison was made of mortality data obtained by two different methods. Adults of *T. confusum* were exposed to low temperatures individually and *en masse.* In the former case only 20 per cent froze normally, the other 80 per cent being inoculated by a liquid secreted by the insects themselves. Each set gave a typical S-shaped mortality curve. Those insects exposed *en masse* gave results intermediate to those from the two sets of individuals.

In the case of insects that can withstand freezing, the minimum lethal temperature is lower than the undercooling point and may be very simply determined by test exposures. For insects that are killed by freezing, the undercooling point is the all-important minimum lethal temperature. If the insect is dry or immune to inoculation by contact moisture, then it is the normal undercooling point that is important. If the insect is inoculated by contact moisture, undercooling is limited, and a new and higher undercooling point is observed. The location of

this new point is dependent on many factors, and may even coincide with the freezing point, in which case undercooling is nil. In any case it becomes the minimum lethal temperature for that particular insect.

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