Studies on the problem of insect damage to keratinaceous materials

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STUDIES ON THE PROBLEM OF INSECT DAMAGE TO KERATINACEOUS MATERIALS

by

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FOREWORD

This thesis is submitted for the degree of Master of Science in the New South Wales University of Technology, N.S.W., Australia. The investigations reported here were carried out at the Division of Entomology, C.S.I.R.O., Canberra, A.C.T.

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I certify that this work has not been submitted to any other University or institution for a higher degree.

R.F. Powning
December, 1952.

SUMMARY

Research on the mechanism of digestion of keratin by insects has recently been intensified and the results presented in this thesis contribute to an understanding of this problem.

Analyses of excreta reveal that the keratin digesting insects eliminate nitrogen as uric acid, urea and ammonia. Uric acid accounts for the bulk of the nitrogen in the clothes moth excreta, but only a small fraction of nitrogen in carpet beetle excreta. Sulphur is excreted by these insects as cystine rather than as sulphate.

In clothes moth larvae cystine appears to be the source of sulphur which is involved in the detoxification of many inorganic poisons by the formation of metallic sulphides. This reaction is rendered possible by the production of H_2S from the digesting food in the gut by an extremely active enzyme, "desulphydrase". Although this enzyme occurs in other insects (including carpet beetle larvae) its activity is relatively very low. The low desulphydrase activity of carpet beetle larvae explains the observed failure to form metallic sulphides in the gut.

Using a specially developed technique, the desulphydrase of the clothes moth larva has been examined.

Comparisons with the desulphydrase from rat liver reveal that the two enzymes are similar with respect to their reaction to various reagents, with the exception of cyanide. The insect enzyme has a pH optimum of 9.1 which is higher than that of rat liver (about pH 7.4), but no differences were observed in substrate specificity or in stability, either in storage or to heat.

Information obtained indicates that urea, high alkalinity, glutathione and certain enzymes (including desulphydrase) probably are not the important factors in the initial degredation of keratin, although they may contribute during the later stages of breakdown. Preliminary experiments on digestion of keratin in vitro by insect enzyme preparations have been carried out and indications have been obtained that enzyme-induced reduction of the keratin desulphide bonds is the key mechanism enabling these insects to digest keratin.

GENERAL INTRODUCTION

In 1946-47 Australian exports totalled about £309 million of which £126 million was earned by wool. It is impossible to estimate the damage done to wool by insects since an appreciable amount occurs in the home, however, when it is considered that under favorable conditions the progeny of one female clothes moth can consume 92 lb. of wool in a year (Clark 1928) it is obvious that large quantities of wool may be lost. Although wool is much superior to synthetic fibers in many ways, its susceptibility to destruction by insect attack still remains a serious disadvantage in its use. Damage may occur in blended materials containing wool, as well as in pure wool. Many mothproofing substances and techniques have been used but most are not cheap, permanent or easy to apply, and many alter the dyeing or wearing properties adversely. The physiological and biochemical processes by which insects utilize keratin, are not clear although a thorough understanding of this is essential for the successful formulation of a mothproofing technique.

Some literature has appeared in overseas publications on the digestion of wool by clothes moths and carpet beetles, however the most recent advances have

occurred in Australia. The present investigation is intended to add to the knowledge of the relationships between wool and its insect pests by chemical and biochemical studies.

It is apparent that the physiological and biochemical processes of insects and the chemistry of keratin are of major importance in an investigation such as this. It is desirable therefore to open this discussion with a short review of the relevant biology of the insects. This is followed by notes on the chemistry of the keratins (Chapter II). A short section is included on mothproofing techniques (Chapter III). Part II deals with the methods (Chapter V) and materials (Chapter VI) used in the investigations, the results of which are described in Part III. Part III is divided into two main Chapters (VII and VIII respectively) in which detailed accounts of the significance of some excretory products of the insects, and desulphydrase activity in insects appear. A short Chapter (IX) details experiments in the digestion of wool in vitro, and the thesis ends with a discussion (Chapter X).

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PART III

PART I

CHAPTER I. THE INSECTS

A. The Important Species

Very few species of animals are able to utilize wool, hair or feather keratin as food. So far as is known this capacity is possessed only by three groups of insects, namely larvae of clothes moths and carpet beetles, and nymphs and adults of the chewing lice (Mallophaga) which infest birds (Waterhouse, 1953). The more important species of clothes moths and carpet beetles causing economic damage include the following:

Tineola bisselliella (Humm.)	- Webbing clothes moth
Tinea pellionella (L.)	- Casemaking clothes moth
Trichophaga tapetzella (L.)	- Tapestry moth
Anthrenus vorax Waterh.	- Carpet beetle
" scrophulariae (L.)	- Common carpet beetle
" verbasci (L.)	- Variegated carpet beetle
Attagenus megatoma (F.)	- Black carpet beetle

Of these, the webbing clothes moth (Tineola bisselliella probably causes more damage to textiles than the remaining species, although the hardy carpet beetles also cause extensive destruction under certain circumstances. Since most of the research on wool digestion has employed the clothes moth, further remarks are mainly confined to this insect, although comparative information on lice and carpet beetles is included where appropriate.

The life cycle of the clothes moth consists of an incubation period of 7-10 days, a larval stage of up to four years depending upon the nature of food and temperature conditions, and a pupal stage of 14-44 days (Hartley, Elsworth and Barritt, 1943). Under laboratory-culture conditions mature larvae are obtained in 5-6 weeks (Chapter VI). The egg, which is not attached by adhesive material to the fibres, weighs about 0.03 mgm., and the mature larva weighs about 8 mgm. Each large larva spins about 0.11 mgm. silk per week or about 1.7 per cent. of its body weight (Hartley, Elsworth and Barritt, 1943). This silk is principally used for building feeding tubes and a cocoon. The larvae are negatively phototropic (Titschack, 1922) and may even be killed by strong light (Reumuth, 1946). The optimal temperature and humidity for growth of the larvae are 80°F. and 50 per cent. respectively (Mosher, 1941).

The carpet beetle, in all stages, is a much hardier insect than the clothes moth. The following details of the life cycle of a carpet beetle (Attagenus piceus) have been taken from Hinton's (1945) monograph on the beetles infesting stored products. The life-cycle, from egg to adult, takes from about six months to three years, but the adult lives only about three weeks. The incubation period for the eggs is 5-24 days, but the larval stage which takes the longest time in development, consists of 7 to 9 instars of 8 to 43 days each, excepting the overwintering instars, which take 194-297 days.

The pupal period, again, is very short and may vary from 5-25 days. Under conditions of adverse temperature, humidity or food the larvae may take up to three years for development. The larvae are free-living and do not spin silk webs like the clothes moth.

B. Nutrition and Ingestion of Food

Although the clothes moth and the carpet beetle are known by the damage they cause to keratinaceous materials, it is by no means true that they are obliged to live on these materials. Further, Titschack (1922) and Crowell and McCay (1937) have found that Tineola larvae do not grow to maturity on pure clean wool. Laboratory cultures of these insects may be kept in good condition if fed on a proteinaceous food such as casein with the addition of yeast, which supplies necessary growth factors. Nevertheless, under natural conditions these pests are found most often in association with keratinaceous materials. All substances containing keratin, such as wool, furs, hair, horn etc., may be attacked by clothes moths or dermestid beetles.

The statements have appeared in the literature that insects can digest silk fibroin (e.g. Scheer, 1948; Abderhalden, 1925) and that finished nylon is more attractive to some beetles than wool (Patton, 1945) but it is clear from the work of Reumuth (1946) that fibres such as silk, viscose,

rayon, cotton and nylon are not digested. He shows that insects can cause considerable damage to fabrics containing wool mixed with other fibres, but only the wool is digested and the others are excreted unchanged. Titschack (1922) has observed that self-spun silk also passes through the digestive tract unchanged. Waterhouse (1952d) has critically re-examined this question and found no indication of breakdown of silk fibroin by insects. He suggested that the previous observations of insects living on silk might be explained by the presence of substances contaminating the silk.

Sitowski (1905) first examined the relationship between the clothes moth and its food. When he observed large quantities of undigested fibres in the excreta, he came to the conclusion that materials adhering to the fibres other than keratin were the true nutritive elements in wool. Although the larvae do prefer soiled wool, Titschack (1922) showed that the wool fibre was actually decomposed in the gut and that the cortex lost its identity first, followed less quickly by the cuticle. He also demonstrated a higher sulphur content in the excreta than in the wool which suggested keratin breakdown. Fat was found to be unimportant for the development of the larvae whilst starch was only very slightly digested (Titschack, 1922).

It is well known that if the disulphide bonds of wool are broken, it becomes easily digestible (Geiger and Harris, 1942). Day (1951a) showed that wool with all disulphide

bonds intact is as easily digestible as reduced wool and it follows that the attacks by insects are not confined to damaged wool.

Studies of the nutritional requirements of the clothes moth have been made by Crowell and McCay (1937) and Fraenkel and Blewett (1946), and the following information has been contributed by these authors. The high proportion of cystine found in wool is not a prerequisite for a suitable food material since the addition of cystine to a casein diet did not markedly improve it. Neither fat nor fat soluble vitamins are necessary but part or all of the vitamin B complex is essential. Cholesterol appears to be necessary for growth, but inositol and p-amino benzoic acid are not required and the value of biotin is doubtful.

A description of the eating habits of the clothes moth is given by Titschack (1922) and Reumuth (1946) made the following observations. The young larva is provided with very strong mandibles which can open to about 90 μ so that fine fibres (less than 90 μ in diameter) are more susceptible to damage than are thick fibres. Generally the top of the fibre is bitten off, but where the fibre is too thick it is bitten through, thus destroying the nap on a fabric.

C. Conditions in the Insect Gut

1. Histology and Tracheation

The alimentary canal of the clothes moth larva is

simple and straight, and may be separated into a short narrow oesophagus, a midgut which forms about two-thirds of the total length and a hindgut. At the junction of the mid- and hindguts the malphigian tubules enter for the excretion of waste products, such as uric acid etc. (Reumuth, 1946).

Lotmar (1941) showed that there are two main types of cells in the midgut of the larva, i.e. columnar and goblet, and that the numbers of these varied along the length of the gut. Waterhouse (1952b) described the goblet cells of Tineola gut and found that they showed differences in form and were highly differentiated compared with the columnar cells. The histology of the Tineola larva is much more specialised than in other Lepidoptera so far examined (Waterhouse, 1952a). The larvae of dermestid beetles, however, have a simpler midgut and the epithelium consists of simple columnar cells together with some regenerative cells (Waterhouse, 1952d).

Day (1951b) studied the tracheation of Tineola larvae and showe d that, in contrast to many other insects, Tineola midgut had a poor tracheal supply. It is suggested that this condition probably contributes to the maintenance of a low oxidation-reduction potential in the midgut, although a number of other insects have an even less well-developed tracheal system. The tracheation of the gut of dermestid larvae (Waterhouse, 1952d) and bird lice (Waterhouse, 1953) is comparable with that of Tineola.

2. Passage of Food

Reumuth (1946) contributed the following information on the passage of food through the digestive tract of the clothes moth. The fibres are bitten off in pieces of roughly equal length depending upon the age of the larva, then are "packeted" in the foregut where no signs of digestion can be seen. When the fibres enter the midgut, the appearance of longitudinal striations between the cortical cells indicate active digestion. As the fibres move through the gut, both scale cells and cortical cells are digested, and finally, in the hindgut, no sign of fibres can be seen unless the larvae are eating rapidly and digestion is not complete. Day (1951a) showed that one of the first signs of digestion of wool in the midgut is the loss of birefringence. Various authors (Day, 1951a; Linderstrøm-Lang and Duspiva, 1936; Reumuth, 1946; Titschack, 1922) agree that the epidermis of the fibre, i.e. the scales, is digestible as well as the corticle cells. The fate of the epicuticle, which is reported to be unaffected by most reagents and enzymes (Lindberg, Mercer, Philip and Gralen, 1949) is unknown. This may explain the higher resistance of scales, compared with the rest of the fibre, to attack by enzymes (Hock, Ramsay and Harris, 1941) or by micro-organisms (Mandels, Stahl and Levinson, 1948).

Food materials have been observed to pass through the alimentary canal of Tineola within eight hours at 27°C. (Day, 1951a).

3. pH and Oxidation-reduction Potential

Sitowski (1905) and Schultz (1925) showed that the midgut of the clothes moth was alkaline, but the hindgut and rectum were acid. Later, Linderstrøm-Lang and Duspiva (1936), using the glass electrode, established that the pH of Tineola midgut was between 9.6 and 10.2 with an average of 9.9. Waterhouse (1952c) extended the work on the pH of Tineola gut using indicators and found that the foregut has a pH of 8.0-8.4, which is followed by the anterior midgut pH 8.5-9.0, the middle region of the midgut pH 9.8-10.0, the posterior midgut pH 7.8-9.0 and the hindgut pH 4.6-5.8. Waterhouse (1952d) has also studied the hydrogen ion concentration of the alimentary canal of dermestid larvae (carpet beetles) and found it to be pH 6.8-7.0 in the midgut and pH 4.4-4.8 in the hindgut. The pH of the midgut of bird lice is about 8.0 (Waterhouse, 1953). Although the pH of Tineola midgut is high when compared with vertebrate gut conditions, it is known that other insects which do not digest keratin (e.g. Bombyx mori (Shinoda, 1930)) have similar pH conditions. In fact this has been shown to be characteristic of all Lepidoptera (Waterhouse, 1949).

In view of the property of wool to become digestible when reduced the low oxidation-reduction potential in the intestinal tract of Tineola is of particular importance. Linderstrøm-Lang and Duspiva (1936) fed Tineola larvae with oxidation-reduction indicators and showed that reducing

conditions occurred in the mid-intestine only, and that the oxidation-reduction potential (Eh) was in the neighbourhood of -0.3 volts. Waterhouse (1952c) has recorded oxidation-reduction potential throughout the whole Tineola larval gut with the aid of indicators. The potential (Eh) is as follows: foregut -20 to + 32 mV., anterior midgut -200 mV., middle region of midgut -250 to -280 mV., posterior midgut -150 mV. to -190 mV. rising to above + 62 mV. The hindgut contents have a potential higher than +250 mV. Dermestid larvae have a midgut potential of about -190 to -250 mV., whilst the hindgut is again oxidising with a potential of +260 mV. The oxidation-reduction potential in the midgut of bird lice is about -200 mV. (Waterhouse, 1953).

No suggestions have been made as to the nature of the system or substances responsible for the maintenance of the unusually high pH in the gut of Tineola, and very little is known of the processes involved in the maintenance of the low oxidation-reduction potential. Concerning the latter, Linderstrøm-Lang and Duspiva (1936) have considered the following three processes as possibly responsible for splitting the disulphide bonds of wool:

(i) reduction by glutathione which is found in the midgut cells;

(ii) a denaturing action by urea, the presence of which can be demonstrated in the gut; and

(iii) the action of a dehydrogenase.

Linderstrøm-Lang and Duspiva (1936) have demonstrated the presence of -SH groups in the gut of Tineola larvae, but only in quantity after the larvae had been feeding on wool. If the gut is exposed to air on dissection, the nitroprusside test for -SH compounds is negative. The authors suggest however that a reducing agent probably free of -SH groups is present in the gut at all times as shown by oxidation-reduction indicators.

4. Enzymes

Very little work has been done on Tineola enzymes other than the protease group. Sitowski (1905) fed starch and cellulose and found them largely undigested indicating that amylase and cellulase are very weak or absent. Day (1951b) showed qualitatively the presence of xanthine oxidase in the gut.

Duspiva (1936) of the Carlsberg Laboratories used special micro-techniques for the study of the proteases of Tineola. He found that Tineola has a proteinase, aminopolypeptidase and a dipeptidase with pH-optima of 9.3, 8.1 and 7.5 respectively. He also worked with the proteases of Galleria (wax moth) and found very similar enzymes. Powning et al. (1951) found the proteinase of Tineola to have an optimum of pH 9.8-10. One difference between Tineola proteinase and others studied was its comparative insensitivity to inhibition

by sulphydryl compounds (Linderstrøm-Lang and Duspiva, 1936). These authors considered that this "special behaviour of the proteinase..... constitutes the adaption of the enzyme to its substrate". Powning, Day and Irzykiewicz (1951) have examined the effects on Tineola proteinase of a series of substances known to activate or inhibit trypsin or papain including sulphydryl compounds. It was found that sulphydryl compounds cyanide, thioglycollate, iodoacetate, ovomucoid, enterokinase and an inhibitor prepared from the intestinal worm Ascaris had no effect, but soybean inhibitor did reduce the activity of Tineola proteinase. Other insect enzymes tested in the same series were also inhibited by soybean inhibitor and unaffected by sulphydryl compounds, so these effects do not represent differences between Tineola and other insects. Changes of oxidation-reduction potential within the biological range had a very small effect on any of the insect enzymes tested. Tests on the stability of the enzymes to heating at their optimum pH showed that the insect enzymes and trypsin were similarly inactivated by heat, but papain was not sensitive under the conditions used. It was concluded that in general the insect enzymes including Tineola are similar, and that they resemble trypsin more closely than papain. No work has been published on the proteolytic enzymes of the carpet beetle.

Day (1949c) has demonstrated a weak alkaline phosphatase in the columnar cells of Tineola midgut, whilst work on the desulphydrase activity in insects is reported in

Chapter VIII. Titschack (1931) reported that larvae reared on sterile diets can still digest wool, thus eliminating digestion by micro-organisms; however, Linderstrøm-Lang and Duspiva (1936) pointed out that Titschack's work did not exclude all organisms.

D. Excretion

Clothes moth excreta generally occurs in the form of rather dry, friable pellets which are roundish in shape, with an exterior surface roughened usually by crystal formations. The colour usually follows the colour of the food materials. Uric acid has been known to be a component of insect excreta since 1846 (Davy, 1846), and its presence has been confirmed in clothes moth excreta and gut by the murexide reaction and its property of double refraction in the crystal form (Sitowski, 1905; Schultz, 1925; Titschack, 1922). Several authors (Babcock, 1912; Schultz, 1925; Hollande and Cordebard, 1926; Mosher, 1941) have analysed the excreta of Tineola or the related genus Tinea.

In view of the large amount of cystine in wool it is important to know how the sulphur is metabolised and excreted. Schultz (1925) examined Tinea excreta and found 0.76 per cent. sulphate sulphur and 1.43 per cent. insoluble sulphur. He found no cystine sulphur or ethereal sulphates and concluded that the cystine sulphur of the keratin appears essentially in the form of sulphate salt. In contrast to this, recent work on Tineola bisselliella and Attagenus piceus has shown that little sulphate sulphur and a considerable amount of cystine

sulphur is excreted by these insects (see Chapter VII).

If clothes moth larvae are fed wool treated with nickel salts they produce black excreta and black granules appear in the cells of the gut. The explanation of this appears to be the formation of nickel sulphide at the expense of the cystine (Waterhouse, 1952a), since the cystine content of the excreta of these insects is lower than that from normally fed insects (Chapter VII). A possible mechanism for the production of the sulphur from the cystine is described in Chapter VIII. This method of elimination of otherwise toxic metals provides an explanation for the failure of some insecticidal techniques when used in moth-proofing (Waterhouse, 1952a).

A small amount (about 3 per cent.) of urea is excreted by both carpet beetle and clothes moth. Some has also been found in the gut of clothes moth, but there is not sufficient to account for the breakdown of wool in the gut through the known reactions of urea with proteins (e.g. Hopkins, 1930) (see Chapter VII).

Decker (1942) has reported the presence of 0.01 per cent. leucopterin in excreta from Tineola larvae. Using the electron microscope, Lagermalm, Philip and Gralen (1950) have observed unusual "fish-net" structures in Tineola excreta, which are probably of insect origin, rather than derived from the wool fibres upon which the insects had been feeding.

CHAPTER II. THE FOOD MATERIALS OF THE INSECTS

A. Types of Food Materials - the Keratins

It has been mentioned in Chapter IB that the insects which damage keratinaceous materials can also live on proteins other than keratins. These insects have the unusual ability to digest unchanged keratin, a material which is normally considered resistant to breakdown (Day, 1951a), although clean scoured wool is not nearly as susceptible to attack as raw wool. Keratin has a very large and general distribution since wool and hair are stored in various conditions, i.e. raw, scoured, yarn, fabric, hide, etc., in stores of which the size varies from home to bulk. The keratins as a chemical group of proteins were characterised in the early days by their high sulphur content; however, they are more truly characterised by their cystine content, since some non-keratins contain large amounts of methionine (Section C of this Chapter).

B. A Physical Examination of Wool

Fibrous proteins such as keratin, myosin and fibrinogen are distinguished from other proteins by exhibiting the α and β types of structure when examined by the X-ray diffraction camera. The keratin fibres are represented by wool, kemp, hair, etc. and vary considerably in their histology and physical and chemical reactions. Histological examination of 600 genera showed that the fibre is made up of three regions,

each with different cell types, i.e. cuticle, cortex and medulla. The structure of these regions is typical of hair from a given genus while in certain cases specific characteristics can be detected (Stoves, 1948). Wool, commercially the most important of the keratins, shows the unusual properties of high elasticity, temporary and permanent "set", and resistance to enzymes which have led to many investigations on its physical and chemical structure.

1. Wool Follicle and Roots

The wool follicle contains groups of actively proliferating cells, richly supplied with nutrients. In the follicle the wool roots are formed, and these are eventually extruded and become "keratinised" to form wool fibres (Marston, 1946). Hock, Ramsay and Harris (1941) found that the wool root was soft and easily crushable, contained roundish cells with granular cytoplasm, and was non-birefringent, whilst the wool fibre was tough, contained fibrous elongated cells, and was birefringent. Chemical examination by these authors has indicated further differences between wool root and fibre. (Section C of this Chapter).

Marston (1946) suggested that the spindle-shaped corticle cells of the wool fibre were derived from columnar cells in the wool follicle, after being forced through the constriction at the top of the follicle. This theory of the formation of fibrils following orientation of the cells by shear has been criticised by Mercer (1949) on the grounds that

fibrils are found in other tissues, in which the phenomenon of shear cannot occur. He suggests that fibril formation is probably a property of a "corpuscular-type" protein to form "spontaneous end to end linkages by low energy bonds" (H bonds and salt linkages).

2. The Wool Fibre

The wool fibre may consist of three main regions: medulla, cuticle and cortex.

Medulla.- None of the fine wools contain a medulla, consequently not a great deal of work has been done on this hair fraction. The medulla contains large air spaces which may be up to 55 per cent. volume of the fibre while the medullary substance is about 5.5 per cent. (Barritt and King, 1931). The protein of the medulla is very rapidly dissolved by enzymes which distinguishes it from some of the other fibre components. It has been suggested that this is due to an almost entire lack of sulphur (Stoves, 1948).

Cuticle.- The cuticle is composed of scale cells, which, on examination by the electron microscope (Lindberg, Mercer, Philip and Gralen, 1949; Mercer and Rees, 1946a; Mercer, Lindberg and Philip, 1949), are shown to be composed of several layers of different chemical reactivity. They are as follows: (a) An epicuticle consisting of a thin continuous membrane on the surface of the fibre. This is extremely resistant to the effects of chemical reagents such as alkalis,

acids, sodium sulphide or enzymes and was first isolated by dissolving wool in sodium sulphide (Lindberg, Philip and Gralen, 1948). Lindberg, Mercer, Philip and Gralen (1949) conclude that the epicuticle is not keratin. (b) An exocuticle layer, described by Mercer and Rees (1946a) as a smooth surfaced layer easily damaged by chemical agents and enzymes. (c) An endocuticle which consists of the "flattened scale remnants resulting from tryptic digestion and is often referred to as cuticle" (Lindberg, Mercer, Philip and Gralen, 1949). This layer has a honey-combed structure and is relatively resistant to enzymes, etc. (Mercer and Rees, 1946a). (d) An inter-cellular cementing substance which binds the cuticle layers and corticle cells together and is probably identical with the exocuticle. This is digestible by trypsin so is probably a "keratin" of different chemical composition to the inner fraction of cuticle (Mercer and Rees, 1946b).

Cortex.- Speakman (1931) postulated, in the cortex, a structure of fibrils embedded in a viscous matrix as an explanation for the elastic properties of wool, but this was abandoned in favour of the "folded molecular chain" theory of Astbury and Woods (1933). However, more recent work (Bull and Gutman, 1944), favours the earlier theory, and this is confirmed by electron microscope studies of Mercer and others. These authors (Mercer and Rees, 1946b; Farrant, Rees and Mercer, 1947; Lindberg, Mercer, Philip and Gralen, 1949) have recorded the following information on the subcellular structure of

cortex. The cortex consists of longitudinally aligned fibrils embedded in an amorphous "matrix" material and the anisotropy of the fibre and the orientated X-ray patterns are probably due to these fibrils. The fibrils (1000-2000 Å wide) show further subdivision into "protofibrils" of fairly uniform size (100 Å wide by 2000 Å long), which appear to be the ultimate fibrous units. These are composed of particles of average diameter 110 Å and it is concluded that these are keratin molecules linked together to give crystals.

Cementing materials.- A large proportion of the wool fibre consists of the "subcellular matrix" and the "intercellular cement". These materials appear to be amorphous but they possess the property of producing threads when stretched (Mercer and Rees 1946b). Farrant, Rees and Mercer (1947) consider that the "matrix " material consists of a keratin which has little tendency to order.

3. Effects of Nutrition on Fiber

Marston (1946) has made the following observations. Nutritional disturbances, such as deficiency of vitamin B or cobalt, lead to failure of appetite in the sheep and thus influence the rate of wool growth. Vitamin A is concerned with the production of soft keratins rather than wool, and copper directly influences the hardening of keratin and is responsible for the crimp. Sullivan, Hess and Howe (1940) found that

maintenance-fed lambs produce wool with approximately 10 per cent. less cystine in the body third of the fibre than do full fed lambs. This shows that wool or keratin are not exactly reproducible materials in nature.

C. Notes on the Chemistry of Wool

1. Chemical Composition

Wool roots.- Various amino acids have been found in wool roots, also Krebs cycle enzymes, phosphatases, dehydrogenase, catalase and esterase (Ellis, Gillespie and Lindley, 1950). These authors also found 0.71 per cent. sulphur as sulphydryl and 1.55 per cent. as disulphide. Marston (1946) showed that the change from the root condition to the shaft took place when the -SH residues were oxidatively closed to disulphide bonds under the catalytic action of copper. Hock, Ramsay and Harris (1941) reported that the wool roots give a positive nucleic acid test, a positive -SH test, and the cell nuclei stain with haemotoxylin, whereas none of these reactions occurred in wool fibre.

Keratin fibres.- Many proteins contain sulphur, which occurs in methionine and cystine, and Block and Bolling (1947) give the following information on the content of these amino acids in some proteins. Blood proteins contain 2-3 per cent. methionine and egg proteins contain up to 5 per cent. Of the cystine-containing proteins, those known as the pseudokeratins

which include skin, spongin, neurokeratin etc. contain only a small percentage of cystine (about 5 per cent.) whilst the eukeratins consisting of horn, hoofs, nails, feathers etc., contain about 8-10 per cent. Sheep's wool contains about 11 per cent. cystine and human hair about 16 per cent. In all of the keratins only a very small percentage of the sulphur is in the form of methionine (about 0.5 per cent.). Table 1 lists the amino acid composition of several keratins and silk fibroin.

In addition to this, Marston (1946) gives 9.4 per cent. serine and 6.7 per cent. threonine (16.8 per cent. N basis) as the best available figures for wool.

Lindley (1947) has shown that the scales of wool contain a higher proportion of proline when separated from the corticle cells. Also, Geiger (1944) has found a higher proportion of cystine in the scales.

Amino acid arrangement.- Harris and Brown (1946) have discussed the importance of the type of amino acids and their arrangement in fibrous proteins such as silk and wool. These authors suggest that the differences in elasticity, and wet and dry strength, between the two fibres are due to the high percentage (more than 80 per cent.) of low molecular weight amino acids in silk, in contrast to the small proportion in wool (about 20 per cent.) (see Table 1). The low molecular weight amino acids in silk permit a "high degree of crystallinity" in the protein, but since "nearly 50 per cent. by weight of wool is in the side chains", crystalline regions would not be expected in this fibre. This should give rise to a very weak fibre; however, the presence of cystine-disulphide bonds contributes considerably to the strength of the fibre.

Table 1

Approximate percentage of amino acids in animal proteins - keratins
(Block and Bolling, 1947)
(Calculated 16 gm. nitrogen)

Amino acids	Hair	Wool	Horn	Feathers	Silk fibroin
Arginine	10.7	10.0	10.4	6.5	0.9
Histidine	1.0	0.7	1	0.7	0.06
Lysine	2.6	3.0	3.2	1.8	0.5
Tyrosine	3.1	5.1	4-6	2-3	11.1
Tryptophane	1.3	1.5	1.5	1	
Phenylalanine	2.7	3.9	4	5	
Cystine	15.9	11.1	7.3	9-10	0
Methionine	1-2	0.6			0
Serine					12.9
Threonine	6.4		5-6		1.3
Leucine	7-10	11*	15		0.8
Isoleucine	3-4		4-5		
Valine	3-6	4-5	5		2.7
Glutamic acid	12.2	15.3	18		
Aspartic acid	3.0	7.3	3		
Glycine	4-5	7	10		36.8
Alanine		4	2		22.2
Proline	8.5	9.3			1

* Includes isoleucine

Two types of carboxyl groups with different bond strengths have been demonstrated in wool, and it is suggested that they are associated with regions of different orientation in the fibre (Lemin and Vickerstaff, 1946).

Rydon (1951) has discussed a new "pleated sheet" structure for fibrous proteins and points out that the properties of native proteins cannot be explained solely on the basis of their formulation as straightforward peptide chains joined by disulphide and other cross-linkages. He states further that there is some regularity of structure which is independent of the side chains and must therefore arise from the coiling or folding of the peptide chains of the polypeptide backbones.

2. The Significance of Cystine

There has been some disagreement in the literature on the sulphur and cystine contents of wool, but it is now known that these are rather variable. Variations can occur along the length of the fibres according to the nutritional state of the animal (Bonsma, 1931) although no correlation has been found between cystine content and tensile strength, fibre fineness, crimping, scaliness, per cent. extension or whiteness (Rossouw and Bosman, 1939).

Geiger (1944) has shown that the cystine content varies in different cell types of the fibre. His sample of wool originally contained 12.2 per cent. cystine, 8.6 per cent. arginine, 6.1 per cent. tyrosine and 9.5 per cent. serine, whereas the scales separated by pepsin digestion contained 20.3 per cent. cystine, 4.8 per cent. arginine, 3.3 per cent. tyrosine and 11.2 per cent. serine. Speakman and Elliott

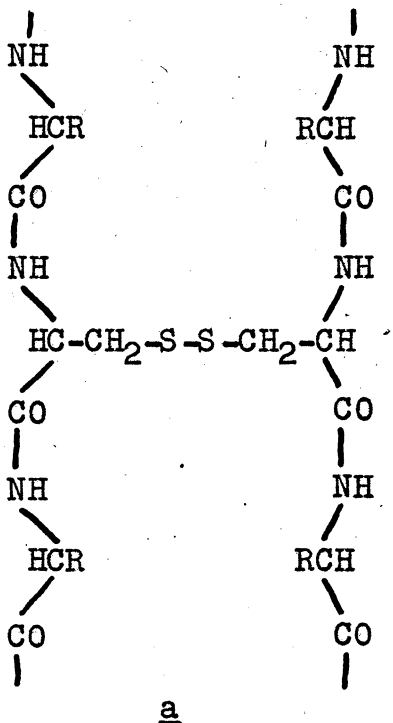
(1946) showed that cells separated from wool, after retting with trypsin, had a higher sulphur content and lower acid binding capacity, suggesting that the part of the fibre most resistant to retting, presumably the most crystalline part, had a high sulphur content and lower content of basic amino acids.

The medulla is devoid of sulphur and thus free of cystine and tests for other amino acids show that this part of the fibre is quite different from the scales or cortex (Becker and King, 1931) as follows:

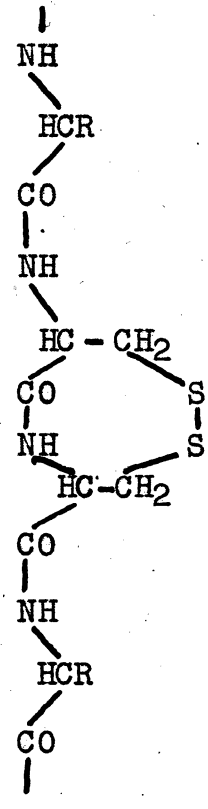
Scales	:	cystine (+) tyrosine (histidine ?) (-)
Cortex	:	cystine (+) tyrosine and histidine (+)
Medulla	:	cystine (-) tyrosine (and histidine) (+)

It was concluded from this that keratinisation is synonymous with cystinisation. This is supported by the observation that medulla is rapidly destroyed by enzymes, and, in contrast to the cortex and cuticle, is not affected by alkalis or reducing agents (Stoves, 1948).

Astbury and Street (1931) pointed out the presence of disulphide cross-links and Marston (1946) proposed several possible structures for the incorporation of disulphide linkages into peptide chains. Available evidence shows that (a) is prevalent in keratin.



a



b

The cystine of wool may be divided into fractions depending on its chemical reactivity. These differences probably depend ultimately on the active groups belonging to neighbouring amino acids in the polypeptide chains. Thus, Phillips (1946) has proposed four subfractions of wool depending upon its reactions with bisulphite and alkalis. Recently, by the use of hypochlorous acid and peracetic acid, it has been shown that cystine reacts again differently, and may be divided into two subfractions which are not related to Phillip's fractions (Alexander, Hudson and Cox, 1950). It is interesting to note that cystine in wool does not necessarily respond to treatments

which affect free cystine (Rimington, 1930).

3. Destruction of Keratin

Digestion by enzymes.- Keratin fibres are not highly resistant to the action of enzymes, except as Geiger, Patterson, Mizell and Harris (1941) suggest, when mechanically sound and chemically unchanged wool is treated with sterile solutions of crystalline enzymes. These authors showed the following effects:

(1) Crystalline pepsin has no effect in 10 days but the acid conditions contribute to attack in 30 days. However, pepsin attacks mechanically damaged fibres at the binding materials leaving the cortical cells and scales.

(2) Crystalline chymotrypsin digests the cortical cells and leaves the scales of reduced fibres but has no effect on untreated fibres.

(3) Papain has no effect on any wool derivative studied.

(4) Crystalline trypsin has no effect on untreated fibres: slight on reduced fibres.

It appears that enzymes do not digest the principal fibrous protein of mechanically damaged wool and that only the intercellular binding substance has been affected.

The best known example of enzymatic breakdown of keratin fibres is provided by the gut enzymes of the clothes

moth, which, according to Reumuth (1946), produce a loosening of the scale cells, digestion of the cortex then finally digestion of the scale cells. This has been confirmed by Day (1951a) who has demonstrated that the clothes moth can digest mechanically and chemically whole fibres.

The characteristic resistance of wool to enzyme action is in part due to the resistance offered by the cuticle scales to diffusion of enzyme into the fibre, and partly to the three-dimensional molecular structure formed by the disulphide cross-links. In connection with the cuticular resistance, Routh and Lewis (1938) and Routh (1940) found that, when wool was powdered by prolonged grinding in a ball mill it was changed chemically as well as physically with the result that it became partly digestible. It has also been shown that powdered keratin materials will support growth in young rats if supplemented with tryptophane, methionine, histidine and lysine (Routh 1942a, 1942b). This effect probably follows a change in the permeability of the cuticle after damage. The cut ends of fibres are always the first to show signs of attack by enzymes (Reumuth, 1946) which provides further evidence of the impermeability of undamaged fibres.

Goddard and Michaelis (1934) suggest that a definite physical pattern imparts to wool its resistance towards enzymes, since they showed that when wool was dissolved in alkaline reducing agents then reoxidised to an amorphous material, it became digestible by enzymes. If wool is reduced under neutral

or acid conditions so that the disulphide bonds are broken, but the fibre still retains its physical identity, it may be reoxidised to regain its original enzyme resistance. Again, if one reoxidises the Goddard and Michaelis solution of keratin at several different concentrations the resulting keratins have different enzyme resistances (Geiger and Harris, 1942). These authors explain these differences as follows. According to the dilution principle (Salomon, 1936), high molecular weight polymers result from highly concentrated solutions and low molecular weight polymers, dimers etc. from low concentrations. Application of this principle suggests that the formation of disulphide cross-links between different polypeptide chains should be favoured when reduced wool fibres are reoxidised because in the solid fibrous form the concentration of reactant (reduced protein) is very high. Reoxidation by the Goddard and Michaelis method in solution would favour formation of disulphide links between different parts of single folded chains as well as between different chains.

Chemically modified wools produced by reduction and alkylation are more stable to enzymic digestion (Brown and Harris, 1948; Geiger, Kobayaski and Harris, 1942; Harris, 1941; Patterson, Geiger, Mizell and Harris, 1941) (see Section 4 following).

Enzymic reduction of disulphide bonds.- Since the reduction of disulphide bonds in keratin is of importance in the digestion of keratin by insects (Chapter X), the following

discussion is included.

The reduction of disulphide bonds in substances rather simpler than keratin has been constantly of interest owing to the importance of -SH groups in a large number of biochemical processes. The chemistry of the oxidation and reduction of sulphur in these compounds is not well known (Oppenheimer and Stern, 1939) and even recently the oxidation-reduction potentials have been in doubt (Freedman and Corwin, 1949). There is some evidence that the potentials of these substances is dependent upon the concentration of the reduced form and the nature of the portion of the molecule other than the -SH groups (Smythe, 1944).

Elliott (1928) was interested in the reduction of glutathione by enzyme systems but found that neither glutathione or dithiodiglycollic acid are reduced by succinoxidase or xanthine oxidase. He concluded that the only natural method of reduction of disulphide bonds was by a thermostable insoluble material in muscles and certain other tissues (Hopkins and Dixon, 1922). Mann (1932) reduced glutathione by glucose plus a dehydrogenase from liver, and Meldrum and Tarr (1935) provided evidence for the occurrence of an enzyme in rat blood and yeast which utilized coenzyme II (triphosphopyridine nucleotide) for the reduction of glutathione. The same enzymatic action has been described in pea seeds by Mapson and Goddard (1951), and in wheat germ by Conn and Vennesland (1951). The latter authors showed an interesting.

substrate specificity as their purified preparations had no action on cystine. However, Nickerson and Romano (1952) have shown that reduction of the two substrates, glutathione and cystine, by a cell-free preparation from baker's yeast depended on the presence of coenzyme II (triphosphopyridine nucleotide) and coenzyme I (diphosphopyridine nucleotide) respectively.

Helferick (1950) has thoroughly surveyed the field of enzyme specificity and it appears that enzymes mostly depend upon the coenzyme for their reaction specificity, and the enzyme proteins (or apoenzymes) for their substrate specificity. In fact, about 35 different substrates enter reactions with coenzyme I and a series of enzyme proteins. It is difficult, with present knowledge, to reconcile with this concept the necessity to have two different coenzymes (although they have many similarities) for the reduction of such similar substrates as glutathione and cystine, since the specific reaction is the same, i.e. reduction of a disulphide bond. However, due to steric factors, the enzymic reduction of the disulphide bonds in such a large molecule as keratin is likely to be rather different from the reduction of these bonds in cystine or glutathione, i.e. it is probable that disulphide bonds will react in various ways depending upon the nearby side chains.

When investigating possible coenzymes it is not at all necessary to assume that the same coenzymes, as are found in vertebrate or plant enzymes, must operate in the insect systems. It is interesting in this connection that a

cytochrome (cytochrome χ) with different characteristics from those already described has been found in the midgut cells of the American silkworm larva (Cecropia), (Sanborn and Williams, 1950). Further, insects may contain lead as well as copper and iron as oxidation catalysts (Rona, Parventjev and Lippman, 1930). This shows that new and perhaps unusual coenzymes and enzymes may be responsible for the reduction of the disulphide bonds of keratin.

Action of alkalis on wool.- Schöberl and Rambacher (1940) have studied the effects of alkali on wool and stress the complications of the reaction. They found that boiling 0.5N NaOH dissolves wool in a few minutes and a large proportion of the wool sulphur appears as H_2S. Under anaerobic conditions at lower temperatures the fibres undergo no superficial changes, but, after hydrolysis with acid, free cysteine and H_2S were found. NH_3 also results from alkali treatment. All of this work was carried out with relatively high concentrations of NaOH above room temperature. Crowder and Harris (1936) also showed considerable changes in the cystine content of keratin after treatment with N/20 NaOH at 65°. However, Stoves (1942) found that the changes in keratin, as reflected in the degree of supercontraction after treatment, were dependent on pH, and that very little effect could be seen below pH 10 even after 24 hours at 35°C.

Schöberl (1941) has examined the effects of boiling water on disulphide bonds of wool. He shows that a considerable

amount of cystine is decomposed and NH_3 and H_2S are produced when wool is boiled in water, but it is a slow process taking several days. Horn, Jones and Ringel (1941) isolated the thioether lanthionine from wool treated with boiling sodium carbonate solution. Mizell and Harris (1943) consider, from their work, that alkali clearage of the -S-S- bond results in a rupture between S and C to yield dehydroalanine and a $-CH_2-S-SH$ residue which loses sulphur and combines with the dehydroalanine to form lanthionine ($-CH_2-S-CH_2-$). Aldehydes may also be formed under some conditions after treatment with alkalis (Schöberl, 1941).

Attack by micro-organisms.- It is known that many micro-organisms including several species of Bacillus and Pseudomonas can damage wool, and it is possible to "educate" some bacteria to degrade keratin in the absence of other sources of nutrition, e.g. B. subtilis, Chr. prodigiosum and P. vulgaris (Race, 1946).

It has been shown (Stahl, McQue, Mandels and Siu, 1949) that, after some time, Microsporum gypseum decreases the sulphur content of the solid residue and increases soluble sulphate sulphur. Also, inorganic sulphate is an end product of fungal destruction of wool, probably after the cystine is converted to cysteine - sulphinic acid. M. gypseum causes fision of the C-S link producing methyl mercaptan from methionine (Stahl, McQue, Mandels and Siu, loc. cit.). The nitrogen metabolism of the microbiological degradation of wool has also been studied (Stahl, McQue and Siu, 1950).

Action of light.- The action of ultra-violet light on wool produces a progressive decrease in strength and increase in swelling in acids and alkalis. This is associated with loss of sulphur and increased reducing power. The total sulphur in weathered fleece tips is 14 per cent. lower than protected tips. It is suggested that sulphydryl groups, aldehydes and H_2S are produced in a similar manner to the action of some alkalis (McMahon and Speakman, 1941; Mease, 1934; Rimington, 1930). The H_2S is readily oxidised to sulphates etc.

4. Modified Wools

Since Goddard and Michaelis (1934) dissolved wool in alkaline sodium sulphide and then reoxidised it, there have been attempts to reconstitute the wool fibre, with the purpose of producing a fibre more stable to the usual industrial processes, e.g. treatments by acid, alkalis etc. Patterson, Geiger, Mizell and Harris (1941) showed that when wool was treated with thioglycollic acid or other mercaptan, below pH 7.0, some of the disulphide bonds were reduced to sulphydryl groups without destroying the fibrous structure, and without detectable changes in other chemical linkages.

$$W\text{-}S\text{-}S\text{-}W + 2\ HSCH_2.COOH \rightarrow 2WSH + \begin{matrix} S\text{-}CH_2\ COOH \\ | \\ S\text{-}CH_2\ COOH \end{matrix}$$

Also they showed that, if this reduced wool was then treated with an alkyl dihalide, the sulphur atoms could be

linked through a short hydrocarbon chain to form new cross-links which were more stable than the original disulphide groups owing to their thioether nature.

$$2W\text{-}SH + (CH_2)_nX_2 \rightarrow W\text{-}S(CH_2)_n\text{-}S\ W + 2HX \quad (X = \text{halogen})$$

An improvement to this process followed the use of calcium thioglycollate instead of the acid (Geiger, Kobayashi and Harris, 1942). By this method, varying proportions of the cystine in the wool could be reduced according to the conditions. These processes are all rather expensive, but a much cheaper method has been described by Brown and Harris (1948) in which an inorganic reducing agent such as hydrosulphite and an alkylating agent such as formaldehyde are used together in a single bath. After this treatment, the fibres have better mechanical strength and greater resistance to alkalis, reducing agents etc. In a single-bath technique there is a greater chance that the new cross-links occur in the positions of the original ones, thus explaining the greater stability of the fibres after this treatment.

Freney (1946) has reviewed the literature on the chemical modification of wool as used in shrink-proofing processes.

CHAPTER III. MOTH-PROOFING

A large volume of literature has accumulated on the subject of moth-proofing, but only a few of the many substances suggested (see reviews of Roark, 1931, 1933, 1936) are used industrially. There are three main types of treatment as pointed out by von Bergen (1946):

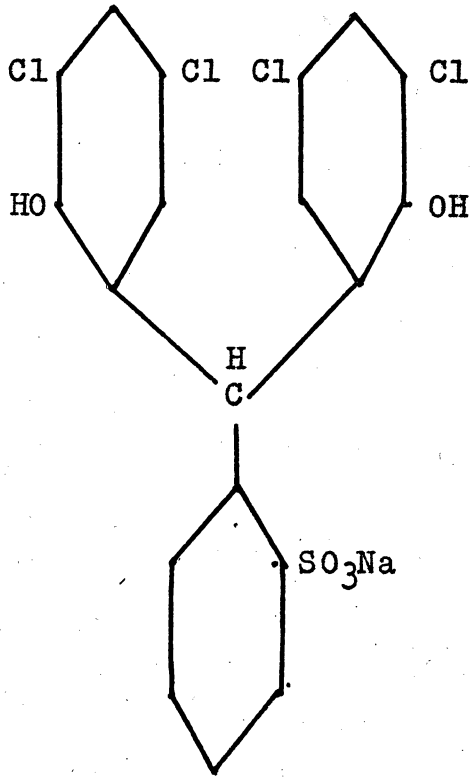
(a) Storage in an atmosphere unsuitable for moth life. This involves storage in a sealed container with a volatile substance such as naphthalene or paradichlorbenzene. This is effective only as long as the vapour concentration is high.

(b) Insecticide sprays containing pyrethrum, DDT etc. This method produces only a limited and comparatively temporary effect; also penetration of some materials is difficult.

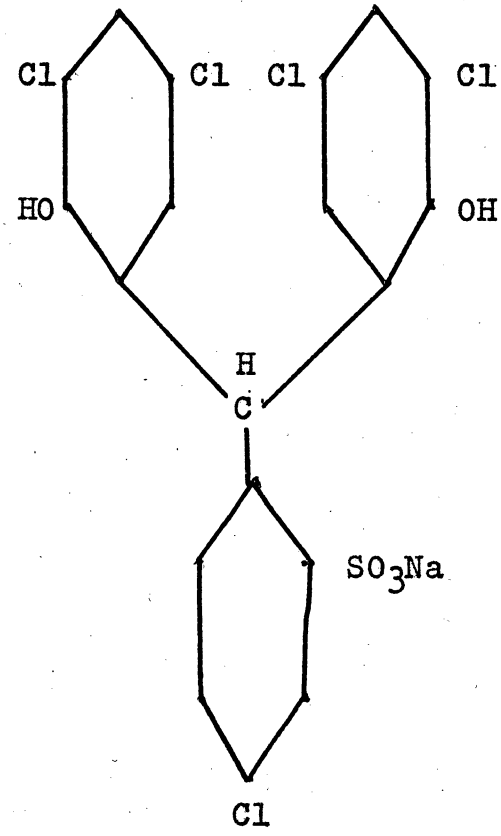
(c) Addition of a repellant or toxicant to the wool. This includes the fluorides such as sodium and potassium fluoride and acid fluorides, also silicofluoride. Chromium fluoride is more wash-resistant than others but is coloured. Pentachlorphenol is good but is washed out in laundering or dry cleaning. Nitro compounds, such as 2:4 dinitronaphthol are effective but highly coloured. DDT can be applied in dry cleaning process but does not withstand washing or dry cleaning. Gammexane has not been used extensively

but would be similar to DDT.

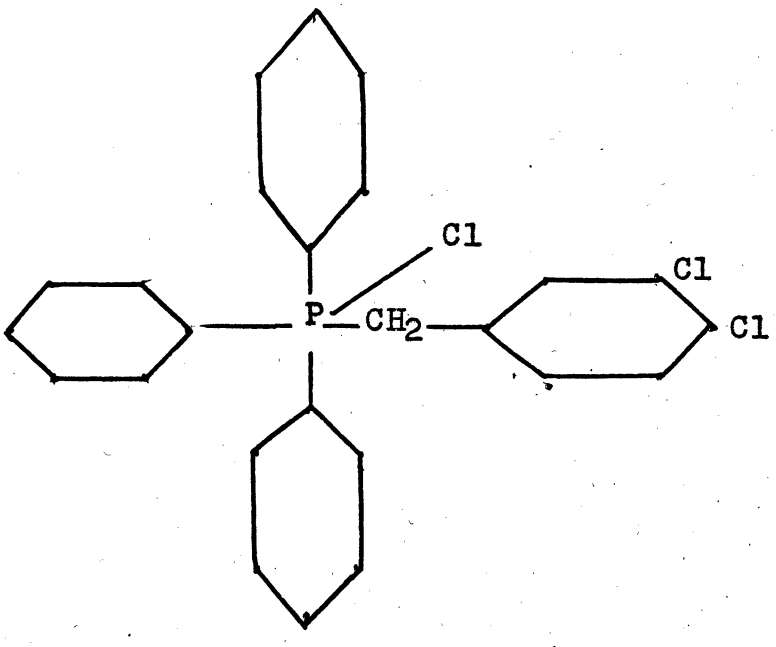
The Eulans developed by I.G. Farbenindustrie and Mitin FF (Geigy) are claimed to be very effective, and represent a departure from the usual moth-proofers, as they are substantive to wool and act like colourless dyes (Crossley, 1946; Lesser, 1949). These are fast to light, washing, dry cleaning etc. and are non-toxic, and may be applied during the normal processing of wool at the rate of about 3 per cent. on the fibre. The chemical structures of some Eulans are shown below (Crossley, 1946) together with that of Mitin FF (Burgess, 1949).



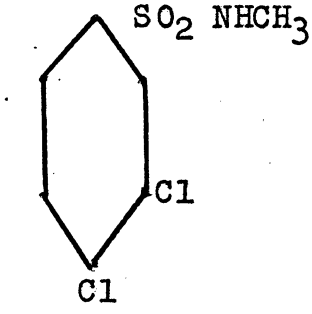
Eulan New



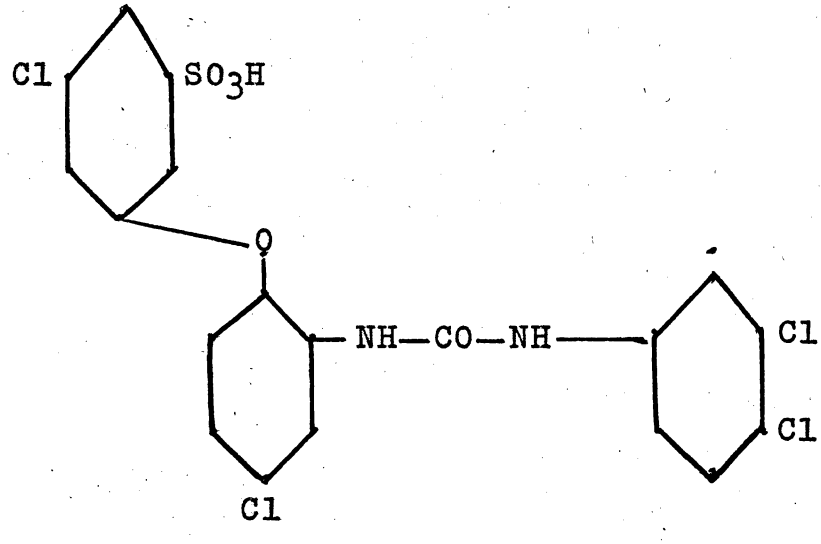
Eulan CN Extra



Eulan NK



Eulan BL



Mitin FF

Luttringhaus (1948) suggests that Eulan BL, a sulphonamide, may act on the digestive enzymes in a similar manner to the action of sulpha drugs on bacteria, and that Eulan NK may act as a cation-active agent on anionic digestive enzymes. Burgess (1949) and von Bergen (1946) consider

Mitin FF as a stomach poison, which is capable of killing young carpet beetles and repelling older ones.

It has been suggested that the use of resins to render the fibres resistant to mechanical disintegration may also be effective in moth-proofing (Powers, 1948), but they have not been found effective so far (Day, M.F., unpublished). Also the incorporation of toxic metals such as mercury, lead, copper etc. into the wool would appear to be ineffective as a moth-proofing technique, since the larvae seem to be able to use excess sulphur from the wool to convert the metals to harmless insoluble sulphides (Waterhouse, 1952a and see Chapters VII and VIII).

A fourth method of moth-proofing, which has not been used commercially to any extent, is the chemical modification of the wool fibre, at the molecular level, to make it resistant to digestion by the clothes moth. The chemistry of this is as follows. The disulphide bonds are relatively weak links in the three-dimensional lattice structure of the keratin molecule, and it is presumably by reduction of these that the clothes moth is able to digest the wool. Harris and his colleagues (Harris, 1941; Geiger, Kobayashi and Harris, 1942; Patterson, Geiger, Mizell and Harris, 1941; Brown and Harris, 1948) strengthened the disulphide bonds to render the fibre more stable to chemical treatments. They did this by first reducing them, and then reacting with alkyl halides to add short hydrocarbon chains between the sulphur atoms. It followed from the work of Linderstrøm-Lang and Duspiva (1936) that this treatment

should produce a moth-proofing effect, since the reconstituted bis-thioether linkages would be more resistant to reduction than the original disulphide linkages. The resistance obtained by this treatment is recorded in Table 2 (Geiger, Kobayashi and Harris, 1942).

Table 2

Effect of "Harris" treatment on insect damage to wool
(Geiger, Kobayashi and Harris, 1942)

	Cystine content of wool %	% weight loss after exposure to moth larvae	Excrement from Black Beetle larvae mgm.
Untreated wool	12.2	15.5	52
Modified wool	10.0	8.0	45
	6.5	0.3	21
	4.0	2.9	34
	2.0	0.1	23

The apparent moth-proofness increased with a decrease in cystine content, but the treatment was not completely satisfactory, particularly for carpet (Black) beetle. Although this is an expensive process and a cheaper modification has been described (Brown and Harris, 1948), little further work has been done on the moth-proofness derived from this process.

CHAPTER IV. SUMMARY OF PART I

A great deal of knowledge has been collected on the physical and chemical properties of wool. This is mostly of industrial interest in the improvement of dyeing and anti-shrink properties etc., but until the use of the electron microscope, the physical structure of wool was not properly realised.

Wool was not considered to be affected by enzymes but it is now known that the keratin fibres consist of several physical components of widely varying resistance to enzyme action and of different chemical composition. Any general conclusions derived from studies of the whole fibre are therefore of limited application.

The resistance of wool to enzyme action depends upon at least two factors:

(a) At the histological level.- The inert "non-keratin" epicuticle on the outside of the fibre which prevents diffusion. This can be damaged or removed, thus allowing the fibre to disintegrate after digestion of some of its components (Chapter 2B).

(b) At the molecular level.- The close packed three-dimensional lattice of the keratin molecule in which cross-links between the polypeptide chains are formed by the disulphide bonds of cystine. These may be broken by reduction when digestion by an otherwise inactive enzyme may proceed (Chapter 2C).

Very few animal species are able to digest keratin materials. The clothes moth, which has received most attention, can digest intact wool, and the process of degradation is very rapid. The whole fibre is digested, with the possible exception of the epicuticle, the fate of which is unknown. Carpet beetles and certain chewing lice can also digest keratin; however, no animals can live on keratin alone (Chapter 1A and B).

In Tineola the first signs of digestion can be seen in the midgut of the larva. This mudgut is alkaline (about pH 10), is probably nearly anaerobic, being poorly supplied with oxygen, has a low red-ox potential (about Eh -300 mV.), contains -SH groups and a proteinase. The gut of carpet beetles and keratinivorous chewing lice is also poorly tracheated, and also possess a low oxidation-reduction potential (about Eh -200 mV.), but the pH is not alkaline (c̄ pH 7) (Chapter 1C).

The proteinase of Tineola gut has a high optimum pH which corresponds to the high pH of the gut in which it is found. This high pH optimum of Tineola proteinase is not unique, however, as other Lepidoptera such as Bombyx are similar in this respect. Tineola proteinase is not greatly affected by the presence of sulphydryl compounds which can be demonstrated in the midgut, but other insect enzymes react similarly on treatment with sulphydryl compounds. The sulphydryl compounds found in Tineola gut are probably cysteine or cysteine peptides from the wool, although sulphide gives a positive nitroprusside test and should be considered together with cysteine,

glutathione etc. These sulphydryl compounds are only present in quantity when the larva has been feeding on wool, and they are rapidly oxidised if exposed to air on dissection of the gut, (Chapter 1C).

Nothing is known of the substances responsible for maintenance of the high pH in the gut, but very little digestion of wool by Tineola proteinase can be seen, in vitro, even at high pH, thus apparently excluding the direct action of alkali (Chapter 2C). It is not clear whether larvae reared on sterile diets can still digest wool (Chapter 1C).

Tineola has been shown to have a proteinase with no special properties, different from that of other insects, which may account for its ability to digest keratin. However, the presence of a reducing agent in the gut, together with a proteinase capable of action under these conditions, constitute a digestive system which has been called a "keratinase". This enzyme, in common with others, cannot act without the intervention of a reducing system, and the latter therefore becomes the key to the problem of insect digestion of keratin. Linderstrøm-Lang and Duspiva (1936) suggested that there are three possibilities for the identity of the reducing agent:

(1) Glutathione which is found in the midgut cells.

(2) A denaturing action by urea, the presence of which can be demonstrated in the gut.

(3) The action of a dehydrogenase.

The first of these was ruled out by these authors because of

the very small quantity of glutathione in the gut. The significance of urea is discussed in Chapter VII and it is shown that the amount present could have very little, if any, effect upon the wool. It seems possible that a hydrogen "transferase" could be responsible for the reducing action on the gut. It would be expected under these conditions that the wool would accept hydrogen by enzyme transfer from a donor which would at the same time be oxidised. The clothes moth excretes a high proportion of uric acid indicating that perhaps xanthine oxidase is the means of hydrogen transfer to keratin. In this connection, however, it is interesting to note that certain disulphide compounds are not reduced by succinoxidase and succinic acid or xanthine oxidase and hypoxanthine (Elliott, 1928). The possible importance of other less usual enzyme systems, such as cysteine desulphydrase (Fromageot, Wookey and Chaix, 1941) should not be overlooked (see Chapter VIII).

Although it has been shown that Tineola proteinase cannot digest natural wool in vitro this test has not been carried out under the same anaerobic conditions as presumably exist in the insect gut. Harris and Crowder (1936) have shown that the reaction of alkali with keratin to produce SH groups is more pronounced under anaerobic conditions, emphasizing the importance of lack of oxygen in these reactions. The extreme rapidity with which the wool fibres disintegrate in the larval gut indicates a very active digestive system.

Very little chemical work has been done on the carpet beetle which is a voracious eater and more hardy than the

clothes moth. Some information on the composition of the excreta and the desulphydrase activity in these insects is presented in Part III.

The most satisfactory practical approach to moth-proofing, at present, appears to be the use of Eulans or Mitin FF which are substantive to wool from aqueous baths, and have little effect on the physical characteristics of the wool. These do not have universal application, however, and are useful mainly in large-scale treatment in the textile mills. Another method which shows promise is the stabilisation of the cross-links between the peptide chains of keratin by modifying the keratin molecule. None of these treatments however are entirely satisfactory as they have no repellent action and it is known that clothes moth larvae and carpet beetles can cause considerable damage before they are killed (Chapter III).

PART II

CHAPTER V. TECHNIQUES

A. Chemical Methods

1. The following methods were employed for the work reported in Chapter VII. Dry matter content: dried to constant weight at 105°C. Water-insoluble matter: excreta heated with water on boiling water bath for one hour, filtered and dried to constant weight at 105°C. Ammonia and urea: urease method of Hawk, Oser, and Summerson (1947, p.822). The xanthydrol method of Engel and Engel (1947) was used as a check on the urea figures. Nitrogen: micro Kjeldahl. Amino nitrogen: micro titrimetric ninhydrin method of Van Slyke, MacFadyen and Hamilton (1941) modified by using Thunberg tubes instead of the recommended U-tubes and flasks, the barium hydroxide being held in the hollow stoppers. Creatine and creatinine: alkaline picrate method of Folin and Wu (1919) using tungstate filtrate. Creatine hydrolysed with HCl in an autoclave at 20 lb./sq. inch pressure for 20 minutes. Total and soluble sulphur: Micro-Carius digestion followed by precipitation of benzidine sulphate (Niederl, Baum, MacCoy and Kuck, 1940). The precipitate was dissolved in hot N/50 NaOH and back titrated with N/50 HCl using neutral red as indicator. Sulphate sulphur: gravimetric as barium sulphate. Cystine: the colorimetric method of Shinohara (1935) using the reagent of Newton (1937).

Total cystine was estimated on the hydrolysate obtained after refluxing for four hours in 5N HCl. Sulphur dioxide: the method of Grant (1947) was applied. All figures in Chapter VII for the soluble constituents of excreta were obtained on hot water extracts with the exception of amino nitrogen, sulphate and cystine, in which cases dilute hydrochloric acid extracts were used.

Paper chromatographic analyses were carried out on Tineola excreta by chromatographing the water-soluble fraction, hydrolysed water-soluble fraction and hydrolysed whole excreta. For the hydrolysis a period of four hours refluxing with 5N HCl was employed. Phenol-water and acetone-water mixtures were used as solvents without the addition of NH_3, HCN etc. (Consden, Gordon and Martin, 1944), and ninhydrin, iodoplatinate (Winegard, Toennies and Block, 1948) and iodine-azide mixture (Chargaff, Levine and Greene, 1948) were used as test reagents.

2. Method for Estimation of Hydrogen Sulphide. The method for estimation of hydrogen sulphide used in the experiments described in Chapter VIII was devised to suit the particular requirements of the work, i.e. the measurement of very small quantities of H_2S produced by the action of a limited quantity of enzyme on cysteine under anaerobic conditions. The amount of H_2S that other workers (Desnuelle and Fromageot, 1939; Smythe, 1942) have estimated, has been of the order of 100-200 γ H_2S. Assuming that insect tissues possessed the same order of

activity as the mammalian tissues that these authors employed, it was calculated that the published methods would require much more insect material than could be considered. Accordingly it was decided to modify existing methods or to devise a new one, by which quantities of H_2S of the order of 10 γ could be estimated with reasonable accuracy.

This problem was divisible into two aspects:

(i) the quantitative recovery of H_2S from the enzyme mixture, and

(ii) the estimation of the H_2S thus obtained.

Smythe's (1942) method entailed carrying out the enzyme action in Warburg vessels under nitrogen, and recovering the H_2S by diffusion and adsorption in cadmium acetate, followed by iodine titration of the precipitated cadmium sulphide, after acidification. Desnuelle and Fromageot's (1939) method consisted of carrying out the enzyme action in special vessels under nitrogen or carbon dioxide then, after acidification, washing the H_2S by a stream of carbon dioxide into a receiver containing zinc acetate. The H_2S thus recovered was estimated by measuring the amount of methylene blue produced by the reaction of H_2S with p-amino dimethyl-aniline sulphate in the presence of iron (Almy, 1925). The method finally adopted was somewhat of a compromise between those outlined above and some details of the work involved in arriving at this method follow.

There are many methods available for estimation of H_2S, for example, spectrophotometric as bismuth or cadmium

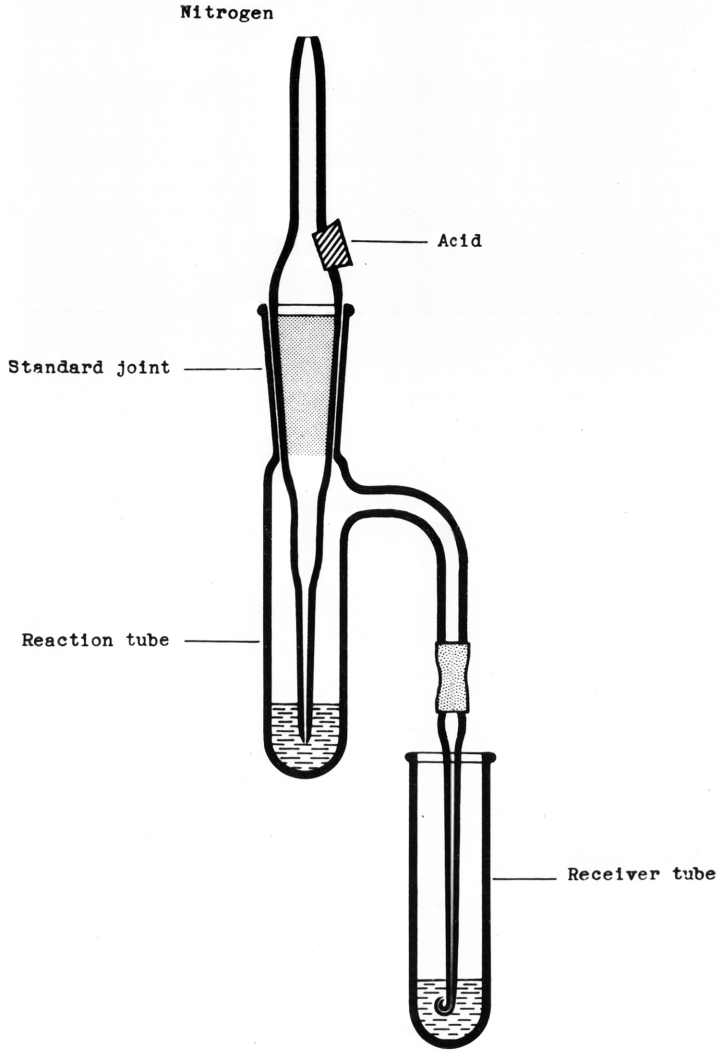


Fig. 1.- Apparatus used in aeration technique for estimation of hydrogen sulphide.

sulphide (Field and Oldach, 1946), titrimetric with sodium hypochlorite (Dunicz and Rosenquist, 1952), titrimetric with iodine (Smythe, 1942) or spectrophotometric as methylene blue (Fogo and Popowsky, 1949). Of these the iodine titrimetric method was chosen for trial in the first experiments.

Warburg apparatus (as used by Smythe, (1942)) was not available, so an attempt was made to use the following aeration technique. In the reaction tube shown in Figure 1 was placed 0.5 ml. diluted standard Na_2S solution (equivalent to about 3 H_2S γ/ml.); in the receiver was placed 0.5 ml. 2 per cent. zinc acetate solution (prepared by neutralising acetic acid with zinc oxide and filtering). Pure nitrogen (prepared by passing cylinder nitrogen through heated copper in a silica tube at about 500°, care being taken to avoid the use of rubber tubing) was passed into the sulphide solution. 0.5 ml. 6N H_2SO_4 was added through the stoppered opening in the nitrogen inlet tube and the released H_2S was swept into the receiver. A simple jet was used for bubbling the gases in the receiver rather than the modified one shown in Figure 1. To the contents of the receiver tubes were added 0.5 ml. 0.5 N HCl and, with the aid of a wash-out micropipette, 25 microlitres of $^N/_{10}$ standard iodine solution. This was then titrated with $^N/_{10}$ $Na_2S_2O_3$ using a 50 microlitre microburette of the Linderstrøm-Lang type (with a Kirk vibrating stirrer) and adding starch indicator towards the end of the titration. Full recovery of the added sulphide was not obtained and the results were inconsistent.

Results of simple tests using slow and rapid titrations of iodine (Table 3) led to the suggestion that the iodine may be evaporating during the titration. This seemed likely as it might be expected that the iodine would not be as soluble in the comparatively highly concentrated salt solutions (zinc acetate and cadmium acetate). This phenomenon has been noted by Kirk (1950).

Table 3

Effect of salts on microtitration of iodine

Titration of 50 μl. N/50 I_2 with N/50 $Na_2S_2O_3$ with addition of the following salt solutions:		
Salt solution	ml. $Na_2S_2O_3$	
	Slow titration	Rapid titration
0.5 ml. 10% zinc acetate	45.2	49.9
" " " "	42.0	49.9
" " " "	42.4	49.8
0.5 ml. water	50.0	50.2

This was further confirmed by mixing during the titration with nitrogen bubbles instead of an electrically vibrated glass rod. The slow titration equalled 33.2 μ l. N/50 $Na_2S_2O_3$ while the rapid titration equalled 48.2 μ l. However, even when the titration was modified so that $Na_2S_2O_3$ was titrated with iodine, losses still occurred.

There seemed to be several possibilities to account for these losses. Fresh sodium sulphide was prepared from pure

H_2S and NaOH in case titratable but non-volatile impurities were present. A double absorber unit was tried to test the possibility of inefficient absorption. The acid added to the sulphide was varied and the aeration with N_2 was varied in speed. None of these procedures improved the recovery of H_2S.

At this stage the titration technique was abandoned in favour of the methylene blue method of Almy (1925). This method is (a) simpler; (b) requires fewer accurate measurements and no standard solutions of doubtful stability; (c) takes less time and (d) eliminates the personal factor in watching for end-points. Details of this method are given at the end of this Section. In all of this work, the term D_{670} refers to optical density at 670 mm. in an 8 x 8 mm. tube, on a Coleman Junior spectrophotometer. It was quickly seen that good results could be obtained directly from standard solutions but in the aerated samples there were still losses amounting to 30 per cent. at 1.5 γ H_2S and 10 per cent. at 6 γ. It was thought that some loss may be due to inefficient absorption so a new type of more efficient microbubbler tip was designed (Fig. 1) (see Appendix); however, an apparatus fitted with the modified bubbler tips again gave disappointing results.

It was concluded after further work that the aeration technique was unsuitable for the recovery of the very small amounts of H_2S anticipated, possibly because of oxidation by very small amounts of oxygen inadvertently introduced during the process. It was felt that certain advantages could be obtained by using a diffusion technique, particularly if this

could be employed in association with a vacuum, providing complete elimination of oxygen from the atmosphere of the diffusion units.

The requirements of a suitable diffusion unit for this work were one which could take enzyme mixture, absorbent solution, and acid for liberation of H_2S from the enzyme mixture, all in separate compartments. A further requirement was that the unit should be easily evacuated and sealed, and the acid should be transferable from its compartment to that of the enzyme mixture without breaking the vacuum. A large amount of enzyme work under anaerobic conditions is carried out in Thunberg tubes, and very satisfactory vacuum diffusion units were made from these by adding an extra limb to hold the NaOH for absorbing the H_2S. These units are illustrated in Figures 2 and 3.

An immediate difficulty with this kind of apparatus was the tendency for leaks to develop owing to the grease melting on the ground glass joints at 37°C. This was overcome by the use of "Lubriseal" [*] which has a melting point about 40°C. and thus does not flow at the temperature of the water bath.

A test was carried out to determine the time necessary for 100 per cent. absorption after diffusion through the vacuum. It was found necessary to gently rock the tubes in the water

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[*] "Lubriseal" is a proprietary product of A.H. Thomas Co. of Philadelphia, U.S.A.

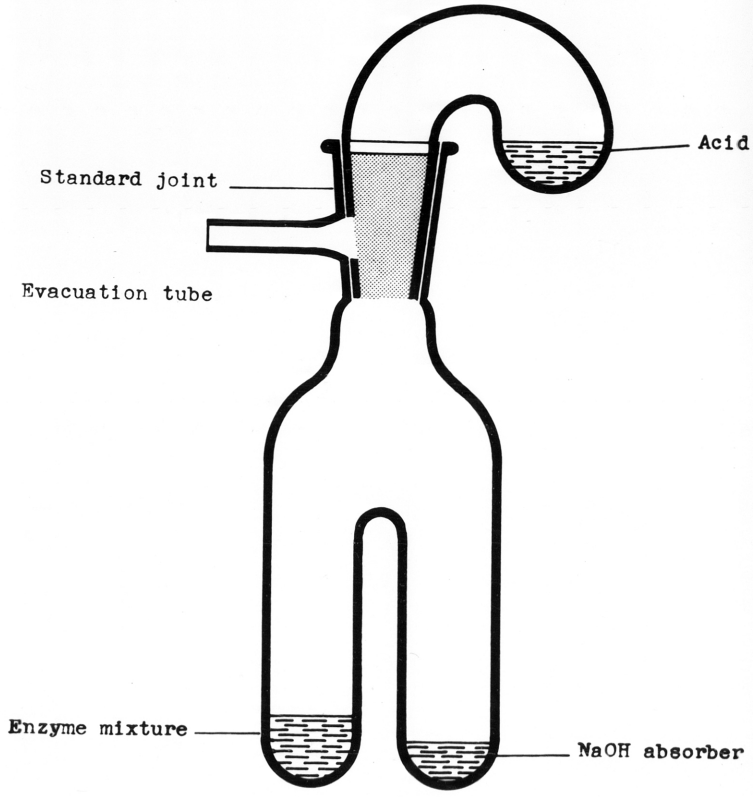

Fig. 2.- Vacuum diffusion unit for estimation of small amounts of hydrogen sulphide.

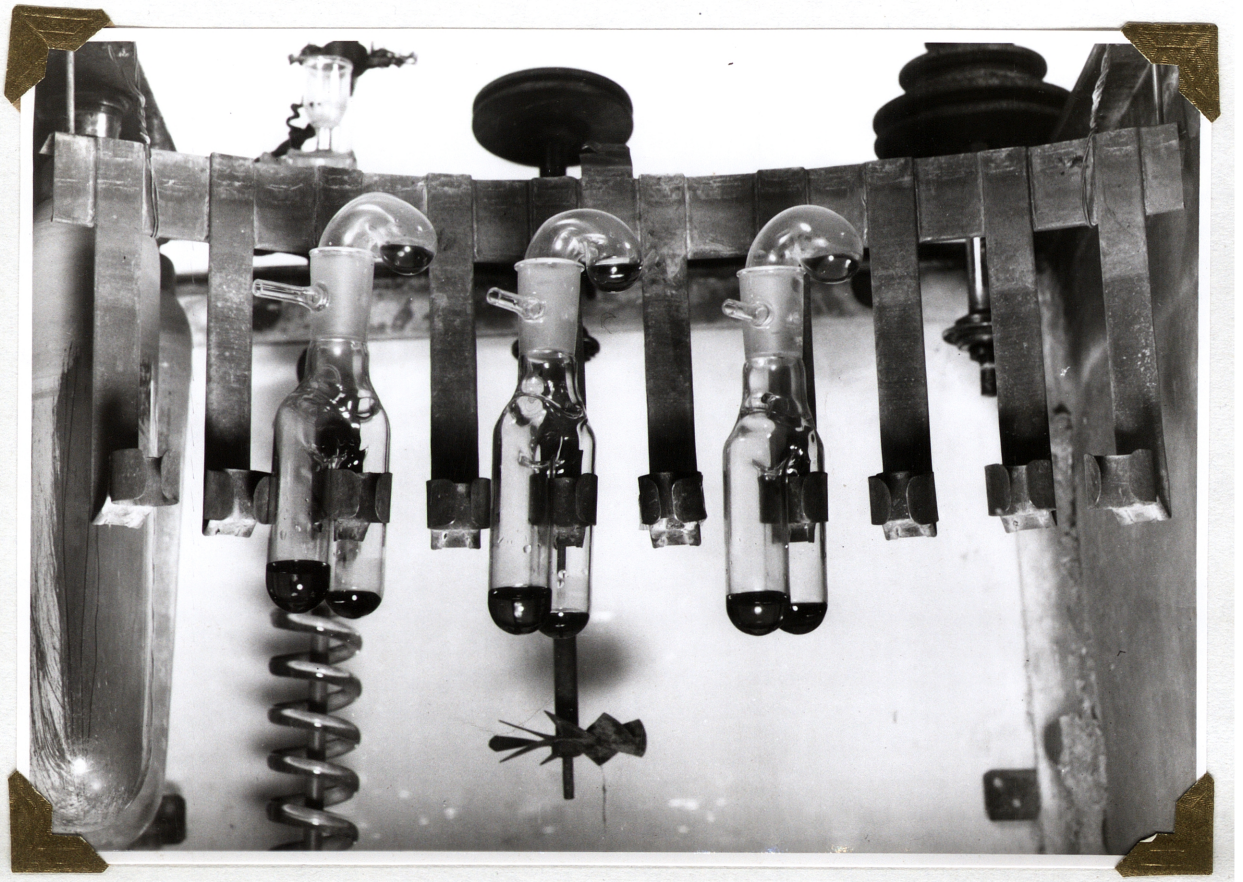

Fig. 3.- Vacuum diffusion units in water bath.

bath to cut absorption time down to a minimum. Using this technique, 30 minutes was sufficient for 100 per cent. absorption (Table 4).

Table 4

Recovery of H_2S

(20 γ H_2S diffused into 0.5 ml. 0.5N NaOH)	D670
10 minutes diffusion	.370
30 " "	.428
60 " "	.448
Standard solution not diffused (1)	.422
(2)	.429

Table 5 shows that the recovery of H_2S in the range 5 to 40 γ by this technique is within about ± 5 per cent. of the true value. The figures in Table 5 were obtained by placing various volumes of standard Na_2S solution together with 0.4 ml. 10 per cent. zinc acetate in the limb of the diffusion units normally containing enzyme mixture, or in a parallel series of 25 ml. volumetric flasks. After diffusion following the technique described later, the H_2S in the absorber solutions of the diffusion units, and in the parallel series of volumetric flasks (standards in Table 6) was estimated. The figures for the recovery of H_2S by the vacuum diffusion method are graphed in Figure 4.

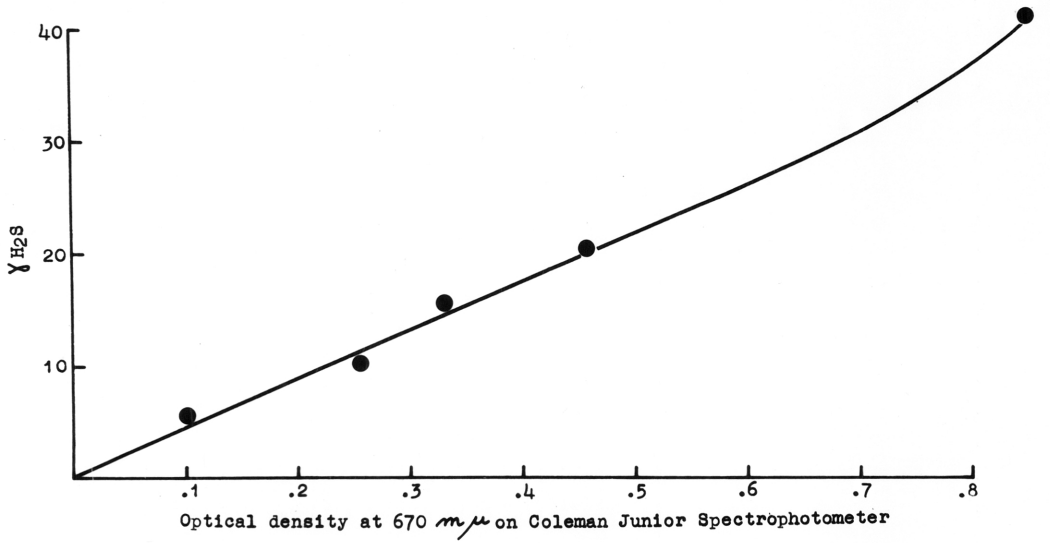

Fig. 4.- Standard curve for spectrophotometric estimation of hydrogen sulphide using vacuum diffusion units.

Table 5

Recovery of H_2S in vacuum diffusion units

γH_2S	Diffused		Standards		% difference from standard
	D_{670}		D_{670}		
5.2	.108 .103	.106	.118 .106	.112	- 5.4
10.4	.254 .250	.252	.240 .235	.238	+ 5.9
15.6	.326 .339	.333	.349 .339	.344	- 3.2
20.8	.456 .469	.463	.430 .472	.451	+ 2.7
41.6	.85 .84	.85	.84 .81	.83	+ 2.4
Blank 0			.002 .001		

Method

The method finally adopted is as follows: 2 ml. of sample solution containing sulphide (≡ 5-30 γH_2S) is placed in one limb of the vacuum diffusion unit (Fig. 2). 0.5 ml. 0.5 N NaOH is placed in the other limb and 0.5 ml. 3.6 N H_2SO_4 in the top (already prepared with Lubriseal). The unit is alternately evacuated and filled with pure nitrogen four or five times and finally evacuated, then the top is turned carefully to ensure a good seal; all solutions to be added to the units are

deoxygenated immediately before using. The unit is then completely submerged in a water bath at 37°C., allowed a few minutes to come to equilibrium, then the acid is tipped from the top into the sulphide solution. A gentle rocking motion, sufficient to renew the surface of the solutions frequently, is imparted mechanically to the rack holding the units. After half an hour, the units are removed from the rack and the NaOH in the receiver is transferred quantitatively to 25 ml. volumetric flasks.

The following details of the estimation of the sulphide by the "methylene blue" method are, with some slight modifications, taken from the method of Fogo and Popowsky (1949) and details of the reagents may be found in their paper. The contents of the 25 ml. flasks mentioned above are diluted to about 20 ml. with water then 2.5 ml. p-amino dimethyl-aniline sulphate reagent is added, mixed quickly and immediately followed by 0.5 ml. ferric chloride reagent. The stopper is replaced as quickly as possible and the solution mixed. After 20 minutes at room temperature (about 20-25°C.) the solution is diluted to the 25 ml. mark, and then the blue colour is measured in a Coleman Junior spectrophotometer at 670 mm. After about 15 minutes the colour is stable for hours, but it changes slightly overnight due probably to adsorption of methylene blue on the glass.

The standard Na_2S solution was prepared from

A.R. grade $Na_2S.9H_2O$ which had been washed with distilled water before use. The solution was standardised iodometrically, and only diluted with oxygen-free water immediately before use.

B. Technique for Estimation of Desulphydrase Activity

In the choice of a method for the estimation of the activity of any enzyme it is desirable to select the method of highest sensitivity. Sensitivity in this sense includes specificity and accuracy. A method which is highly accurate but which at the same time measures the activity of other enzymes of entirely different function is sometimes unavoidable. Materials, other than H_2S, can be estimated as a measure of desulphydrase activity, i.e. NH_3 and pyruvic acid (Greenstein and Leuthardt, 1944); however it is not possible to assume that the products of action of the insect enzymes are the same as those from the mammalian enzyme. Since the liberation of H_2S from cysteine was of prime importance it was desirable that the method for estimation of activity should depend upon the estimation of H_2S. Although H_2S may be removed from an enzyme mixture, e.g. by reaction with pyruvic acid (Smythe, 1942), the possibility of the production of large amounts of H_2S, from other sources than the added substrate, is small.

Other workers who have examined desulphydrase activity have found that more H_2S was recovered under anaerobic than under aerobic conditions (e.g. Smythe, 1942). This appears to

be due to the oxidation of the H_2S, in a secondary reaction, rather than any specific inhibiting effect upon the enzyme. In support of this, Smythe (loc. cit.) showed that the NH_3 and pyruvic acid production was not affected when the experiment was carried out in aerobic conditions, whereas the H_2S was considerably reduced. Because of these considerations, it was decided to carry out all work on the desulphydrase under anaerobic conditions. As it happens the method for estimation of small quantities of H_2S, as outlined in Section A of this Chapter, is ideally adapted for this purpose, as the solutions and apparatus in any case must be completely deoxygenated for satisfactory results.

The method for estimation of desulphydrase is as follows. Into one limb of a vacuum diffusion unit (Fig. 2) is placed 1 ml. enzyme solution, 1 ml. suitable buffer, 0.2 ml. 0.1M l-cysteine (freshly prepared daily) and 0.2 ml. water or other solution such as inhibitors etc. In the other limb and the top is placed the usual 0.5 ml. 0.5N NaOH and 0.5 ml. 3.6N H_2SO_4 respectively. All solutions are stored in Thunberg tubes after deoxygenating by evacuating while warm and replacing the atmosphere in the tubes with pure nitrogen. The diffusion units are evacuated and filled with pure nitrogen several times, then finally evacuated and immersed in the water bath for two hours at 37°C. After this period the usual technique for the estimation of H_2S is followed (Section A of this Chapter).

The effect of enzyme concentration on the yield of H_2S is shown in Figure 5. An enzyme concentration yielding

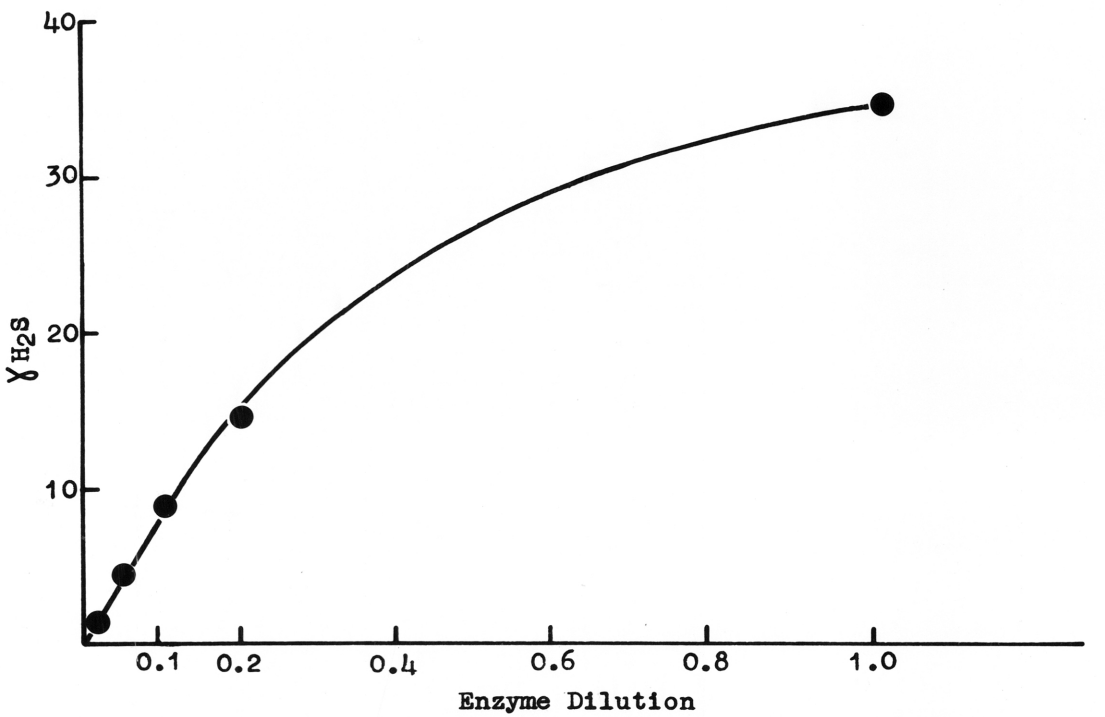

Fig. 5.- Effect of enzyme dilution on the production of hydrogen sulphide by desulphydrase.

lower than about 13 γ H_2S yields a straight line relationship, but above this the graph is not linear with the technique employed.

The effect of substrate concentration on yield of H_2S is reported in Table 6. Since the concentration of cystine used in the experiments in Chapter VIII was 8.3×10^{-3}M, it can be seen from Table 6 that the substrate concentration was adequate to give maximum activity, with an enzyme concentration yielding about 10 γ H_2S per test.

Table 6

Effect of substrate concentration on desulphydrase activity

Molar concentration l-cysteine	γ H_2S
1.66×10^{-2}	9.2
8.3×10^{-3}	9.8
4.2×10^{-3}	6.6
8.3×10^{-4}	2.0
1.66×10^{-4}	0.4
1 ml. Tineola enzyme, 1 ml. glycine buffer pH 9.08, 0.2 ml. H_2O, 0.2 ml. l-cysteine. Held in water bath at 37°C. for 2 hours.	

CHAPTER VI. MATERIALS

A. Insect and Other Animal Material

Cultures of Tineola bisselliella (Humm.) using a method developed by M.F. Day were maintained in the laboratory as follows. Adult moths are kept overnight in quart size jars (with wire gauze lids for ventilation) with a piece of pure woollen fabric upon which they lay eggs. The fabric with the eggs is transferred on the following day to a similar jar containing artificial larval food material, consisting of finely ground yeast and casein. After about 5-6 weeks, the larvae are mature and measure about 8-10 mm. long and up to 1 mm. in diameter. If not used, the larvae soon pupate and emerge as adults. All of this culture work is carried out in a room controlled at 80 per cent. relative humidity and 80°C. To prevent mites causing trouble in these cultures, an effective miticide "Dimite" * is dusted on to the lids of the culture jars.

The separation of the larvae for experimental purposes presents the problem of removing them not only from their food, but also from their feeding tubes. These are tough tubular structures, spun from silk, in which the larvae spend a great deal of their life. Fortunately the larvae are negatively phototropic and, as they become quite active if warmed slightly,

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* "Dimite" is a proprietary product of Sherwill-Williams Co. U.S.A.

it only requires warm conditions and a strong light to make them leave the tubes.

When required, the gut of the clothes moth larva may be dissected out by the following technique which is somewhat similar to that described by Reumuth (1946). With a pair of fine forceps and a scalpel the head and rectum are severed and then, if the larva is fairly large, the gut protruding from the severed ends of the body is gripped by the forceps and withdrawn. Although the use of a dissecting microscope is not necessary, the work is nevertheless very slow.

The analyses discussed in Chapter VII were carried out on excreta from clothes moth larvae, Tineola bisselliella (Humm.) which had been bred on white woollen fabric alone, or on dead beetles (Aphodius howitti), from larvae of the carpet beetle, Attagenus piceus (Oliv.) feeding on wool and from the potato moth, Gnorimoschema operculella (Zell.) feeding on potato tubers. The faecal pellets from Tineola and Attagenus were separated from the loose wool fragments, as well as possible, by a process of sieving and winnowing with a small air jet. Microscopic examination of the excreta showed that an excellent separation had been effected, and that the only wool fragments present in the samples were a small number actually adhering to the faecal pellets. The excreta from the other two insect samples were separated from lighter debris in the same way.

Insect material required for the urea and ammonia estimations in the gut was drawn from laboratory cultures of

Tineola bisselliella (Humm.), Locusta migratoria L., Agrotis infusa (Boisd.) and Tribolium confusum J. du V., and from field collected Aphodius, Melalonthid and Dynastid larvae. The insects used in the work on desulphydrase were as follows: Ephestia kühniella Zell., Tribolium confusum J. du V., Rhizopertha dominica F., Sitophilus granarius L., Sitophilus oryzae L., Oryzaephilus surinamensis L., Nasutitermes exitiosus (Hill), Coptotermes acinaciformis (Frogatt) and Musca domestica L.

The rat livers from which the preparation of mammalian desulphydrase was made, were taken from male laboratory bred rats after chloroforming.

B. Chemical and Other Substances Used.

All chemical reagents used in this work were of the purest quality available, mostly analytical reagent grade:

(a) Buffers - The buffers used in Chapter VIII are as follows:

Phosphate.- (pH 5.6 to 8.04 at 18°C. Sorensen) M/15 KH_2PO_4 and M/15 $Na_2HPO_4.2H_2O$ in varying proportions.

Glycine-NaOH.- (pH 8.18 to 12.45 at 37°C. Sorensen) 0.1N NaOH and 0.1M glycine (Eastman Kodak) in 0.1N NaCl in varying proportions.

Veronal-HCl.- (pH 7.00 to 9.20 at 25°C. Michaelis) 0.1N sodium salt of veronal and 0.1N HCl in varying proportions.

Borax.- (pH 9.08 at 37°C.) 0.05M solution of $Na_2B_4O_7.10\ H_2O$.

Histidine.- (pH 9.08 at 37°C.) 0.1M histidine hydrochloride monohydrate (Eastman Kodak) adjusted to pH with NaOH.

Most of the buffers were checked on a Cambridge pH Meter at 37°C., using a normal electrode or an "Alki" electrode where necessary.

(b) Inhibitors, Activators, etc.- All solutions of inhibitors and activators used in Chapter VIII were used freshly prepared and adjusted to the pH of the enzyme reaction with the aid of a pH meter, with the exception of the concentrated salt solutions which are discussed in Chapter VIII B 6.

(c) Substrates.- l-cysteine (E. Light & Co.), dl-methionine (Eastman Kodak), glutathione (B.D.H.), L(-) cysteine (General Biochemicals), dl-homocystine (General Biochemicals).

C. Enzyme Preparations

Owing to the uncertainty of effects of salts, glycerine etc. on the insect enzymes, distilled water only was used for extraction. All the insect enzyme preparations were obtained by first grinding the material well in a mortar; water was then added, followed by further grinding until the brei was as homogeneous as possible. After standing for half an hour, this was centrifuged at about 2,000 revs. for five minutes, mainly to separate large lumps and fat which rose to the surface.

A second extraction was carried out by adding more water, stirring and centrifuging. The two extracts were bulked and then spun again in a high speed semi-micro "angle head" centrifuge at 10,000 revs. for 10 minutes to give a solution fairly free of suspended material. It was found that preparations of Tineola gut enzyme were conveniently made in a Potter glass homogeniser followed by centrifugation. All extracts were kept under deep freeze conditions so far as possible because of the instability of the enzyme. In some extracts it was noticed that a considerable clearing and flocculation of residual suspended material occurred on thawing after deep freezing. This material was removed with little effect on the activity of the extract either by centrifuging or filtering. Before use in the tests a few drops of toluene and a drop of octyl alcohol were added to the enzyme preparations as a preservative and antifoam agent respectively. Smythe (1942) found that toluene could be used without effect on rat liver desulphydrase, and Fromageot, Wookey and Chaix (1941) showed that octyl alcohol also had very little effect.

The rat liver desulphydrase was prepared essentially as described by Smythe (1942). Some of the saline extract was purified further by shaking with chloroform (Smythe, loc. cit.). These preparations were also kept under deep freeze conditions.

The concentrated enzyme solution used in the tests on digestion of wool in vitro (Chapter IX) was prepared by homogenizing 20 gms. whole Tineola larvae with 20 ml. water

in a Waring Blendor with a few drops of octyl alcohol and toluene. This was centrifuged to yield about 10 ml. of enzyme solution, which was diluted with 10 ml. N/10 glycine-NaOH buffer of pH 9.08 (37°C.)

PART III

CHAPTER VII. THE SIGNIFICANCE OF CERTAIN EXCRETORY PRODUCTS OF THE CLOTHES MOTH *Tineola bisselliella* AND THE CARPET BEETLE *Attagenus piceus*

A. Introduction

An examination of the end products of digestion in keratin-feeding insects should provide valuable data for the study of the digestive processes in these insects. Although some analyses do appear in the literature, a number of questions, some assuming greater importance in the light of recent work, remain unanswered. The fate of the keratin sulphur, after digestion by insects, is of considerable interest as the presence of sulphur is essential to the structure and stability of the keratin molecule (Geiger and Harris, 1942). Babcock (1912) suggested that sulphur dioxide might be a metabolic product of clothes moth larvae feeding on wool, while Schultz (1925) concluded that the sulphur of keratin is excreted essentially as sulphate. The source of the sulphur required for the formation of metallic sulphides in *Tineola* gut (Waterhouse, 1952a) is also of interest.

Wool is chemically changed with formation of lanthionine after treatment with alkalis (Cuthbertson and Phillips, 1945), and recent work of Blackburn (1950) shows that wool thus treated becomes more easily digested by papain-bisulphite mixture than is normal wool. It was suggested that

digestion of wool in the alkaline Tineola gut (about pH 10, Linderstrøm-Lang and Duspiva, 1936; Waterhouse, 1952b) might depend to a large extent on this reaction. Linderstrøm-Lang and Duspiva (1936) drew attention to the presence of urea in the gut of Tineola and inferred that urea might be involved in the digestion of wool in the larvae through some denaturation of the keratin. No quantitative data however were presented with which to determine the significance of the amounts of urea found in the larval gut.

Results are presented in this chapter to show that the digestion of wool by clothes moths and carpet beetles gives rise to certain excretory materials, the presence of which gives added information of value in the interpretation of the processes of digestion of wool.

B. Experimental and Results

1. Nitrogen Excretion

For the purpose of this discussion, nitrogen excretion is taken to mean excretion of such materials as uric acid, ammonia, urea etc. Figures of ash and dry-matter content are included in this part (Table 7), whilst materials containing sulphur are treated separately in Part 2 of this Section (Table 9). All results are quoted to the nearest significant figures.

The three common nitrogenous excretory products, namely uric acid, urea and ammonia, are all present in Tineola,

Attagenus and Gnorimoschema excreta. The figures for the latter are quite low compared with those for Tineola and Attagenus probably owing to the relatively low protein content of the food of the potato moth. After the ingestion by an insect of a high sulphur-containing diet such as keratin, it might be expected that ammonia would be required to assist in the excretion of sulphate (Wigglesworth, 1931). A calculation from the figures in Tables 7 and 9, however, shows that only about 0.1 per cent. ammonia would be required to form ammonium sulphate with the small quantity of sulphate in Tineola excreta. Although the water-soluble nitrogen figures for both Tineola and Attagenus are of the same order (21.1 and 17.8 per cent. respectively), ammonia, urea and uric acid can account for as much as 86 per cent. of soluble nitrogen from Tineola but only about 17 per cent. from Attagenus. The low uric acid figure is notable in Attagenus and it was therefore thought that nitrogen might be excreted in the form of allantoin as in certain other insects, e.g. Lucilia (Brown, 1938). However, no allantoin was found and no further attempts were made to establish the identity of the nitrogenous excretory products.

Linderstrøm-Lang and Duspiva (1936) suggested that reduction of wool in the insect gut might be due to the presence of a hydrogen transferring enzyme such as the Schardinger enzyme. It seemed possible, in view of the high uric acid excretion in Tineola (Table 7) that xanthine oxidase, an enzyme responsible for the production of uric acid, might be involved.

While this may be true in Tineola it seems much less likely in Attagenus which has a low uric acid excretion although it digests wool readily. The 41 per cent. uric acid excreted by Tineola appears to be high, but it is not exceptionally so, as the dried urine of the blood-sucking bug Rhodnius contains 64-84 per cent. (Wigglesworth, 1932). The possibility still remains that dehydrogenase activity takes part in the digestive processes of some keratin-feeding insects.

Three per cent. of urea was found in both Tineola and Attagenus excreta when feeding on wool, and Tineola feeding on Aphodius (beetle) remains excreted 1.5 per cent. (Table 7). On general grounds one would not expect urea to be a "favourable vehicle" for the excretion of nitrogen in these keratin-feeding insects because of the relatively dry diet (Wigglesworth, 1931). However, it is clear that an appreciable amount of urea is a normal constituent of the excreta of these insects, and that it is of metabolic origin in contrast to that of Rhodnius, which is largely derived from the blood upon which the insects feed (Wigglesworth, 1931).

The nitrogen and ammonia figures in Table 7 are of the same order as those previously published for Tineola bisselliella (Hollande and Cordebard, 1926) and the related Tinea pellionella (Babcock, 1912); however, the uric acid figures from the present work are slightly higher than those published, i.e. 28 per cent. (Hollande and Cordebard, 1926) and 38 per cent. (Babcock, 1912). The urea figures for both

Table 7

Analyses of insect excreta. (All results reported as per cent. of dry excreta).

	Tineola bisselliella		Attagenus piceus	Gnorimoschema operculella
	Wool	Beetle		
Dry matter	95.2	91.2	93.3	93.8
Water insoluble	25	30	31	59
Ash	1.06	9.92		11.8
Total nitrogen	24.9	19.0	22.5	1.80
Soluble nitrogen	21.1	16.2	17.8	1.03
Purine bases nitrogen				
Amino nitrogen	1.2			
Uric acid	41	31	2.8	2.0
Ammonia	4.1	2.8	0.97	0.05
Urea	3.0	1.5	3.0	0.06
Creatinine	< 0.1			
Creatine	<0.1			
Allantoin			<0.1	

Tineola and Attagenus are considerably higher than Hollande and Cordebard's (1926) (0.4 per cent.) and rather lower than Babcock's (1912) 10.1 per cent., although Wigglesworth (1950) has queried this last figure. Schultz (1925) reports the presence of purine bases in Tinea excreta, but no analyses for these materials were carried out in the present work.

Small quantities of urea were found in whole Tineola larvae (Table 8). Both the clothes moth and the grasshopper (Locusta) were divided up into appropriate tissues for the analyses. Tineola was divided into (a) the hind part of the body together with the hindgut; (b) the midgut, and (c) the remainder of the body including head, whilst the Locusta gut was separated into (a) crop; (b) caeca, and (c) hindgut.

It was at first thought that the presence of urea in the whole insect could be accounted for by the urea content of the faecal pellets in the hindgut. However, on dissection, it was shown that, although the ammonia concentrations were comparable, the Tineola midgut contains 0.14 per cent. urea, whilst other species examined contained none. The presence of this urea in Tineola gut might appear to be of significance in the digestion of wool by insects in view of the known effects of urea on proteins, e.g. the denaturation of proteins (Hopkins, 1930) and the depolymerisation of actin (Szent-Gyorgi and Joseph, 1951). However, these authors show that high concentrations of urea (about 3-5M) are required for the effects to appear

Table 8

Urea and ammonia content of insect tissues.
All results reported as per cent. of fresh tissues.

Insect material	Urea	NH_3
Tineola, whole larvae	0.14	0.04
Tineola, hind (a)	0.16	0.20
Tineola, midgut (b)	0.14	0.05
Tineola, body (c)	0.05	0.03
Agrotis larvae, entire gut	Nil	0.01
Locusta, crop (a)	"	0.02
Locusta, caeca (b)	"	0.06
Locusta, hindgut (c)	"	0.05
Aphodius larvae, entire gut	"	0.04
Melalonthid larvae, entire gut	"	0.07
Dynastid larvae, entire gut	"	0.02
Tribolium, whole larvae	"	0.01

Assuming that the gut contents occupy one half the volume of the total gut, the urea concentration may become about 0.28 per cent. or about 0.05M in the contents. It is known that certain changes in the contents of the gut, such as the loss of birefringence of wool and other indications of breakdown occur quite rapidly in the Tineola midgut (Day, 1951a). If all of the urea found in Tineola gut were concentrated in the region of this noticeable activity, approximately one tenth of the length of the gut, the maximum local concentration of urea would still only be about 0.5M, which is very much lower than that required for any denaturing action.

It is also interesting to note, in view of the

restricted air supply to the Tineola gut (Day, 1951b), that the speed of induction of gels in concentrated albumin solutions by urea is increased under anaerobic conditions (Huggins, Tapley and Jensen, 1951). However, since these authors have shown that gelation follows the formation of a lattice structure by a rearrangement of sulphydryl and disulphide groups, it would seem that the action of concentrated urea under these conditions would tend to preserve the three-dimensional molecular structure of keratin rather than to disrupt it. Although the effect of dilute urea solutions on proteins under anaerobic conditions is not known, it is unlikely that the concentrations of urea in Tineola gut could contribute to the digestion of wool by any direct and independent action on the fibres. This is supported by Mercer's (1949) observation that urea solutions dissolve the "unstabilized lower levels" of the wool root but have no effect on hardened keratin. No analysis of Attagenus gut has been made for urea concentration but it can be expected to be similar to Tineola in view of the similar concentrations in the excreta.

Whenconsidering the combined effects of urea and enzyme systems it is notable that Steinhardt (1938), de Jaffe (1947) and others have found that pepsin, trypsin and some other enzymes are not inhibited by up to 10 per cent. urea. Furthermore, Lennox (1952) has found that urea increases the rate of digestion of wool by some enzymes in the presence of reducing agents. The gut of both Tineola and Attagenus are both very reducing, but nothing is known of the effect of urea on the

activity of the proteinases of these insects. However, the maximum local concentration of about 0.5M would seem to be of little significance when the rate of digestion of wool by the urea-activated papain only increases about one third at this urea concentration (Lennox, 1952).

2. Sulphur Excretion

The figure for total sulphur in clothes moth excreta (Table 9) confirms that of Titschack (1922) (4.03 to 4.62 per cent.). Schultz (1925) observed some crystalline material in the faecal pellets of Tinea which resembled cystine crystals, but since he found total sulphur corresponded with sulphate sulphur he concluded that the sulphur of keratin is excreted mainly as inorganic sulphate and that cystine was absent. Pradhan (1949) examined the excreta of Anthrenus fasciatus and, on the grounds of crystal structure and solubility, expressed the opinion that cystine was present. It may be calculated from Table 9 that 55 per cent. of the total water-soluble sulphur excreted by Tineola is cystine sulphur, but only 8 per cent. is sulphate sulphur. Cystine is also excreted in high concentration (11.8 per cent.) by Attagenus. It is clear that cystine rather than sulphate is the important sulphur-containing excretory product in Tineola and Attagenus and probably also in the closely related genera Tinea and Anthrenus.

The presence of cystine in keratin-feeding insects is interesting in view of the paucity of information on the

excretion of amino acids by insects in general (Wigglesworth, 1950). Since the keratin ingested by the larvae of clothes moth and carpet beetles is digested under highly reducing conditions, one would expect the production in the gut of cysteine rather than cystine. As we have seen, the sulphur of this cysteine is excreted principally as cystine and very little appears as sulphate. This provides an interesting parallel to the work on vertebrate cystinuria (Brand, Cahill and Harris, 1935) which shows that any cysteine in the gut is excreted as cystine, whereas the sulphur of ingested cystine appears as sulphate through a different catabolic process.

The black carpet beetle (Attagenus) excretes almost twice as much cystine as Tineola when feeding on a similar diet (Table 9). This is of interest since Waterhouse (1952d) has found that Attagenus, in contrast to Tineola, does not form metallic sulphides in the gut when fed wool treated with metallic salts. The discussion in the following Section (4) of this chapter shows that the cystine content is considerably lower in the excreta of Tineola larvae fed wool treated with a metallic salt. Since sulphides are formed, this suggests that the cystine provides the necessary sulphur. The carpet beetle does not produce sulphides in the gut (Waterhouse, 1952d), and it follows that the process of cystine breakdown is probably lacking in carpet beetle (Chapter VIII).

A possible product of the action of alkali on wool is lanthionine, ($NH_2.COOH.CH.CH_2.S.CH_2.CH.COOH.NH_2$)

Table 9

Analyses of insect excreta (all results reported as per cent. of dry excreta).

	Tineola bisselliella		Attagenus piceus	Gnorimoschema operculella
	(Wool)	(Beetle)		
Total sulphur	4.5	0.95		0.28
Soluble sulphur	3.1			
Soluble SO_4 sulphur	0.24	Nil		Nil
Ethereal sulphate sulphur	Nil			
Insoluble sulphur	1.4 (by diff.)			
Total cystine	10.0	1.4		Nil
Soluble cystine	6.2		11.8	
Soluble lanthionine	Nil			
Insoluble lanthionine	Nil			

(Cuthbertson and Phillips, 1945) and as the proteinase of Tineola has no special characteristics which could explain the digestion of wool by the larvae, it was thought that the presence of lanthionine in the excreta would indicate that the highly alkaline conditions in the gut contributed in a direct manner. The larva of Tineola has a midgut pH of about 10 (Linderstrøm-Lang and Duspiva, 1936; Waterhouse, 1952c) which is unusually high, although midgut alkalinity has been shown to be characteristic of Lepidoptera whether carnivorous, nectar- or wax-feeding (Waterhouse, 1949). A paper chromatographic examination failed to show the presence of lanthionine in Tineola excreta either before or after hydrolysis. Also, the excreted undigested wool fragments constituting the water-insoluble fraction (Table 10), contained more rather than less cystine than the woollen fabric, upon which the insects had been feeding, thus excluding lanthionine formation in the insoluble material. It may be concluded that neither Attagenus, which has a midgut pH of about 7 (Waterhouse, 1952d), nor Tineola depend solely upon gut alkalinity for the digestion of wool.

The possible excretion of sulphur dioxide by clothes moth larvae was checked by analysis of the atmosphere passing over a group of 300 Tineola larvae feeding on wool. The air was drawn over the larvae at the rate of 5 litres in two days and passed through an absorbent of dilute alkali. No sulphur dioxide was detectable (< 0.2 microgram).

3. The Water-Insoluble Fraction of Excreta

The water-insoluble fraction of a sample of Tineola larval excreta was examined by microscope and appeared to consist entirely of small fragments of undigested or partly digested wool fibres. This insoluble fraction, together with a sample of the original woollen fabric was analysed for cystine content.

Table 10

Cystine content of water-insoluble material from Tineola excreta. Results expressed as per cent. of dried material.

	Cystine		
	1	2	Mean
Insoluble matter from Tineola excreta	12.1	12.8	12.5
Woollen fabric	10.5	10.9	10.7

It has been shown that the scales of wool contain a higher proportion of proline (Lindley, 1947) and cystine (Geiger, 1944), and that they are less easily attacked by enzymes than the cortex (Hock, Ramsey and Harris, 1941); Reumuth, 1946). However, other authors are of the opinion that the scales are as easily digested by Tineola as the rest of the fiber (Day, 1951a; Reumuth, 1946), but undoubtedly there is at least some differential digestion, as is easily seen in the contents of the middle region of the Tineola larval midgut.

One of the first signs of digestion is a fraying at the ends of the fibre pieces, and is probably due to a primary attack on the less well keratinised cementing materials.

Table 10 shows that the water-insoluble fraction of Tineola excreta (undigested wool fragments) contains slightly more cystine than the original woollen fabric. This may be a result of the effects of the differential digestion described above.

4. Examination of Excreta from Nickel-fed Larvae

An examination was made of a sample of excreta from Tineola larvae fed on woollen fabric treated with nickel salts (Table 11). The standard white fabric was used as in the other experiments but it contained about 25 per cent. of its original weight of nickel sulphate, and a control test was carried out with untreated fabric. The analytical method for cystine (Shinohara, 1935), was shown to be unaffected by the presence of nickel sulphate or nickel sulphide in the amounts anticipated.

Table 11

Analyses of excreta from Tineola larvae on treated wool. Results reported as per cent. of dried excreta.

Treatment	Cystine (Soluble	Nitrogen in dilute HCl)	Nitrogen (Total)
Nickel-treated fabric	1.4	4.6	19.1
Untreated fabric	6.7	7.8	26.0

It is notable that when Tineola excretes black faecal pellets after feeding on nickel-treated fabric, the cystine content is much lower than after feeding on normal fabric (Table 11). Any dilution of the cystine in this experiment by nickel compounds should be reflected in a drop in nitrogen content; however, this occurs to a much smaller extent than the drop in cystine content. These differences between the insects may be explained by the presence, in Tineola, of an enzyme which decomposes cysteine with the production of hydrogen sulphide, and its absence in Attagenus. Such an enzyme is cysteine desulphydrase as found in the liver of the rat and dog (Fromageot, Wookey and Chaix, 1941). One of the products of the action of this enzyme is pyruvic acid and this has been demonstrated in both Tineola and Attagenus excreta, using the 2:4 dinitrophenyl hydrazine derivative (Lu, 1939). Preliminary tests in vivo (Waterhouse, 1952d) have indicated that the clothes moth larva does contain an enzyme of this type. A description of this enzyme and its possible significance is presented in Chapter VIII.

C. Summary of Chapter VII

Analyses of the excreta of the clothes moth Tineola bisselliella and the carpet beetle Attagenus piceus reveal that a high proportion of the excreted nitrogen can be accounted for as uric acid in Tineola, but less is excreted by the carpet beetle. Little is known of the identity of

the nitrogenous compounds, excreted by the carpet beetle. Both species of insect excrete a small amount of urea which led to the suggestion that urea might be involved in the breakdown of keratin, however analyses of Tineola gut showed that the quantity of urea present was not enough to have any large effect directly on the keratin, or on the enzyme action.

An examination of sulphur excretion showed that cystine sulphur rather than sulphate sulphur was the main excretory product. The carpet beetle excreted twice as much cystine as the clothes moth. Further evidence was presented to show that the clothes moth does not rely wholely on the high alkalinity of its gut for the breakdown of wool since lanthionine, a product of the action of alkalis on wool, was not found in the water soluble fraction of excreta, and the water insoluble fraction actually had a higher cystine content than the original wool.

Clothes moth larvae, fed wool treated with a nickel salt, produced black excreta, and the cystine content of this material was found to be much lower than that from normally fed larvae. This suggested that the cystine was being broken down and the sulphur used for the production of nickel sulphide. An enzymic mechanism for this breakdown has been suggested.

CHAPTER VIII. DESULPHYDRASE ACTIVITY IN INSECTS

A. Introduction

Reducing systems which occur in the gut of keratin digesting insects are of extreme interest since they may be concerned in disulphide-bond breakdown, a reaction which presumably must occur before keratin can be digested. There are a large number of reducing agents which can split the disulphide bond, but few of them are likely to occur in the insect gut. They include sulphite, bisulphite, cyanide, hydrosulphite, thioglycollate, hydrogen sulphide etc. Of these, only hydrogen sulphide is thought to occur, the evidence being the formation of metallic sulphides when Tineola larvae are fed wool treated with metallic salts (Waterhouse, 1952a). The excreta from Tineola larvae feeding on nickel-treated wool contained much less cystine than normal excreta (see Chapter VII) suggesting that some of the deficiency might be accounted for by the production of nickel sulphide. It was thought that H_2S might be produced by an enzyme similar to the "cysteinase" of B. coli (Desnuelle, 1939) or the "desulphydrase" of Smythe (1942).

Waterhouse (1952d, 1953) showed that carpet beetle larvae and certain chewing lice did not produce metallic sulphides in the gut after being fed on metal impregnated wool or feathers. Furthermore, the carpet beetle was found to have a much higher percentage of cystine in the excreta

than the clothes moth (Chapter VII). This implies the absence of H_2S in the gut of carpet beetle and consequently the absence of an enzyme producing it.

Although an overall theory for digestion of keratin by insects cannot be formulated yet, it is thought that the possible presence of H_2S in the gut of Tineola larvae may be of some importance in view of the known reaction of H_2S with disulphide bonds (Smythe, 1942) namely : $RSSR + H_2S = 2RSH + S$. The following account shows that Tineola possesses an enzyme which liberates H_2S from cysteine and that its concentration is very much higher than in any other insect tested. Some of the properties of this enzyme are also discussed.

B. Experimental and Results

1. Preliminary Tests

Since the presence of a desulphydrase has not been recorded before in insects, and since it is well known that enzymes with similar action from different sources may have very different properties (e.g. effects of heat, change of pH etc.), it was not to be expected that the results obtained from the work on rat-liver enzyme (e.g. Smythe, 1942) could be repeated entirely in experiments on insects. A few preliminary tests were therefore carried out to indicate suitable methods for handling the insect material.

(a) In order to determine the variability to be expected from the method for enzyme estimation, and the effects of storage, the following test was carried out. An enzyme solution was tested (at pH 7.4 by the method outlined in Chapter VI) on one day, kept in the refrigerator overnight and tested again, then finally tested after a further five days in the refrigerator.

Table 12

Effect of storage in refrigerator on enzyme activity

Time of storage:	0 days D670	1 day D670	6 days D670 *
Replicate No. 1	0.240	0.113	0.048
2	0.249	0.120	
3	0.253	0.126	
4	0.238		
5	0.230		

*D670 denotes optical density at 670 mμ

It can be seen from Table 12 that the results may be expected to vary about ± 5 per cent. of the mean, and also that the enzyme appears to be quite unstable, even in the refrigerator. It was decided that enzyme solutions should in future be used immediately, or kept under deep freeze conditions.

(b) As a regular supply of amounts of tissue from clothes moth larvae comparable with those used in work with mammalian tissues was a serious problem, it was important to conserve the material as much as practicable. The following test was therefore carried out to determine if there was any great difference between young and old larvae. Three hundred larvae of each of two different age groups were ground with water in a mortar and, after centrifuging, diluted to 100 mgm. original tissue /ml. The results were, for young larvae D670 = 0.74, and for old larvae D670 = 0.61, showing that there is little difference between the two groups.

(c) To determine if the H_2S produced by extracts of ground larvae was due to (i) endogenous substrate, or (ii) adhering yeast from the food of the larvae, the test outlined in Table 13 was carried out.

Table 13

Desulphydrase activity from outside agencies

	D670*
Tineola enzyme alone	0.010
Tineola enzyme + cysteine	0.225
100 mgm./ml. solution Tineola food (yeast etc.) alone	0.010
100 mgm./ml. solution Tineola food (yeast etc.) + cysteine	0.012

*D670 denotes optical density at 670 mμ

It is clear that the H_2S derived from the enzyme extract of Tineola is not due to the small particles of food (yeast etc.) adhering to the larvae, nor to any endogenous substrate. To check on the possible production of H_2S from the cysteine substrate by the alkaline conditions of the test alone, various concentrations of l-cysteine from 1×10^{-1} to 1×10^{-3} were carried through the test with phosphate buffer (pH 7.4) and glycine buffer (pH 9.08). In none of the tests was any H_2S detected.

It was decided following these tests to pick medium-sized larvae, to ignore the very small amount of contamination with food materials and to store enzyme solutions under deep freeze conditions.

2. Stability and Purification of the Desulphydrase

Table 12 shows that even in the refrigerator a rapid loss of activity can be expected in the insect enzyme preparations. At elevated temperatures the destruction of the enzyme is rapid, experiments on one enzyme preparation showing that when held at 50°C. for 5 minutes 63 per cent. of its activity was lost, and 100 per cent. loss occurred in the same time at higher temperatures. It was important to determine the rate of breakdown of the enzyme during a normal test period, i.e. 2 hours at 37°C. This was tested by making extracts

of three different enzyme preparations and then holding them at 37°C. under anaerobic conditions for 1, 2 or 3 hours before addition of the cysteine substrate. The first preparation was a water extract of separated larval guts (50 mgm./ml.), the second a water extract of acetone dried whole larvae (20 mgm./ml.), and the third a water extract of fresh whole larvae (64 mgm./ml.).

Table 14

Breakdown of desulphydrase at 37°C. without substrate

Time without cysteine at 37°C.	Larval guts	Acetone dried whole larvae	Fresh whole larvae
	γH_2S	γH_2S	γH_2S
0 hour	7.5	10.0	26.5
1 "	2.8	8.2	23.5
2 "	0.7	8.1	23.2
3 "	0.9	6.4	22.8

Table 14 shows that some decomposition apparently does occur at 37°C. without substrate, especially in the extract of dissected guts, but that it is far less important in the case of an extract of fresh whole larvae.

It was considered that the original differences in activity between the extracts were not important and that the greater stability of the extract of gut only may be due to a higher proportion of proteins in the latter extract, thus affording a larger amount of alternative

substrate for proteinase which is known to be present (Linderstrøm-Lang and Duspiva, 1936). It is interesting to note in this connection that Eadie and Bernheim (1952) found proteinase interfering in their work by destruction of sarcosine oxidase of liver. It was not possible with the materials at hand to carry out an extensive purification program, but two simple processes were tried:

(a) Firstly, an attempt was made to eliminate proteinase by selective adsorption on to insoluble casein (substrate) followed by centrifugation, as suggested in Hawk, Oser and Summerson (1947). Two lots of 1 ml. of enzyme were each treated with 20 mgm. reprecipitated casein, then these solutions, together with untreated samples, were stored overnight at room temperature and in the deep freeze. The residual activity was determined the next day.

Table 15

Effect of casein adsorption on enzyme activity

Storage	Casein treatment	Activity γH_2S/ml.
Deep freeze	Untreated	21.0
" "	Treated	4.5
Room temperature	Untreated	8.5
" "	Treated	2.5

Table 15 shows that no increase of stability of enzyme preparation could be obtained by treatment with casein, and that casein actually causes loss of desulphydrase activity.

(b) The second method was used by Lawrence and Smythe (1943) and consisted of shaking 2 ml. enzyme solution with 2 ml. chloroform for 10 minutes. This was centrifuged and the supernatant examined for activity. The original enzyme solution had an activity of 19 γ H_2S per ml., but the treated solution had the considerably lower activity of 3 γ/ml.

Reference to Table 18 will show that a crude aqueous extract from whole Tineola larvae yields a comparatively pure enzyme. Most further work was carried out using such an extract.

3. Desulphydrase Activity in Various Insects and Tissues

The presence of desulphydrase activity was expected in the gut of Tineola, as explained in the introduction to this chapter, however it was also of interest to determine if the rest of the insect body had any activity. A large number of Tineola larvae were dissected and separated into two groups (a) bodies and (b) guts, and, as this took some days, the material was kept at freezing temperature during its collection. The two lots of tissue were then ground

thoroughly in water, centrifuged, and finally diluted to 25 ml. each. All the activity in Tineola larvae was found to be localised in the gut (Table 16).

Table 16

Desulphydrase activities of Tineola larval tissues

Tissue	Wet weight of tissue in 25 ml. extract	Activity of extract γH_2S/ml.
Gut	1.24 gms.	60
Body	6.42 gms.	0

A number of insects representing several different orders were collected and extracts of these were tested for desulphydrase activity at pH 7.4 (Table 17).

Table 17 shows that the larva of the clothes moth Tineola has a highly active desulphydrase with cysteine as substrate under the conditions of the test. The other insects, with the exception of Tribolium adults and larvae (which are discussed in Section B6 of this chapter) and Oryzaephilus, also have desulphydrase activity, but it is considerably lower than that of Tineola.

One important feature of the results in Table 17 is that the carpet beetle, Anthrenus vorax has only a weak desulphydrase with the same order of activity as those from most insects tested other than Tineola. The production of metallic sulphides in the gut of Tineola larvae, but not

Table 17

Desulphydrase activities of various insects

Insect	Gms. tissue/ test	Weight/ insect mgms.	γH_2S	γH_2S/ gm. tissue	γH_2S/ mgm.N
LEPIDOPTERA					
Tineola bisselliella (Larvae)	0.0109	4.10	2.3	209	34
" " "	0.011	4.16	10.8	979	85
Ephestia kühniella (Larvae)	0.303	11.2	8.3	27.4	
DIPTERA					
Musca domestica (Larvae)	0.132	19.8	7.5	57	5.4
COLEOPTERA					
Tribolium confusum (Larvae)	0.0557	1.70	0	0	
" " (Adults)	0.2960	1.80	0	0	
Rhizopertha dominica (Adults)	0.1730	1.15	5.8	33.5	5.1
Sitophilus oryzae (Adults)	0.230	1.20	9.6	42	11.4
Oryzaephilus surinamensis (Adults)	0.113	0.45	0	0	0
Anthrenus vorax (Larvae)	0.0677	4.98	2.0	29	2.7
ISOPTERA					
Nasutitermes exitiosus (Workers)	0.2100	5.72	2.6	12.4	2.9
Coptotermes acinaciformis (")	0.249	5.17	0.6	2.4	0.5

by Anthrenus when fed metal salts (Waterhouse, 1952d) thus becomes more understandable, since Tineola is capable of producing very much larger quantities of H_2S than Anthrenus.

Rat liver desulphydrase prepared according to the technique of Smythe (1942) was tested for activity, together with a partially purified extract after chloroform treatment. The activities per gram of tissue and per milligram Kjeldahl nitrogen by the method used in this work are presented in Table 18 together with comparative figures on Tineola extracts.

Table 18

Desulphydrase activity of rat liver and insect tissues

Tissue	Extract	Activity	
		γH_2S/ gm. tissue	γH_2S/ mgm. N
Rat Liver	Crude (saline)	122 (pH 7.4)	7.6
" "	$CHCl_3$ treated	104 (pH 7.4)	17.9
Tineola gut only	Water	1200 (pH 7.4)	80
Tineola whole larvae	Water	704 (pH 7.4)	49
Tineola whole larvae	Water	450 (pH 8.9)	68
Tineola whole larvae	Water	285 (pH 8.9)	45

The desulphydrase of Tineola is apparently considerably more active than that from rat-liver preparations, although some of the insect preparations were not tested at their optimum pH (about 9.1, see Section 4 of this Chapter).

One preparation from the gut only, even at pH 7.4, had ten times the activity of a comparable rat-liver preparation. The large variations in the figures in Table 18 are due to the difficulty in handling the enzyme without destruction, and the figures only indicate the order of activity. The specific activity or purity recorded as γH_2S per mgm. nitrogen in all crude insect enzyme preparations tested is also considerably higher than that of rat-liver preparation. Lawrence and Smythe (1943) obtained rather better figures for their rat-liver enzymes than are shown in Table 18. Their saline extract had an activity of about 30γH_2S per mgm. Kjeldahl N, and a chloroform treated extract about 70 γH_2S per mgm. N, however, the insect extracts from the present work were in all instances of higher purity than these crude rat-liver extracts.

4. Effects of pH on Desulphydrase Activity

It was assumed in the initial stages of this work that the pH optimum of insect desulphydrase would be about the same as that of rat-liver enzyme (pH 7.4-7.8, Smythe, 1942); however, a preliminary test indicated that Tineola at least had a rather higher optimum. Although no attempt at this stage could be made to determine the pH optima of all the insect preparations tested, it was felt that some indication of the change of activity with pH should be obtained, owing to its importance when comparing various insects and tissues.

Table 19

Effect of pH on desulphydrase activity in insects

Insect Tissue	Activity at various pH values (γH_2S per gm. tissue)							
	pH 5.9(1)	7.0(1)	7.4(1)	8.0(1)	8.5(2)	8.9(2)	9.3(2)	10.5(2)
LEPIDOPTERA								
Tineola bisselliella (Larvae)	54	108	270	270	324		750	608
Ephestia kühniella (Larvae)			27			34 (pH 9.08)		
DIPTERA								
Musca domestica (Larvae)	13	42	50	55	42		45	44
COLEOPTERA								
Rhizopertha dominica (Adults)	0		12			34		
Anthrenus vorax (Larvae)	0		22			32		
ISOPTERA								
Nasutitermes exitiosus (Workers)	2.4		9.5			8.5		

(1)Phosphate buffer (2)Glycine-NaOH buffer

It appears from Table 19 that Anthrenus vorax larvae, Rhizopertha dominica adults, Ephestia kühniella larvae and Tineola bisselliella larvae possess desulphydrases with optima of pH 9 in contrast to the desulphydrase of Nasutitermes exitiosus workers, Musca domestica larvae and Sitophilus oryzae adults whose optima appear to lie in the region about pH 8.

A further examination of the pH optimum of Tineola desulphydrase (Fig. 6) illustrated the phenomenon of buffer specificity which is found in some enzymes. Howell and Sumner (1934) have shown this phenomenon to occur with crystalline urease; phosphate buffer indicates a pH optimum 0.5 to 1 pH unit higher than citrate or acetate depending upon the concentration of the substrate. It can be seen that there is a distinct break in the curve corresponding to the change in type of buffer from phosphate to glycine-NaOH. Owing to the impracticability of using a single buffer for the whole range at first desired (pH 5-10) and the desirability of avoiding the use of borates which inactivate some enzymes, the present two buffers were chosen.

No explanation for these differences can be advanced but the possible part played by these buffers in removing metals from the reaction cannot be ignored. In this connection Albert (1950, 1952) has shown that amino acids including glycine rapidly from complexes with metals.

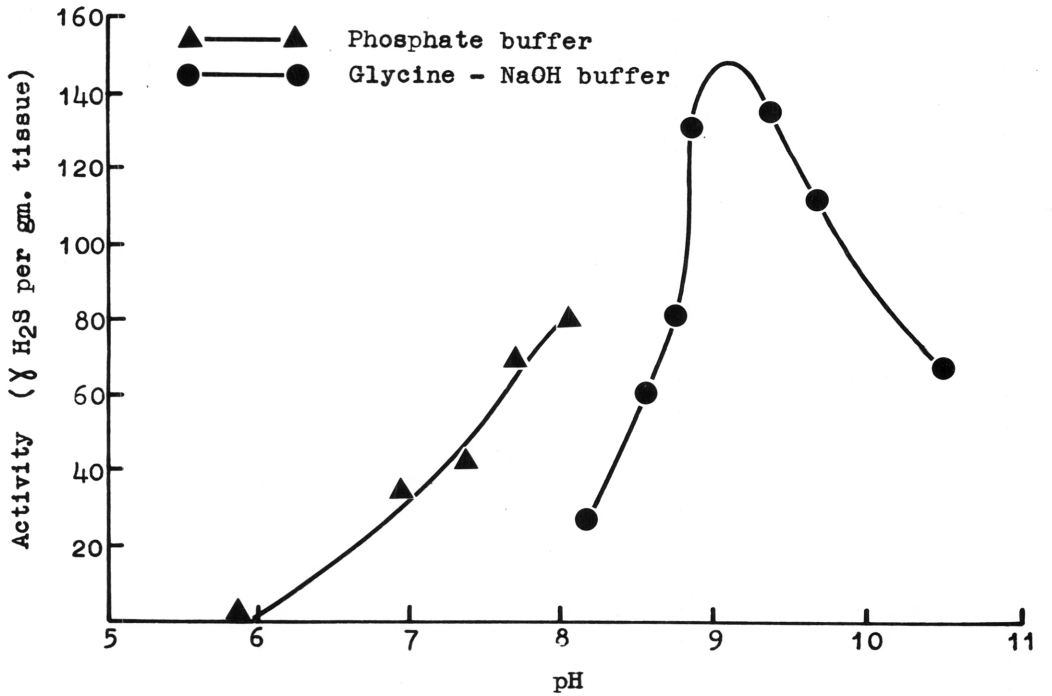


Fig. 6.- Specific effects of two buffers on Tineola desulphydrase.

A further test using five different buffer mixtures all brought to the same pH (9.08) at 37°C. was carried out (Table 20). Glycine-NaOH buffer was compared with the same mixture containing Na_2HPO_4 but no activating effect due to phosphate was found. Comparison was also made with veronal, borax and histidine and it was found that the enzyme was similarly active in glycine and veronal, but has a greater activity in borax and histidine. This is somewhat surprising in view of the depressing effects of borates on urease (Howell and Summer 1934) and on insect adenosine deaminase (Lennox, 1941).

Table 20

Activity of Tineola desulphydrase with various buffers

Buffer mixture (pH 9.08 37°C.)	Activity γH_2S/gm. tissue
0.075 M glycine	200
0.075 M glycine + 0.1 M Na_2HPO_4	200
0.1 M Veronal	190
0.05 M borax	254
0.1M histidine	334

It was decided to adopt the conservative view that the glycine-NaOH buffer would probably interfer least in any inhibitor-activator studies, so this was used in further work on Tineola instead of the buffers which produced the highest activity.

Figure 67 shows the pH optimum curve for Tineola desulphydrase. The optimum in glycine-NaOH buffer when acting on 0.0083 M l-cysteine for 2 hours at 37°C. is pH 9.1

5. Action of Desulphydrase on Various Possible Substrates

The production of H_2S from cysteine by micro-organisms is well known (e.g. by B. coli, Desnuelle and Fromageot, 1938) and the enzyme responsible was called "cysteinase" by these authors. Subsequently, Fromageot, Wookey and Chaix (1941) showed the presence of an enzyme in dog and rat liver which liberated H_2S from cysteine under anaerobic conditions. This they termed a "desulphurase" to distinguish it from cysteinase of B. coli. Smythe (1942) however showed that this enzyme could degrade both cystine and cysteine under either aerobic or anaerobic conditions, thus widening the substrate specificity. He pointed out certain inconsistencies in the term "desulphurase" and suggested "desulphydrase" as a better term (Smythe, 1944). Subsequently Greenstein and Leuthardt (1944) have extended this work to show that the enzyme in liver not only degrades cystine and cysteine but also certain peptides of cystine. All the peptides degraded by this enzyme contained the cystine at the end of the peptide chain. Any deviation from this, such as the central position of cystine occurring in the tripeptide glutathione, prevents the action of the enzyme. This enzyme was named by Greenstein and Leuthardt (1944)

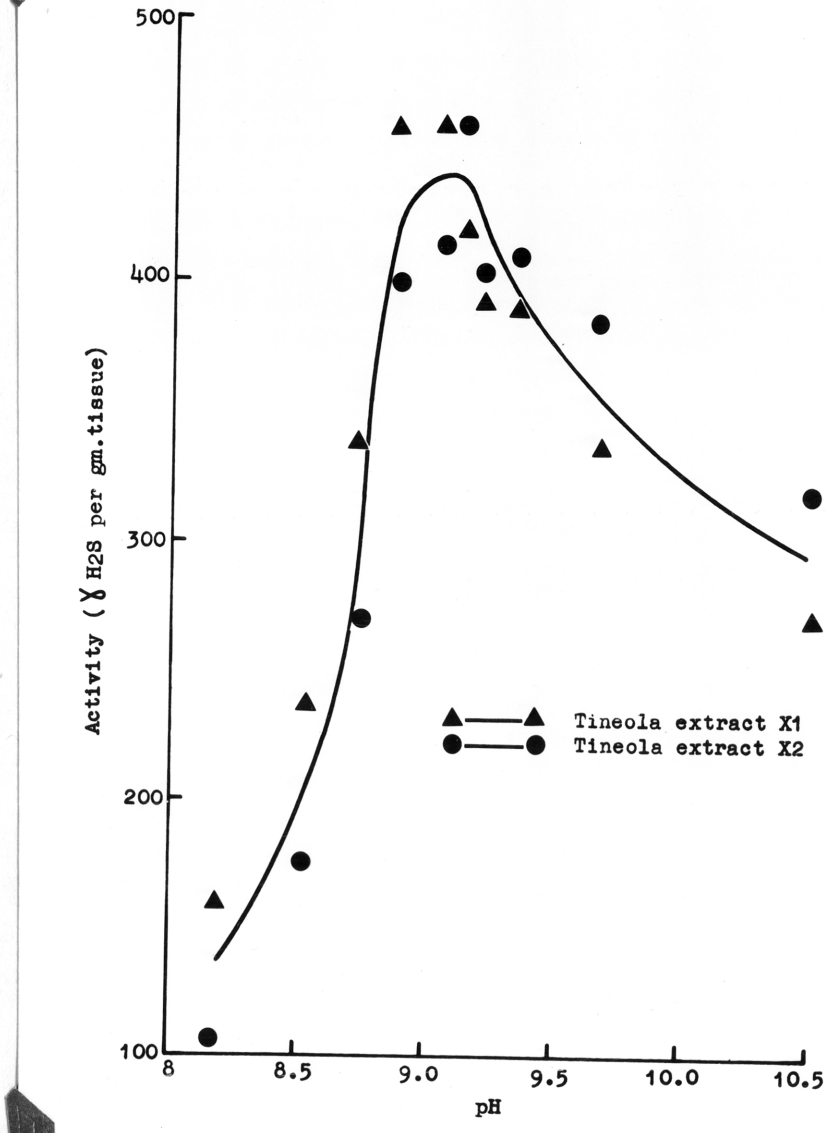


Fig. 7.- Effect of change of pH on activity of Tineola desulphydrase.

an "exocystine desulphurase". Throughout the present work the non-specific term desulphydrase has been used, although most of the results have been obtained with cysteine only.

Smythe (1942) showed that only very small quantities of H_2S were obtained from glutathione, methionine, thioglycollic acid and dl-homocysteine when compared with cysteine as substrate. Similar results were obtained in a series of tests on alternative substrates with Tineola desulphydrase (Table 21). The cystine and homocystine were added in acid solution, followed immediately by the exact quantity of NaOH required to neutralise the acid. A white precipitate resulted with cystine. No test was carried out with homocysteine.

Table 21

Action of Tineola desulphydrase on alternative substrates

Substrate	Concentration	Activity (γ H_2S/gm. tissue)
l-cysteine	8.3×10^{-3}M	440
l-cystine	"	0
dl-homocystine	"	0
dl-methionine	"	0
glutathione	"	0
sodium thioglycollate	"	0

It has been found by Smythe (1942) and Fromageot, Wookey and Chaix (1941) that their enzyme preparations showed a

specificity for the l- form of cysteine, although the latter authors suspected the presence of a "d-cysteine desulfurase" in some of their preparations. Unfortunately, no d-cysteine was available so the optical specificity of the insect enzyme could not be examined.

The complete lack of action on methionine or glutathione is perhaps surprising in view of the production (although limited) of metal sulphides in the gut of clothes moth larvae when fed silk plus these substances and metal salts (Waterhouse, 1952a). It is possible that the H_2S required to form the sulphides when methionine and glutathione are fed is derived through another reaction distinct from desulphydrase activity. In this connection Fromageot and Moubacher (1937) using acetone extracts of B. coli reported the formation of H_2S from glutathione but not from cysteine.

6. Effects of Various Substances on the Enzyme Activity

Studies of activation and inhibition of enzyme systems have yielded much information on the mode of action of the enzyme, on enzyme substrate complex formation, on the primary products of enzyme action etc. The results which follow record some of the properties of Tineola desulphydrase and enable comparisons to be made with the rat-liver enzyme. All the results were obtained on two similar enzyme preparations made up at the same time and kept

under deep freeze conditions.

(a) Effect of salts.- Rather high concentrations of salts (0.42 M) gave varying degrees of inhibition when tested against rat-liver enzyme (Lawrence and Smythe, 1943). Table 22 compares the rat-liver enzyme with Tineola enzyme.

Table 22

Effect of salts on desulphydrase

Salt	Concentration	Per cent. inhibition	
		Tineola	Rat liver (Lawrence & Smythe, 1943)
NaCl	0.42 M	9	35
KCl	0.42 M	6	42
NH_4Cl	0.42 M	52	63
KNO_3	0.42 M	35	100

A check on the effect of these high concentrations of salts on the pH of the final mixture was carried out by mixing 10 ml. of the glycine-NaOH buffer with 12 ml. H_2O then either 2 ml. of water or 2 ml. of the salt solutions were added.

Table 23

Effect of salts on pH of glycine buffer at 22°C.

	pH
Glycine-NaOH buffer + water	9.50
0.42 M NaCl	9.48
0.42 M KCl	9.50
0.42 M NH_4Cl	8.50
0.42 M KNO_3	9.50

It can be seen in Table 23 that the hydrolysis of NH_4Cl has affected the pH of the glycine NaOH buffer, dropping it approximately 1 pH unit. Reference to Figure 6 will show that a change in pH of 1 unit would cause approximately 50 per cent. reduction in activity of the enzyme. This therefore accounts for the apparent effect of NH_4Cl in the present work. The small effects due to NaCl and KCl are negligible, thus leaving KNO_3 as the only salt tested which affects Tineola desulphydrase, whereas all the salts had considerable effect upon rat-liver enzyme.

(b) Effect of other sulphur compounds.- It might be expected that some compounds containing sulphur, and having a structure similar to cystine, would inhibit the action of desulphydrase. Table 24 shows the effects of some compounds containing sulphur.

Table 24

Effect of sulphur compounds

Inhibitor	Per cent. inhibition
.0083 M methionine	5
.0083 M glutathione	67
.0083 M cystine	31
.0083 M homocystine	39
.0083 M sodium thio-glycollate	32

The sample of sodium thioglycollate used in this experiment yielded 2.1 γ H_2S on its own under the conditions of the test, and the figure in Table 24 has been corrected for this. Methionine was the only sulphur compound which did not inhibit the action of Tineola desulphydrase. The inhibition of rat-liver desulphydrase by sodium thioglycollate and glutathione, 27 per cent. and 37 per cent. respectively (Lawrence and Smythe, 1943), is not so great as that for Tineola. It seems likely that the apparent inhibition by glutathione, cystine and homocystine, and possibly sodium thioglycollate could be due to the reduction of disulphides by H_2S with the production of sulphur (Smythe, 1942)

$$RSS + H_2S = 2RSH + S$$

If this were so the effect of these materials is then not an inhibition but a secondary reaction with H_2S, the product of the first reaction . No tests have been carried out as yet to confirm this possibility. Fromageot, Wookey and Chaix (1941) have shown a similar effect by adding cystine to cysteine to form a mixed substrate.

(c) Effect of metal-complex forming compounds.- In order to make further comparisons with Lawrence and Smythe's (1943) data the effect of a number of compounds known to form complexes with metals was tested.

Table 25

Effect of metal-complex forming compounds

Inhibitor	Per cent. inhibition	
	Tineola	Rat liver (Lawrence & Smythe 1943)
0.01 M sodium pyrosphosphate	4	0
0.01 M thiourea	0	8
0.01 M sodium azide	-	20
0.01 M sodium diethyldithiocarbamate	0	18

The figure for effect of sodium azide on the action of Tineola desulphydrase was not included, as it was apparent that the reagent had an independent action upon the development of colour in the method for estimation of H_2S. The sodium diethyldithiocarbamate, on addition of acid, yielded not only carbon bisulphide but also H_2S; however, this was estimated and the figures in Table 25 corrected. The lack of effect of these metal-complex forming compounds is perhaps not surprising, at least in the Tineola experiments, as the amino acids already present as buffer and substrate (glycine and cysteine respectively) also form very strong complexes with metals (Albert, 1952). Lawrence and Smythe (1943) tested this type of compound because of a strong inhibition of the enzyme by cyanide, however, in the presence of cysteine, one would not expect a very great effect by metal-complex

forming compounds. This leads to the interesting conclusion that this enzyme probably does not require the intervention of metals for its action. Nevertheless, Binkley (1943) and Lawrence and Smythe (1943) consider that magnesium is a component of the enzyme and Chargaff and Sprinson's (1943) work suggests that other metals may also take part. It is extremely difficult to test the effect of metals with the present methods, as any metal which forms insoluble sulphides will remove H_2S from the reaction and the overall effect will appear as an inhibition; e.g. in one experiment, 10^{-4} M Ag NO_3 produced a 64 per cent. inhibition. The effects of metals on this enzyme requires further work, although Fromageot, Wookey and Chaix (1941) showed no significant effect of Fe, Mn and Ti on liver desulphydrase.

(d) Effect of various compounds which affect carbonyl groups.- When it was found that rat-liver desulphydrase was inhibited by cyanide and metal-complex forming substances had no effect, Lawrence and Smythe (1943) decided to test the effect of carbonyl reagents. Since cyanide activates rather than inhibits Tineola enzyme (Table 26) it is again interesting to compare the effect on Tineola.

The Tineola preparation appears to be less sensitive (in the order of 1/100th) to concentrations of phenylhydrazine, hydroxylamine and semicarbazide than is rat-liver enzyme, but both enzymes are equally sensitive to As_2O_3 (Table 26).

Table 26

Effect of carbonyl reagents on desulphydrase

Inhibitor	Molar concentration	Per cent inhibition	
		Tineola	Rat-liver (Lawrence & Smythe, 1943)
KCN	2.5×10^{-2}	91 (activation)	82
	1×10^{-2}	126 "	65
	5.0×10^{-3}	129 "	54
	2.5×10^{-3}	122 "	41
As_2O_3	1×10^{-2}	100	100
	5×10^{-3}	100	100
	2.5×10^{-3}	94	97
	1×10^{-4}	88	54
Phenylhydrazine	1×10^{-2}	33	100
	2.5×10^{-3}	15	100
	5×10^{-4}	2	93
	1×10^{-4}	0	53
Hydroxylamine	1×10^{-2}	100	-
	2.5×10^{-3}	94	-
	5×10^{-4}	58	100
	1×10^{-4}	21	96
Semicarbazide	1×10^{-2}	13	100
	5×10^{-3}	8	-
	1×10^{-3}	12	90
	1×10^{-4}	7	61

The activation by cyanide on the Tineola enzyme represents a difference between the insect and rat-liver desulphydrase. In order to confirm Lawrence & Smythe's (1943) work using

the present methods, a rat-liver preparation was tested for cyanide inhibition. Table 27 shows that cyanide inhibition, observed by Lawrence and Smythe (1943) and Fromageot, Wookey and Chaix (1941), is confirmed under the conditions used in these comperiments.

Table 27

Cyanide inhibition of rat-liver desulphydrase

Cyanide concentration	Per cent. inhibition
2.5×10^{-2}M	100
1×10^{-2}M	100
5×10^{-3}M	87
2.5×10^{-3}M	85

A single additional test was carried out to enable some comparisons to be made between insects. A preparation of desulphydrase from the lepidopterous larva, Ephestia kühniella was tested both with and without 1×10^{-2}M KCN, and showed the somewhat unexpected result of 22 per cent. inhibition. It appears that Tineola desulphydrase may be different in this respect from other desulphydrases in general, although further work is required to confirm this. Included in the various ways in which cyanide can activate an enzyme are (i) formation of a complex with the substrate, e.g. uric acid (Griffiths, 1952); (ii) formation of a complex with the enzyme, e.g. papain (Fruton and Bergman,

(1940), or (iii) formation of a complex with a metal inhibitor. No work on these possibilities has been carried out, but it would seem that (iii) is not likely because of the strongly complex-forming substances already present, i.e. glycine and cysteine, and the lack of effect of other metal-complex forming substances (Table 25).

An interesting phenomenon was seen when the reasons were sought for the apparent inactivity of Tribolium extracts (Table 17). For the preparation of extracts of most insects it was only possible to grind whole insects in a mortar and make a brei which was centrifuged fairly free of suspended matter. In doing this one must extract soluble materials from parts of the body other than the gut. During the preparation of the extracts of Tribolium adults it was noticed that the solutions rapidly took on a deep reddish-brown colour. This formation of colour was stopped by keeping at freezing temperatures and not only stopped but reversed to yield a colourless solution under anaerobic conditions. A similar darkening of extracts of Sitophilus granarius adults was noticed, and this was also seen to be reversed by addition of cysteine or by keeping under anaerobic conditions for a few minutes. This production of colour is due to the tyrosine-tyrosinase reaction or the tanning of proteins by quinones from the cuticle of the insect. The first reaction is strongly inhibited by glutathione (Figge and Allen, 1941) and the second reaction

is inhibited and reversed by reducing compounds (Hackman, 1948). It appears that the reduction and reversal of colour observed in Tribolium extracts may be an enzymatic reduction by a dehydrogenase system which transfers hydrogen to the coloured compounds under anaerobic conditions.

Quinones are known to be toxic to enzymes (Sumner and Somers, 1943) and to react with sulphydryl groups and since Tribolium adults produce considerable amounts of quinones from their repugnatorial glands (Alexander and Barton, 1943), one would expect a crude extract of Tribolium to show little enzyme action and even act as an inhibitor. Tineola extracts with quite active desulphydrase were strongly inhibited by crude Tribolium extracts (82 per cent. in one test). No further work was done to identify the substances responsible responsible for this inhibition or their mode of action.

C. Discussion and Summary of Chapter VIII

It has been shown that insect tissues have the ability to liberate H_2S from cysteine. In most insects tested so far this ability is very small but in the gut of the clothes moth larva, Tineola, it is much greater even than that derived from the most concentrated source known at present, i.e. rat liver. The activity appears to be enzymic in nature since it is soluble in water and destroyed

by heat. This enzyme, "desulphydrase", is different in some respects to that obtained from rat liver. The effect of pH is broadly similar to the effect on rat-liver enzyme since the Tineola enzyme is rapidly inhibited by acid conditions but more slowly by alkaline conditions; however, the optimum pH of the Tineola enzyme (pH 9.1) is considerably higher than that from rat liver (pH 7.4-7.8). This is interesting in view of the high pH in the insect gut (about pH 8.5-10, Waterhouse, 1952) and the fact that the Tineola proteinase also has a high pH optimum (Linderstrøm-Lang and Duspiva, 1936). However, indications have been obtained that the desulphydrase of other insects may share this high pH optimum.

Agreement with rat-liver enzyme is obtained when studying the substrate specificity and stability of the insect enzyme. Both enzymes are unstable and lose their activity even in the refrigerator, and neither enzyme has any action on glutathione or methionine. Also, both enzymes are inhibited by certain sulphur compounds, notably glutathione, cystine and sodium thioglycollate. Neither Tineola enzyme nor rat-liver enzyme are greatly affected by metal-complex forming substances, indicating that heavy metals are probably not involved in the action. A suggestion by Lawrence and Smythe (1943) that magnesium may be involved in rat-liver activity has not been investigated further.

Efforts to establish a common mode of action of the two enzymes were negated by the entirely different reaction to

cyanide addition, i.e. the rat-liver enzyme was inhibited by cyanide but the Tineola enzyme was activated. Other substances such as As_2O_3, phenylhydrazine, semicarbazide and hydroxylamine had similar inhibiting effects on the two enzymes, but generally the insect enzyme was inhibited to a far smaller degree by these substances. The effects of high concentrations of salts on the insect enzyme was also much less than on rat-liver enzyme. An interesting inhibition was found in extracts of whole Tribolium adults and the suggestion has been made that it is due to the presence of quinones in the insects.

The presence of a high desulphydrase activity in Tineola and a low activity in the carpet beetle (Anthrenus vorax) provides an explanation for the ability of the clothes moth but not the carpet beetle to form metallic sulphides in the gut (Waterhouse, 1952a and d).

That an enzyme such as desulphydrase is active in the gut lumen of Tineola is interesting in view of the reduction of wool by sodium sulphide. It is not clear what part this plays in Tineola digestion, but it would seem that this could not contribute greatly to the breakdown of disulphide bonds in the carpet beetle in which the desulphydrase is very weak. However, the phenomenon of stimulation to secretion of these enzymes by the food cannot be ignored. It is possible that, at the time the carpet beetles were examined, only a weak desulphydrase was present,

but this has not been examined further. No conclusive results have been obtained from the examination of digestion of wool *in vitro* by *Tineola* enzyme preparations (Chapter IX), indicating that perhaps the mechanism for the production of H_2S from cysteine in the gut is only involved in the final degradation of cystine, one of the products of wool digestion.

It seems possible that the H_2S produced by this enzyme would contribute to the strongly positive nitroprusside tests obtained in the gut of wool-digesting insects. This is supported by the fact that the amount of colour obtained in the tests varied as the amount of food eaten and was very small unless the food contained sulphur, i.e. wool or cystine. Linderstrøm-Lang and Duspiva (1936) discuss the possibility of sulphides giving a positive test but come to the conclusion, after testing with lead acetate, that the amounts of sulphide are only very small and that the positive tests are due to cysteine or cysteine peptides. The role of pyridoxine or pyridoxal phosphate as coenzyme in the reactions involving transfer or removal of sulphur from organic compounds has attracted considerable attention recently (Braunstein and Azarkh, 1950, Ohigashi, Tsunetoshi and Ichihara, 1951, Binkley, Christensen and Jensen, 1952). Pyridoxal phosphate has been stated to be important in the deamination of serine and threonine (Reissig, 1952) also pyridoxal-5-phosphate acts as coenzyme in the degredation

of kynurenine whilst pyridoxine-3-phosphate is not active (Wiss, 1952). This suggests considerable specificity in the coenzyme activity of these compounds. No pyridoxine compounds were available at the time of the present work, so it was not possible to determine whether any of them were coenzymes for the insect desulphydrase.

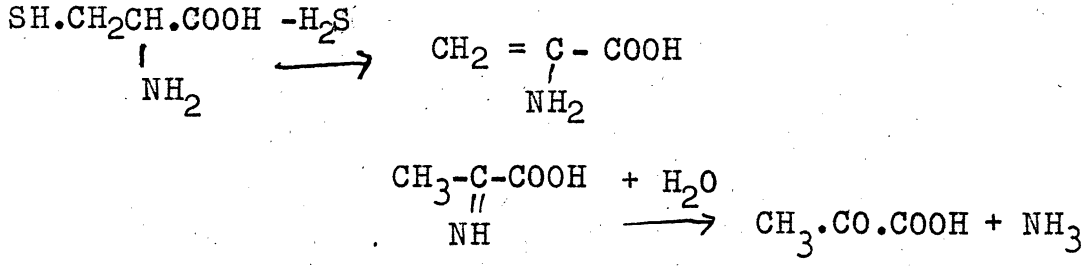
Chargaff and Sprinson (1943) presented evidence that serine deaminase is different to other deaminases (e.g. alanine) in its mode of action, since it produces pyruvic acid as well as ammonia. These authors suggest that the reaction proceeds firstly through the action of a dehydrase to produce water and amino acrylic acid, then the unstable aminoacrylic acid breakdown to pyruvic acid and ammonia as follows:

$$OH.CH_2.\underset{NH_2}{CH}.COOH \xrightarrow{-H_2O} CH_2 = \underset{NH_2}{C}-COOH$$

$$CH_3-\underset{NH}{C}-COOH + H_2O \longrightarrow CH_3.CO.COOH + NH_3$$

The striking similarity of this reaction to that proposed for the desulphydrase action on cysteine (as follows) led Binkley (1943) to present evidence which indicates that cysteine desulphydrase, serine deaminase and enolase (acting on phosphoglyceric acid) may be identical. This was supported by the fact that the three reactions all produced pyruvate or

phospyruvate and ammonia. No experiments as yet have been carried out to examine the action of insect preparations on serine or phosphoglyceric acid.



CHAPTER IX. DIGESTION OF WOOL IN VITRO

Wool may be digested *in vitro* by a number of enzymes although the process is rather slow and incomplete. Geiger, Patterson, Mizell and Harris (1941), using crystalline enzymes, showed that the greatest effect was apparently on the cementing materials which bind the corticle cells and scales together. However, if the wool is first reduced and dissolved by sodium sulphide then reprecipitated in an amorphous form it becomes digestible (Goddard and Michaelis, 1934). When wool is reduced with alkaline thioglycollate it becomes digestible by enzymes, either in the reduced state or in the reoxidised state, showing that the process of reduction and reoxidation destroyes the physical structure which protects it against enzyme attack. Also the clothes moth proteinase partially digests wool at pH 10 in the presence of thioglycollate; heat inactivated enzyme solutions have very little effect under these conditions. No signs of active digestion could be seen in the contents of a dissected larval gut in a moist chamber (Linderstrøm-Lang and Duspiva, 1936), although oxidising conditions would be established here.

Lennox (1952) discussed the digestion of wool *in vitro* by enzymes in the presence of reducing agents and pointed out that urea has an activating effect. It is extremely doubtful, however, if urea, which is known to

be present in the gut of Tineola has any appreciable effect on the digestion of wool by this insect (Chapter VII).

The results reported in Chapter VIII suggested that, at least in Tineola, the H_2S liberated by the desulphydrase may be in some way connected with the reduction of disulphide bonds of wool and it was thought that some indication of this might be obtained by in vitro tests with Tineola enzyme. A concentrated enzyme solution was prepared, and the experiments outlined in Table 28 were carried out in the vacuum diffusion units designed for the desulphydrase estimations. After 18 hours, the H_2S produced in the enzyme mixture was diffused and colorimetrically estimated in the usual manner. Owing to the high concentration of enzyme it was not possible to estimate the H_2S accurately but merely to indicate its presence in abundance. The wool used in the experiments was white fabric cut into approximately 0.5 mm. lengths. Bacterial action was prevented in all tubes with toluene. After the digestion period the wool was examined microscopically and its physical state noted.

The amount of digestion as revealed by microscopic examination was not great, even when H_2S was present (Table 28) and appeared to be confined to the cementing materials of the fibres. No H_2S was observed in the tubes without the presence of cysteine.

Table 28

Digestion of wool *in vitro* (18 hours at 37°C.)

Reaction mixture	H_2S	Digestion
1. Wool + 1 ml. buffer	-	-
2. Wool + 1 ml. buffer + 1 ml. enzyme	-	+
3. Wool + 1 ml. buffer + 1 ml. enzyme + 0.2 ml. cysteine	+ +	+
4. 1 ml. buffer + 1 ml. enzyme + 0.2 ml. cysteine	+ + +	
5. 1 ml. buffer + 1 ml. enzyme	-	

All mixtures diluted to final volume of 2.2 ml. Approximately 20 mgm. wool were used for tubes 1, 2 and 3. The buffer was M. veronal at pH 9.1 and the l-cysteine solution was 0.1 M.

A further experiment under the same conditions as shown in Table 28 produced about the same degree of breakdown of the wool. In addition, an indication of the ammonia and pyruvate production was obtained together with a test for SH groups (nitroprusside). After removal of H_2S, the ammonia was diffused under vacuum into acid and estimated by nesslerization. The pyruvate was tested for qualitatively by reaction of the enzyme mixture with an acid solution of 2:4 dinitrophenylhydrazine, extraction with ethyl acetate, extraction back into sodium carbonate solution, then addition of NaOH.

Table 29

Digestion of wool in vitro at 37°C. for 18 hours

Reaction mixture	H_2S	NH_3	Pyruvate	SH
1. Wool + 1 ml. enzyme + 1 ml. buffer + 0.2 ml. cysteine	+	+	-	+
2. Wool + 1 ml. enzyme + 1 ml. buffer	-	+	+	-
3. Wool + 1 ml. buffer	-	-	-	-

All mixtures were diluted to 2.2 ml. Approximately 20 mgm. wool were used. The buffer was M. veronal and the l-cysteine was 0.1 M.

A small amount of digestion of the fibres could be seen in tubes 1 and 2 (Table 29). The presence of H_2S only when cysteine was added confirms the previous result obtained in Table 28. The presence of NH_3 in both tubes 1 and 2 is to be expected owing to the crude nature of the enzyme preparation. Pyruvate should also be expected in tubes 1 and 2 but appeared in tube 2 only. This may be due to a reaction with the cysteine (in tube 1) as suggested by Smythe (1942). The nitroprusside test was positive in tube 1 only, showing that no large quantity of sulphydryl was formed from the wool by the enzyme.

These results can only be regarded as indicative of the role that desulphydrase and H_2S play in the breakdown of wool by clothes moth, and further work is necessary to

provide a full understanding of the problem. However, it appears that the presence of H_2S, following the desulphydrase action, has only a small effect, if any at all, on the digestion of wool by Tineola enzymes.

CHAPTER X. DISCUSSION

As a study in applied chemistry the question of damage to keratinaceous materials by insects has provided many problems, some of which have been satisfactorily solved, but many are still awaiting investigation. As in all work involving biological material, the research can take up the labors of scientists in many different fields, such as physical chemistry, organic chemistry, biochemistry, physiology and entomology. In the present work the results of chemical and biochemical studies in the broad field as described in the title are presented, and discussion on knowledge gleaned from research in other scientific fields is included.

The first major question which arises is how some insects are able to digest the protein, keratin, which is generally recognised as being a highly indigestible material. When one examines the chemistry of keratin in wool it becomes clear that, if wool is treated with reducing agents so that the disulphide bonds are broken, it is possible to break it down further by enzyme action. The larva of the clothes moth has been shown to have strong reducing conditions and a high pH in the gut, and a proteinase which can act under these conditions (Linderstrøm-Lang and Duspiva, 1936). Powning, Day and Irzykiewicz (1951) however, showed that several other insects have an apparently similar proteinase system, and Waterhouse (1949) has shown that

other insects also have a high pH in the gut. Very little is known about the conditions of oxidation-reduction in the gut of other insects; however, this condition is apparently of prime importance in the digestion of keratin, as native wool has not yet been digested *in vitro* with clothes moth enzymes at any greater rate than with other enzymes (Chapter IX).

It follows then from the above that the process of reduction of keratin represents an important physiological difference between keratinivorous and other insects. All keratin-digesting insects possess highly reducing conditions in the gut, but so far, the process responsible for the maintenance of these reducing conditions has not been described. It should be pointed out, however, that there is no *a priori* reason why a single reducing system should be common to all keratin digesting insects because of the tremendous variation between insects found in other research.

There are two groups of insects among those which digest keratin: (a) the clothes moth, and (b) certain dermestid beetles and chewing lice. When comparisons are made it appears that none of the similarities or differences between these groups yield sufficient data to provide a theory for the digestion of keratin. The two groups have the following similar characteristics. Poor midgut tracheation and consequently a small oxygen supply to the midgut, a neutral to alkaline midgut, a low midgut oxidation-

reduction potential and a positive nitroprusside test (Chapter I). Also they have similar excretion of urea (Chapter VII) and the desulphydrase in the gut has a similar pH optimum (Chapter VIII). The differences between the groups are greater variation in the midgut histology and pH values in Tineola than in the other group, higher pH in Tineola midgut, formation of metal sulphides in Tineola and not in the other group (Chapter I). Also it has been shown that Tineola excretes more uric acid and less cystine (Chapter VII) and contains a much stronger desulphydrase activity (Chapter VIII).

The possibility that the initial reduction of keratin is caused by one of a variety of substances or systems has been investigated. Sulphur dioxide is a strong reducing agent but was not found in Tineola (Chapter VII). Urea is known to have the effect of denaturing some proteins and also accelerates the digestion of wool by certain enzyme systems, but the amount of urea present in the gut is not sufficient to assist greatly in this fashion, although it may contribute (Chapter VII). The action of alkali under certain circumstances is known to liberate -SH groups from proteins but little digestion can be obtained in vitro with Tineola enzyme at the same pH as is found in Tineola gut, or even up to pH 11 (Linderstrøm-Lang and Duspiva, 1936). A possible product of decomposition of keratin through the

action of alkali is lanthionine but none was found after digestion of wool by Tineola. The gut of dermestids and certain chewing lice is almost neutral (Waterhouse, 1953) so digestion of wool depending upon high pH cannot be invoked for these insects.

Keratin is known to be reduced by reaction with sulphide at an alkaline pH. An enzyme, desulphydrase, which can produce H_2S from cysteine in the gut of Tineola has been described (Chapter VIII), but this has not yet been thoroughly investigated to determine how important the enzyme is in the digestion of wool by Tineola. However it is interesting to note that anaerobic in vitro tests with Tineola preparations did not produce any noticeable increase in digestion of wool (Chapter IX). The desulphydrase from the carpet beetle is much weaker than that from Tineola and probably would not contribute greatly to their digestive process.

Linderstrøm-Lang and Duspiva (1936) considered the possible effect of glutathione as a reducing substance but found it difficult to account for digestion on the basis of the very small amounts which could be detected in the cells of the midgut. They preferred the view that the midgut contains a reducing agent lacking -SH groups. This may be true but it is probable that large quantities of the reducing substance would not be required, providing it were recovered by absorption in the hindgut and circulated by the haemolymph

for re-use. It is interesting to note that insect blood contains some reducing substances, of which only a quarter has been accounted for as reducing sugars, whilst nothing is known of the composition of the remainder (Wigglesworth, 1950). The reducing agent in Tineola would be expected to be in the oxidised condition when reabsorbed and may not be estimated by the methods used for detecting reducing substances. However, in this connection it is interesting that Mann (1932) was able to reduce glutathione by glucose plus a dehydrogenase preparation from liver, and Meldrum and Tarr (1935) suggested the reduction of glutathione in rat blood.

The possibility of an enzyme system dehydrogenating an unknown substance and a carrier system then transferring hydrogen to the sulphur bridges of the keratin was mentioned by Linderstrøm-Lang and Duspiva (1936). Owing to the large amount of uric acid in the gut of Tineola it was thought that possibly xanthine oxidase might play a part indirectly (Chapter VII). However, this is less likely in the carpet beetle owing to the smaller quantity of uric acid excreted. Further, an examination of the biochemistry of xanthine oxidase shows that it probably requires the cytochrome system as a hydrogen acceptor and may not be able to transfer hydrogen to other substances. This is supported by Elliott (1928) who showed that glutathione was not reduced

by xanthine oxidase and hypoxanthine. However, an enzyme, which is able to reduce glutathione in rat blood (Meldrum and Tarr, 1935), has been described, and Nickerson and Rowano (1952) have shown the reduction of both glutathione and cystine by yeast preparations, since the reduction of the disulphide bonds of keratin is probably the key to the digestion of keratin by insects, it is considered that an investigation of this process would be the most profitable line of research for the future.

APPENDIX

Modified Micro Bubbler Tips

The modified bubbler tips described below were designed for use in an aeration technique for recovery of H_2S as explain in Chapter V.

It was noticed that, when the normal type bubbler tip (Fig. 7A) was immersed in a liquid and gas passed through, bubbles of a certain size appeared, but when the tip was gently pressed against the bottom of the tube the bubbles broke into very much smaller bubbles. Figure 7B shows the construction of the modified tip which can be made from the normal tip quite easily in the pilot flame of a gas burner. The tip is bent around upon itself and rested on or kept slightly away from the shaft of the bubbler as desired. No measurements of bubble size have been made, but by a visual estimate it would seem that the modification of the tip breaks the bubbles into 4 or 5 of smaller size. A further advantage of the modified tip is that it is considerably more rugged than that in Fig. 7A and can be washed and handled more conveniently.

A thorough search of the literature has not been carried out to discover if this modification is new, but a modified bubbler tip of different design has been described by Gordon (1951). Gordon's modification requires the embedding of fine metallic wires in the end of a glass tube and the production of fine holes following the solution of the wires in an acid.

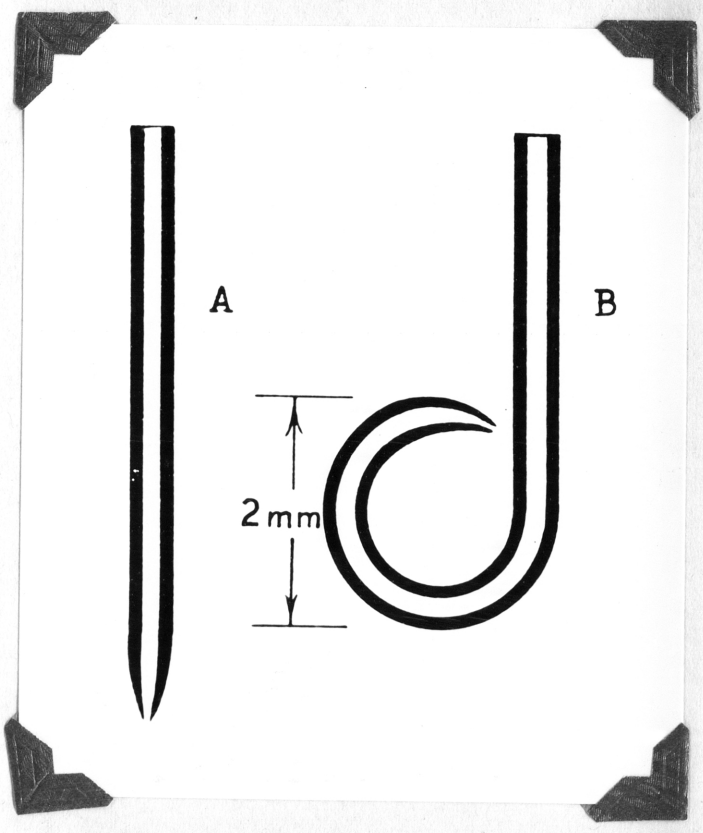


Fig. 8.- Design of bubbler tips

A. Normal tip

B. Modified tip

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