131. THE PHYSIOLOGICAL ACTION OF ABNORMALLY HIGH TEMPERATURES ON POIKILOTHERMIC ANIMALS

1. TEMPERATURE ADAPTATION AND THE DEGREE OF SATURATION OF THE PHOSPHATIDES

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At temperatures in the region of 40–45° most animals die within a few hours. This applies equally to hot- and to cold-blooded animals. Often, however, temperatures lethal to cold-blooded animals are considerably lower than that limit. The cause of death by heat and the mechanism of heat injury at temperatures below those where a visible destruction of the tissues (e.g. coagulation of protein) takes place have given rise to a considerable amount of speculation and numerous hypotheses have been advanced to explain the phenomena observed.

In the older literature on the subject death by heat was attributed mostly to the coagulation of proteins; more recently other hypotheses [recently reviewed by Belehradek, 1935] have been put forward, such as destruction of enzymes, asphyxiation or other disturbance in the equilibrium of protoplasm through accumulation of waste products. While none of these hypotheses is yet proved experimentally the one most deserving further investigation is the "lipoid liberation theory" since it is the only one where evidence has been provided linking the phenomenon of heat adaptation—i.e. the capability of an organism to increase its heat resistance—with data on the constitution of an important component of the protoplasm.

It has been shown by various authors that the melting point of fats of micro-organisms and of higher plants and animals varies with the temperature at which they have been laid down. When examined at a given temperature fat formed at a higher temperature is found to be more solid than fat formed at a lower temperature. Since the melting point of a fat depends largely on its degree of saturation, the iodine value (number) of a fat as a measure of double bonds in the fatty acid chains is a good measure for its melting point. Thus the iodine number of the visceral fat of a mammal is lower than that of the subcutaneous fat. The number is also lower in oils from tropical plants than from plants grown in a cooler climate. (Numerous other examples in Belehradek [1935].)

Various workers observed changes occurring in the lipid constituents of the cell on heating. Fauré-Fremiet [1924] found in the eggs of *Sabellaria* granules appearing on heating which seemed to be of lipid character. A similar "liberation" of lipins was found by Heilbrunn [1924] who centrifuged eggs of *Arbacia* after heat treatment. Koeppe [1903] and Rywosch [1911] attributed haemolysis of erythrocytes to a melting of the superficial lipid membrane. Heilbrunn [1924] first drew special attention to the correlation between heat resistance and the

melting point of protoplasmic fats, a point which has later been enlarged upon by Belehradsek [1931]. The latter, formulating the "lipoid liberation theory", links up the heat "adaptability" of the protoplasmic fats, as expressed by the correlation between the melting points—degree of unsaturation—of fats and the temperature of their formation, with the adaptability of the whole organism to high temperatures. According to him heat injury, whether reversible or irreversible, is caused by the melting of lipid constituents in the cell or in the cellular membranes. Fat formed at a higher temperature and consequently of a higher melting point would render an organism more resistant to the damaging effect of high temperatures.

As already mentioned, there are well-established examples available about the comparative degree of saturation of lipins found in plants and animals from different climates or of different habitat. The literature contains only a few, but well-established examples in which changes in the iodine number of fats have been demonstrated by growing an organism at different temperatures. [Terroine *et al.* 1927; 1930] on bacteria, Pearson & Raper [1927] on *Aspergillus*.] Although the phenomenon of adaptability of the living organism to the temperature of its environment has been regarded almost as an axiom by ecologists, it is supported by surprisingly few certain examples in the literature. The most striking examples of temperature adaptation are found in the work of Hathaway [1927], Wells [1935, 1, 2], and Sumner & Doudoroff [1938], all relating to fishes. The phenomena of heat adaptation on the one hand and changing melting point of lipins on the other have, however, never been investigated in the same organism and their combination in the lipid liberation theory is based on mere analogy.

The object of our work is to fill this gap in our knowledge by breeding an organism at different temperatures and subsequently testing for differences in its heat resistance and in the saturation degree of lipins of individuals bred under otherwise comparable conditions at different temperatures.

Adaptation to high temperatures

The larvae of two species of flesh flies, *Calliphora erythrocephala* Meigen and *Phormia terra-novae* R.D., differ widely in their tolerance of high and low temperatures. *Calliphora* develops from the egg to the fully grown larva at any temperature between 12 and 31°, the closely related *Phormia* only develops at temperatures between 18 and 36°. To attain full growth the larvae of both species require about 3 days at the higher and 16 days at the lower end of the respective ranges. The larvae, thus bred at different temperatures to their full size, were submitted simultaneously to a test for their resistance to a constant high temperature. The method adopted was similar to that described by Mansbridge [1936]. The larvae were dropped suddenly into the temperature required and the time after which approximately 50 % of them were still undamaged, i.e. would pupate and develop to normal flies, was determined. The following tables present the summary of the experiments.

Table 1. *Heat resistance of Phormia*

Tested at	Period in hr. after which 50 % were killed	
	(Reared at 18°)	(Reared at 36°)
46°	$\frac{1}{2}$ – $\frac{3}{4}$	2 $\frac{1}{2}$
45	3 $\frac{1}{2}$	>7, <15
44	7–11, 15	15, 18
43	19	24

Table 2. *Heat resistance of Calliphora*

Tested at	Period in hr. after which 50 % were killed	
	(Reared at 12°)	(Reared at 30°)
41°	1	1½
40	1½	2½
39	3	5
38	>4 <10	13

From the figures in Tables 1 and 2 it is clear that larvae which were bred at a higher temperature could resist a given high temperature for a considerably longer period than larvae which were bred at a lower temperature. Or, considering the temperature which heat-adapted larvae and non-adapted specimens could tolerate for a given time, it appears that the difference is approximately 1°. Thus, for *Phormia*, reared at 36°, a temperature of 46° for 2½ hr. has about the same effect as a temperature of 45° for the same period for larvae bred at 18°.

The correlation between the iodine values of the fatty acids of the phosphatides and the temperature of breeding

For these experiments the same species of larvae were bred under identical conditions as in the heat resistance experiments described.

It had been proposed at first to work with the total fats of the body for these investigations. This, however, was abandoned for the following reasons. The work of Evans [1932] has shown a selective utilization of the reserve fats in blowfly larvae in the period preceding pupation, when the insects have stopped feeding (so-called prepupae), and it was feared that this selective utilization would constitute a considerable source of error, as these larvae are at that stage of their life history entirely dependent upon the reserve materials of their body. This source of error would be especially large, as the known difference in duration of the life history at different temperatures makes it evident that, for instance, 1 hr. at 36° does not at all correspond to 1 hr. at 18°, and that even the smallest differences in "physiological" age would greatly add to the possible error.

The phosphatides, on the other hand, are now generally considered as the "vital" fats, as opposed to the reserve fats. Though very little is known about their function so far, we do know that they occur in every cell and that their presence is apparently essential to the continued existence of protoplasm. It is therefore improbable that they would be utilized as reserve fats, though a certain proportion might always be found in a transit stage engaged in metabolic processes. But, whatever their alleged "vital" role may be, it is most likely that they would be involved in the protoplasmic processes connected with heat injury.

In the method of analysis the procedure of Terroine *et al.* [1930] was followed with certain modifications.

At least 500 fully grown larvae, with their guts empty, were extracted for 48 hr. in a Soxhlet apparatus with absolute alcohol. From the extract obtained, the alcohol was evaporated off, and the residue extracted several times with ether of sp.gr. 0.720, to which, after concentration, 4 vol. acetone were added. The resulting precipitate was allowed to stand for 15 min., centrifuged, the liquid decanted off, and then redissolved with ether. The precipitation was repeated four times in the early part of the work, when it was assumed that the material thus obtained would be fairly pure phosphatide, or at least phosphatide of a comparable standard of purity. The results of the determinations obtained with

material prepared by this method are given in Table 3, under the heading "Iodine values by first method".

A comparatively large divergence in the properties of material thus obtained, led to an investigation of its purity, and it was found that the phosphorus/nitrogen ratio still varied considerably in materials prepared in the above way. It was therefore decided to work in future on material showing a constant percentage of phosphorus, and to continue precipitation until this was obtained.

Phosphorus was estimated by the colorimetric method of Martland & Robison [1926], measuring the total phosphorus as inorganic phosphate to within ± 0.005 mg.

The results for the highest degree of purity obtainable were as follows:

	Theoretical value, pure lecithin + kephalin %	Actual value %
Phosphorus	4.8	3.4-3.6
Nitrogen	2.4	2.8-3.1

The material finally worked with was therefore by no means pure phosphatide, but was in all probability still adulterated with protein or urea. However, the degree of purity obtained need not be regarded as unsatisfactory, as commercial technical lecithin, which was tested for comparison, only contained about 2.8% phosphorus.

For the second series of estimations (called "second method" in Table 3), the phosphorus in the acetone precipitation was estimated and precipitation repeated until the phosphorus percentage remained constant at the above value (approx. 3.5%). The "phosphatide" thus obtained was then saponified with 10% alcoholic KOH on a boiling water bath under reflux.

The further procedure was then as used by Evans [1932] and Rainey [1938] in their work on blow-fly larvae. Saponification was carried out for 11 hr. at least, after which time most of the alcohol was boiled off under reduced pressure and the resultant syrupy solution was taken up with a little water, from which the unsaponifiable matter was removed by shaking five times with ether in a separating funnel. From the ethereal solution thus obtained any remaining soaps were shaken out with water, which was united with the original soap solution. From this the fatty acids were precipitated by adding hydrochloric acid (1 part of conc. HCl to one part of water). These were removed by shaking out five times with ether. The solvent was removed, the residue, which still contained a certain amount of impurity, was taken up with light petroleum (B.P. 40-60°), and the fatty acids recovered by evaporation of the solvent in a weighed flask. The iodine values were measured by the method described by Dam [1924], and the results are as follows:

Table 3. *Iodine values of phosphatides*

Species	Temp. of breeding	Iodine values by	
		First method	Second method
<i>Phormia</i>	18°	88, 89, 92, 86	91, 92
	27	64.5, 84, 76, 78	71, 72, 73.5
	36	69, 60.5, 65.8, 66.7	69, 62, 63
<i>Calliphora</i>	12	78, 88, 89, 88, 82.7, 84, 85	—
	15	—	96, 95.8
	27	72, 76, 72.5, 84, 83, 75.5	72.7, 75
	30-31	65.2, 66.2, 70	70, 70, 67

The results obtained by the "first method" show more scatter than those from determination of phosphatides with constant phosphorus content, but there is no significant difference between the two sets.

The results are graphically represented in Fig. 1. It is apparent that the degree of saturation of the phosphatides is directly dependent upon the tem-

Fig. 1. The relation of the iodine values of the phosphatides of the larvae of *Calliphora* and *Phormia* with the temperature of breeding.

perature of breeding in the two closely allied species investigated. The fatty acids of the phosphatides laid down at the higher temperature in both species are more saturated than those produced at lower temperatures, proved by a lower iodine value. The difference for the extreme temperatures of breeding, a temperature difference of about 18°, is about 26 units in each species.

Discussion

It is shown that blow-fly larvae, reared at higher temperatures, not only become more resistant to high temperatures than larvae reared at a lower temperature, but that the melting point of their cellular fats is also "adapted". In both species of insects the range of change of the iodine value is about equal. The mean difference between the values at the highest and lowest temperatures of breeding in *Phormia* is about 26 units, and in *Calliphora* also 26 units, with a range of breeding temperature of 18°, in both cases. This amount of adaptation may appear rather high, but the values are well in agreement with those found by other workers. Pearson & Raper [1927], working on the total fat of two species of fungus, found the mean iodine value of *Aspergillus niger* to be 149 if bred at 18°, 129 at 25° and 95 if the cultures were kept at 35°. In *Rhizopus nigricans* the corresponding figures were 88 for 12° and 78 for 25°. Terroine *et al.* [1930] found that the micro-organism *Sterigmatocystis nigra* in cultures grown at 18° had an iodine value of 135 for the fatty acids of the phosphatides, and the same organism grown at 38° gave a mean of 99. "Bacille de la Fleole" gave mean values of 51 and 31 for cultures bred at 18 and 38° respectively. The values for the two species of fly larvae can therefore be regarded as well within the range to be expected from other work.

No figures are available for the degree of saturation of fatty acids of the phosphatides in other insects. Ackerman [1926] cultivated the aphid *Rhopalosiphum prunifolia* at different temperatures and found that the solidification

point of fatty globules in the body fluid increased with the temperature of cultivation. Rainey [1938] reared the larva of another flesh fly, *Lucilia sericata*, which is closely related to the species used by us, at temperatures of 15, 25 and 35° and found the iodine values of the total fatty acids to be 75.8, 72.2 and 71.0 respectively. These figures, although showing a clear tendency to falling iodine number with increasing temperature, are not regarded as significant by Rainey, the difference in iodine value being just outside the experimental error and the larvae having been used for the determinations at a different physiological age. As the physiological role of the phosphatides is so different from that of the greater part of the total fat, which constitutes storage material, no conclusions can be drawn from a comparison of Rainey's figures and our own.

The many data now available of which those quoted above constitute only a fraction makes it clear beyond doubt that the rule that the constitution of fats depends on the temperature at which they are laid down applies to all living matter. Leathes & Raper [1925] and Pearson & Raper [1927] point out that lipid substances are formed not by a single chemical process but by a catenary series of changes. The unsaturated compounds are formed first, and the saturated ones arise in turn from these. At low temperature this process cannot be completed but stops at a higher degree of unsaturation than would have been the case if the formation had taken place at a higher temperature.

Table 3 and Fig. 1 show that the mean iodine values for the two flies used by us vary only according to temperature and not to species within the same range. However, it is evident that this is not applicable to all living matter and that completely different sets of results are obtained with different sorts of organisms. This is shown clearly by comparing our results with those of Terroine, quoted above. Whether the similarity in the two closely related insects examined is merely accidental or whether phylogenetic relationships will also lead to a similarity in this respect is a question demanding further investigation. That fats derived directly from the food are often laid down unchanged as reserve material is a well-known fact in vertebrates. Yuill & Craig showed [1937] that larvae of *Lucilia sericata* reared on different diets showed a difference in the iodine value of their fats according to the food offered to them. However, as the fly larvae in our own experiments were grown at different temperatures on the same food (lean meat), the differences found in the constitution of the phosphatides cannot be due to the food. It is generally assumed that phosphatides, e.g. lecithin, are not obtained directly from the food material in unchanged form but are products of synthesis within the body itself.

The results obtained for *Phormia* and *Calliphora* support the lipid liberation theory in so far that for the first time there has been found a direct correlation between the saturation degree of the phosphatides and the heat resistance of the whole organism. However, the results obtained make it very unlikely that death at moderately high temperatures is due simply to a melting of the protoplasmic fats. Otherwise there would be no explanation of the fact that the lowest temperature which each of the two species of fly larvae can tolerate for a given period is approximately 7 degrees lower for *Calliphora* than for *Phormia*. Adaptation consists only of pushing the lowest lethal temperature for a given period up by approximately 1 degree. *Calliphora* and *Phormia*, bred at 27°, are killed at an exposure of 5 hr. at 39 and 45° respectively and yet the iodine values of the phosphatides of both species are the same, namely 75.

From this the conclusion may be drawn that it is unlikely that a breakdown of lipins in a purely physical way, causing for instance a change in cell permeability, is the cause of heat injury at the comparatively low temperatures

required. The lipid liberation theory of adaptation to high temperatures, while certainly substantiated by the present work in so far as such striking differences in the constitution of lipins bear out the predictions made by it, cannot explain fully and satisfactorily the phenomena of heat injury and heat adaptation. Other factors must play their role, but the striking adaptation of the saturation degree of lipins described in this work leads to the tentative suggestion that the physical nature of these fats may have a decisive influence on the chains of physiological processes involved.

SUMMARY

The adaptability to high temperature and the nature of the phosphatides have been studied in fully grown larvae of the blow-flies *Calliphora erythrocephala* Meig. and *Phormia terra-novae* R.D.

The results are as follows:

1. In both species of insect the amount of heat adaptation is such that larvae bred 18° higher than others will be able to withstand the injurious effects of high temperature for one degree more for the same time of exposure than those bred at the lower end of the temperature range.

2. The degree of unsaturation of the phosphatides of these larvae, measured by their iodine values, is dependent upon the temperature of breeding only, and was found to show a difference of 26 units in both species for a difference in the breeding temperature of 18°.

3. These results are discussed in the light of the "lipoid liberation theory", as advanced by Heilbrunn and Belehradek. While the strong adaptation of the phosphatides to the temperature at which they have been laid down suggests a role for these substances in the mechanism of heat adaptation, the fact that two closely allied species of insects, bred at the same temperature and having the same iodine value for the fatty acids of their phosphatides have different resistances to heat, shows that a physical breakdown of the fatty substances cannot be the direct cause of heat injury.

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