

## Unmasking of Sudanophil Lipid in the Testis of the House-cricket, *Acheta domesticus*

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### SUMMARY

1. When the testis of *Acheta domesticus* is fixed in Flemming's fluid and embedded in gelatine, Sudan black reveals scarcely any lipid in the externum of the acroblast.

2. It can be shown that the externum of the acroblast contains much lipid in a masked (bound) form.

3. Experiments were performed to find what substances were effective, after Flemming fixation, in unmasking the lipid of the externum and thus making it colourable by Sudan black.

4. The following substances were found to act as unmasking agents: ethanol, dioxane, phenol, resorcinol, hydroquinone, pyrogallol, tannic acid.

5. Under the conditions of the experiment, the most effective unmasking agents were 90% ethanol and 5% hydroquinone.

### INTRODUCTION

WHILE a cytochemical study of spermatogenesis in the house-cricket, *Acheta domesticus* (L.), was being carried out, it was noticed that certain cytoplasmic inclusions (pro-acroblast, acroblast, mitochondria) were almost entirely negative (absence of blue to blue-black coloration) to Sudan black. This negative result was obtained after the testes had been subjected to fixation by strong Flemming's fluid, washing, and gelatine embedding. On the other hand, it was found that the same inclusions were strongly positive to Sudan black if paraffin sections were used. The fixed material was passed through the alcohols and chloroform into paraffin; the wax was removed from the sections by soaking for 10 min in dioxane at 60°.

The almost negative results obtained by using gelatine-embedded tissue, and the very positive results obtained after embedding in paraffin, suggested that, in effect, one or other of the various compounds employed after fixation was acting as a lipid 'unmasking' agent: the lipid, having been in some manner split from the associated protein, was now rendered sensitive to detection by Sudan black.

For the above reasons, it was thought worth while to make a further, more detailed study in order to find out what compound (or compounds) was acting in this manner.

In this connexion it was considered that, as ethyl alcohol is known to act as an unmasker when used simultaneously with the fixative (Ciaccio, 1926), it was also acting in this capacity after (Flemming) fixation.

Furthermore, as phenol is known to be a lipid unmasker when used after

fixation (Ciaccio, 1926), certain other phenolic compounds (hydroquinone, pyrogallol, resorcinol, and tannic acid) were chosen for experimentation. Finally, as dioxane had been used, in the initial work, for dewaxing sections preparatory to staining, it was also included.

As a test object for the demonstration of unmasked lipid, a cytoplasmic inclusion, the acroblast, was chosen. This structure was selected because the chemistry of its constituents had already been studied fairly extensively by Clayton, Deutsch, and Jordan-Luke (1958). This work demonstrates that the acroblast consists of an outer and an inner part. The outer, or externum, appears to be made up of parallel lamellae, arranged (in electron micrographs) in the form of a horseshoe. It gives a reaction (among others) for a lipid. The externum is used in the present study to assess the effect of unmasking agents. As the inner part, the internum, appears almost structureless (by light microscopy) and gives only a very feeble (or no) reaction for lipid, reference to it is omitted.

#### MATERIALS AND METHODS

Testes of the house-cricket were rapidly dissected out of lightly anaesthetized animals and were fixed in Flemming's fluid; thereafter, they were washed and (usually) prior to gelatine embedding, were subjected to the treatments listed under tables 1 and 2 in the appendix. With reference to these experiments, it is to be understood that each one is concerned with one piece of tissue only: each experiment was self-contained and was planned to demonstrate the effect (in lipid unmasking) of one compound only. Furthermore, for the sake of clarity in interpretation of the tables, some notes are added below.

(1) Both ethyl alcohol (of various concentrations) and dioxane were used on the tissue after fixation and washing and before embedding.

(2) All the other compounds (hydroquinone, phenol, pyrogallol, resorcinol, and tannic acid) were tested on gelatine sections.

(3) The period in pyrogallol was restricted to one half-hour, because, after this time, a general darkening of the tissue impeded interpretation of the results obtained by Sudan black.

(4) A control was carried out by applying the Sudan black technique to tissue fixed in Flemming's fluid but not subjected to any other unmasking treatment.

(5) In order to confirm the original observation—sudanophilia after Flemming fixation and paraffin embedding—the Sudan black technique was again carried out on tissue fixed and embedded in this manner. The results were again positive.

(6) The Sudan black, used in all experiments, was prepared and employed according to Baker (1949). Solutions not more than 3 weeks old were used. For this technique, two periods were chosen:  $2\frac{1}{2}$  min at room temperature, and 10 min at 60° C.

(7) Sudan black lability was tested by placing sections coloured by Sudan black in 70% ethanol for  $\frac{1}{2}$  h and noting whether the colour disappeared.

## RESULTS

The results of the individual experiments are listed under tables 1 (effect of compounds used as lipid unmasking agents) and 2 (effect of lipid extraction on sudanophilia) (see the appendix). An examination of these tables will show the following facts.

(1) All the compounds tested as unmasking agents give sudanophilia to a greater or less degree. Both ethanol (90%) and hydroquinone (5%) afford results optimal in effect and clearest in interpretation. With respect to the latter point, dioxane, pyrogallol, and tannin all tend to give a general cytoplasmic darkening.

(2) In the control (table 1) the results are almost entirely negative. The weak sudanophilia shown by the externum of the acroblast may well be due to the unmasking action of the ethanol (70%) used as the solvent for the Sudan black.

(3) Extraction by xylene slightly inhibits the sudanophilia and that by pyridine abolishes it, perhaps by solution of the lipid in the unmasking.

## DISCUSSION

Ciaccio (1926) long ago demonstrated that, among other substances, both ethanol and phenol could act as lipid unmasking agents; of these two compounds, he used ethanol simultaneously with the fixative and phenol after it. Until very recently no other workers appear to have carried Ciaccio's experiments further. Lately, however, Gupta (1958) has shown that phenol also acts as an unmasking agent after fixation. Bradbury and Clayton (1958) have also given evidence that Flemming's fluid itself, without any post-fixation treatment, acts very efficiently on masked lipids in mammalian tissue (basal region of the acinar cells of the mouse pancreas).

The present study supports both the unmasking techniques mentioned above, and, in addition, gives further evidence of compounds, other than ethanol and phenol, acting after fixation.

Until recently, very little was known about the structure of lipoproteins and, consequently, about the bonding between the components (Macheboeuf, 1938; Dawson, 1957); lately, however, it has been suggested (Engström and Finean, 1958) that the protein is almost certainly linked with the polar groups of the ionic lipids (lecithin, cephalin, &c.). But, even though this fact seems now fairly well established, our knowledge, in general, concerning these compounds (lipoproteins) is still too fragmentary to allow any serious attempt at explaining the actual mechanism whereby lipid unmasking agents disrupt the lipoprotein bonding.

As a result of the present study, it is suggested that ethyl alcohol at a concentration of 90% be employed as a convenient and simple method for lipid unmasking. Moreover, this method is in accordance with that long used in biochemical extraction of lipoproteins (Dawson, 1957); even though, in cytochemistry, it is employed after fixation.

The writer wishes to acknowledge with pleasure much valuable advice and criticism, which, in the course of this work, arose during discussions with Dr. J. R. Baker, F.R.S., and also suggestions and technical help contributed by Mrs. A. Przelecka and by Mrs. Barbara Jordan-Luke. She also wishes to thank Professor A. C. Hardy, F.R.S., in whose department this work was carried out.

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## APPENDIX

TABLE I

*A summary of the effects of compounds used as unmasking agents (when employed after fixation) on bound lipids in the acroblast of the spermatid of the house-cricket, Acheta domesticus*

<i>Treatment subsequent to fixation and prior to the use of Sudan black</i>	<i>Sudan black technique</i>	<i>Degree of sudanophilia shown by the extermum of the acroblast</i>
Ethanol, 50%	2½ min	O
6 h	10 min, 60° C	++ to +++
Ethanol, 70%	2½ min	+ to ++
4 h	10 min, 60° C	+++
Ethanol, 50%, 70%, 50%	2½ min	++
6 h	10 min, 60° C	+++
Ethanol, 90%	2½ min	++ to +++
6 h	10 min, 60° C	+++
Ethanol, 100%	2½ min	+ to ++
6 h	10 min, 60° C	+++
Dioxane, 100%	2½ min	+++
½ h	10 min, 60° C	+++
Hydroquinone, 5%	2½ min	+ to ++
1 h, 60° C	10 min, 60° C	+++
Phenol, 1%	2½ min	++
24 h	10 min, 60° C	+++
Pyrogallol, 5%	2½ min	+
½ h, 60° C	10 min, 60° C	++ to +++
Resorcinol, 5%	2½ min	+
1 h, 60° C	10 min, 60° C	++
Tannic acid, 20%	2½ min	+
	10 min, 60° C	++ to +++
No treatment	2½ min	O
	10 min, 60° C	+

*Note:* the treatment above designated as 'ethanol, 100%' actually comprised passage of the tissue up and down alcoholic solutions of the following concentrations: 50%, 70%, 80%, 94%, 100%, 94%, 80%, 70%, 50%.

TABLE 2

*A summary of the effects of extraction by xylene and pyridine on lipids unmasked by ethyl alcohol*

<i>Treatment subsequent to fixation and prior to the use of Sudan black</i>	<i>Sudan black technique</i>	<i>Degree of sudanophilia shown by the externum of the acroblast</i>
Alcohol series, xylene, 4 h	2½ min 10 min, 60° C	+
Alcohol series, xylene (boiling), 4 h	2½ min 10 min, 60° C	+
Alcohol series, pyridine, 60° C, 15 h	2½ min 10 min, 60° C	+
		+
		O
		O

KEY TO TABLES 1 AND 2. +++ = strong reaction; ++ = moderate reaction;  
+ = weak reaction; O = negative.