sich nach dem Zustand des Tieres richten. Die erste Reaktion auf die Injektion äußert sich meist durch Erbrechen von Mageninhalt und Häutung. Die Kopulationsbereitschaft erkennt man beim ♂ an der Schwärzung der Vorderpfoten, an Umklammerungsbewegungen und Quaken unter Wasser. Vor der Kopulation sind noch weitere 0,6 cm³ zu injizieren.

Behandlung der ♀. * Vorspritzen ist meist nur im Winter nötig; es geschieht 4–7 Tage vor der Kopulation mit 0,3–0,5 cm³ oder, wenn die Kopulationsbereitschaft (erkennbar an den leicht geröteten Analpapillen) etwas weiter fortgeschritten ist, nur mit 0,1–0,3 cm³ 1–2 Tage vor der Paarung. Unmittelbar vor dieser erhält das ♀ eine Spritze von 0,5–0,6 cm³, wenn es vorbehandelt ist, sonst etwa 0,7 cm³.

Zur Paarung sollte man nur ♀ mit ausladenden, prallen Flanken verwenden. Die größte und oft nicht beachtete Schwierigkeit der Behandlung besteht darin, ♀ und ♂ zur gleichen Zeit kopulationsreif zu machen.

So ist es notwendig, die Partner während der Vorbehandlung in Einzelgefäßen zu halten, um eine vorzeitige Kopulation mit ungenügender Spermaabgabe zu verhindern. Die Weibchen werden von uns nur alle 4–6 Monate zur Eiablage veranlaßt.

3. *Eier.* Bei richtig abgestimmter Hormonbehandlung und Schutz vor Störungen bei der Kopulation sind oft mehr als 90% der abgelegten Eier befruchtet; der Durchschnitt bewegt sich zwischen 70 und 80%, liegt also wesentlich höher als bei OCHSÉ.

Nach beendeter Eiablage (etwa nach einem Tag) müssen die Adulten entfernt und das durch sie verunreinigte Wasser vorsichtig (Temperaturschwankungen und Schädigung durch mechanische Einwirkung vermeiden!) gewechselt werden. Der Laich ist evtl. auf mehrere Aquarien zu verteilen und darf nicht in dicken Ballen zusammenliegen. Leere, faulende Eihüllen und unbefruchtete Eier sind zu entfernen, denn *starke Fäulnis* (auch des Futters) *schädigt die jungen Larven* nachhaltig (siehe auch OCHSÉ, 1948).

4. *Larven.* Sobald die Larven frei umherschwimmen und zu fressen beginnen, werden sie gegen * *chlorhaltiges Leitungswasser stark empfindlich.* Eine Chlorkonzentration von 1:20000000 wirkt noch tödlich, bei 1:50000000 ist die Entwicklung normal. Die Aquarien sind daher nicht mit frischem chlorhaltigen Leitungswasser zu beschicken, sondern mit Wasser, das einige Tage in offenen Behältern gestanden war. Mit der Fütterung wird begonnen, wenn die Kaulquappen frei umherschwimmen und regelmäßig schnappen. Gefüttert wird täglich mit Brennesselpulver (GASCHE, 1943), so daß das Wasser gleichmäßig trüb ist.

* *Zubereitung des Futters.* Brennesselpulver wird durch ein feines Weißmehlsieb gesiebt. Nur der feine Anteil wird mit heißem Wasser aufgeschwemmt und abfiltriert. Das grünlichgelbe Filtrat, das Kahmhautbildung und Fäulnis fördert, wird weggeschüttet. Der Filtrierrückstand wird erneut mit lauem Wasser aufgeschwemmt und als Futter verwendet. Bakterien- und Pilzentwicklung werden so auf ein Minimum reduziert.

Die Aquarien sollen wöchentlich einmal (nach OCHSÉ, 1948, öfter) gereinigt werden. * Hierbei werden die Larven sorgfältig durch ein in ein Aquarium getauchtes Sieb oder Netz gegossen, das den Unrat durchläßt, die Larven jedoch zurückhält, und dann in ein vorbereitetes reines Aquarium gebracht.

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Zoologisches Institut der Universität Bern, Abteilung für Zoophysiologie, den 9. September 1948.

Summary

In order to obtain a high percentage of fertilized eggs and to reduce losses of larvæ to a minimum several not yet generally known improvements of raising methods are given in our note. A double treatment of males and females during a few days with gonadotropic chorionhormone is especially in winter time successful and results in high numbers of fertilized eggs.

A Histochemical Method for the Demonstration of Fat Peroxides[1]

It has been shown that certain symptoms in chicks (exudative diathesis, brown coloration of the adipose tissue, and encephalomalacia) and in rats (yellow-brown coloration of the adipose tissue, and depigmentation of the incisors) depend upon the feeding of vitamin E deficient diets containing highly unsaturated fatty acids[2]. Formation of peroxides preceded and, in some instances paralleled the brown coloration, in the adipose tissue of these animals[3,4]. The pigment responsible for the coloration of the adipose tissue was found to consist of at least 2 fractions: one of them is fat soluble, while the other, which occurs in larger amounts, is not extracted by lipid solvents. A preliminary chemical characterization of the latter pigment showed it to agree with the description of "Ceroid", the pigment found in various tissues in experimental liver cirrhosis in rats[3,5].

Histologically, the gross pigment (non-fat-soluble fraction) in the adipose tissue was found to be represented by an acid-fast pigment in the fat cells at different stages of development. Furthermore, the histopathological changes that occur in the adipose and other tissues have been studied to a certain extent[6].

In several of the works reviewed above[7,8,9] it has been suggested that the mentioned symptoms found in chicks and rats could be due to an abnormal oxidation of the highly unsaturated fatty acids, in absence of the antioxidant effect of vitamin E in the tissues. It was also suggested that such a peroxidation could cause the changes which lead to the development of the acid-fast pigment in the adipose tissue. The finding of a considerable formation of peroxides in the tissues affected[7,10] supported these suggestions. However, in order to further the study of the relationship between the peroxidation and the development of the above-mentioned symptoms, it has been proved fundamental to develop a method for the histological demonstration of peroxides. Such a method should prove also important for the understanding of the

[1] A preliminary report of this paper was presented at the 6th Scandinavian Physiological Congress, Acta Physiol. Scand. *16*, suppl. 53, 29 (1948).

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[3] H. DAM and H. GRANADOS, Acta Physiol. Scand. *10*, 162 (1945).

[4] H. DAM and H. GRANADOS, Science *102*, 327 (1945).

[5] P. GYORGY and H. GOLDBLATT, J. Exp. Med. *75*, 355 (1942). – K. M. ENDICOTT and R. D. LILLIE, Amer. J. Pathol. *20*, 149 (1944).

[6] H. DAM and K. E. MASON, Fed. Proc. *4*, 153 (1945). – K. E. MASON, H. DAM, and H. GRANADOS, Anat. Rec. *94*, 265 (1946). – H. GRANADOS, K. E. MASON, and H. DAM, Acta Pathol. et Microbiol. Scand. *24*, 86 (1947).

[7] H. DAM, J. Nutrition *28*, 297 (1944).

[8] H. DAM and H. GRANADOS, Acta Physiol. Scand. *10*, 162 (1945).

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[10] H. DAM and H. GRANADOS, Science *102*, 327 (1945).

actual role played by the peroxides in the formation of the acid-fast pigment.

The method is based upon our study of the heme-catalyzed reactions of organic peroxides[1]. It was observed that under suitable conditions peroxidized fats and fatty acids reacted with a number of oxidizable substances, among them benzidine, leuco malachite green, and leuco dichlorophenolindophenol. Hemin, with a peroxidase-like action, catalyzed such reactions, having a greater effect when used in combination with pyridine, forming so a pyridine-hemochromogen. It was also observed that heating, as well as the presence of a suitable amount of an acid, e.g. acetic acid, accelerated the reaction.

Applying these observations to the development of the histological method, it was found that also cellular peroxides were able to oxidize a reduced substance, especially when the reaction was catalyzed by hemin in combination with pyridine and glacial acetic acid. The leuco base of 2,6-dichlorophenolindophenol was found to be the most suitable compound for the histochemical reaction, since the indophenol formed by the reaction is fat-soluble and will, therefore, tend to remain in the cellular fat instead of going into the staining fluid. When catalyzed by hemin, and in the presence of alcohol, the reaction acquires a velocity sufficient to make possible its carrying out at ordinary temperatures. The alcohol should, however, be diluted with an amount of water enough to prevent the indophenol formed from going into the staining solution; but this amount of water should not be so high as to bring about a precipitation of the hemin or of the leuco-dye. After some preliminary studies of the reaction in frozen sections of fresh and peroxidized bacon, the method was developed on frozen sections of adipose tissue of rats fed vitamin E-deficient diets rich in cod liver oil, with their respective controls.

Two staining solutions are prepared separately. Solution A: 40 mg of hemin[2] are dissolved in a mixture of 10 cc of pyridine and 20 cc of glacial acetic acid. This solution is stable and can be kept for a long time. Solution B: 25 mg of leuco 2,6-dichlorophenolindophenol (3,5-dichloro-4-4′-dihydroxyphenylenediamine[3]) are dissolved in 3·5 cc of absolute alcohol, and then mixed with 5 cc of distilled water. This solution should be made just before being used, since it rapidly becomes oxidized by the atmospheric oxygen. Frozen-cut sections of the tissue are brought from the dishes of water onto slides, which then are blot dried to exclude the excess of water. Then 0·74 ml of solution A are mixed with the total amount of solution B and applied for 3 to 5 minutes on the sections to be stained. The sections are then thoroughly washed with distilled water and covered with glass slips using an aqueous medium such as FARRANT's, or glycerin jelly, and sealed with a cement, e.g. asphaltum. The blot drying of the excess of water that remains on the slides after the transfer of the sections from the dishes of water, prior to the staining, may be a necessary step for sometimes this excess increases the volume of water in the final mixture of solutions A and B to such an extent that the hemin becomes precipitated, and the reaction does not take place. However, when this occurs, it can be overcome by the addition of more alcohol, which causes a redissolution of the hemin. The frozen-cut sections may be fixed with a mixture of 1 part of 40% formalin and 3 parts of 40% ethyl alcohol for about 5 minutes, before or after the staining. The fixation before staining seems to give better results.

By this method the leuco dichlorophenolindophenol is oxidized by the cellular peroxides, and the red color of the indophenol formed reveals itself and remains in those cells and cellular structures in which peroxides are present. The intensity of the red colour is proportional to the degree of cellular peroxides. Likewise, the amount of cells which exhibit peroxides is proportional to the amount of tissue peroxides as determined by the chemical methods. This has proved to be a fact by taking a portion of adipose tissue from a given region, treating some pieces of it by the histochemical method, and determining the peroxide values of other pieces by two chemical methods[1].

This method, as developed so far, serves to demonstrate peroxides present in fat cells; it should prove, however, with suitable modifications, applicable for the demonstration of peroxides in other tissues. In this way, such a method would make possible the study of the relationship that may exist between peroxides and the formation of the fluorescent and acid-fast pigments found in the liver of rats suffering from experimental cirrhosis[2], in the uterus and other organs of vitamin E-deficient laboratory animals[3], and in certain tissues in human pathology[4]. Furthermore, the method may also prove helpful for the further study of the suggested relationship between the oxidation of unsaturated fats and the pigment present in the mitochondria and submicroscopic particles of normal liver cells[5].

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Department of Biology, Polytechnic Institute, Copenhagen, September 29, 1948.

Zusammenfassung

Es wird ein histochemisches Verfahren zum Nachweis von Fettperoxyden beschrieben. Als Objekte dienen einerseits gefrorene Fettgewebsschnitte von Ratten, die eine vitamin-E-freie und an Dorschlebertran reiche Kost erhalten hatten, und andererseits entsprechende Präparate von Kontrolltieren. Die Methode basiert auf der Oxydation der Leukobase des 2,6-Dichlorphenolindophenols durch Zellperoxyde. Die Reaktion wird durch Hämin in Gegenwart von Essigsäure und Alkohol katalysiert. Die rote Farbe des bei der Reaktion gebildeten Indophenols erscheint in allen denjenigen Zellen und Zellstrukturen, in denen Peroxyde vorhanden sind. Das Verfahren dürfte in abgeänderter Form auch für den Nachweis von Peroxyden in anderen Geweben verwendbar sein.

[1] J. GLAVIND and S. HARTMANN, Acta Physiol. Scand. *16*, suppl. 53, 26 (1948).

[2] We thank F. Hoffmann-La Roche & Co., Basle (Switzerland), for the kind supply of the hemin used in these experiments.

[3] The leuco base can be prepared by the method of GIBBS *et al.* (H. D. GIBBS, B. COHEN, and R. K. CANNAN, Public Health Reports *40*, 649 (1925), or by reducing a solution of dichlorophenolindophenol in 50% alcohol with the stoichiometric amount of ascorbic acid, followed by precipitation as a sodium salt by means of sodium chloride. It is purified by repeated dissolutions in alcohol, followed by precipitations with water. It must not contain traces of ascorbic acid, which will delay the reaction until it is oxidized.

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