A STUDY OF CYSTEINE DESULPHYDRASE IN CERTAIN INSECTS

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Summary

An enzyme capable of liberating H_2S from *l*-cysteine *in vitro* (cysteine desulphydrase) is present in insect tissues. The clothes moth (*Tineola bisselliella* Humm.) has a highly active desulphydrase apparently concentrated in the gut, whilst some other insects have little or no activity. Aqueous enzyme solutions from insects are unstable, their optimum pH ranges between pH 7·8 and 8·9, they are inhibited by NaCl and KNO_3 (both at 0·42M), and As_2O_3, $NaHSO_3$, and phenylhydrazine (all at 10^{-2}M), but they are not affected by semicarbazide, thiourea, sodium pyrophosphate, or sodium fluoride (all at 10^{-2}M). KCN (10^{-2}M) activates clothes moth preparations, has no effect on those of carpet beetle, and inhibits some other insect enzymes.

Cystine, methionine, homocysteine, glutathione, or thioglycollate do not function as substrates for *Tineola* desulphydrase. The Michaelis constant for *Tineola* desulphydrase under the conditions used is $3{\cdot}8 \times 10^{-2}$M. Dialysis of the clothes moth enzyme against water results in a considerable loss of activity but this did not occur to the same extent in buffered solutions. Activity cannot be restored by the addition of Mn, Zn, or Mg ions (2×10^{-2}M), vitamin B_6 derivatives, folic acid, or boiled undialysed enzyme.

I. Introduction

It has been shown by Waterhouse (1952*a*) that larvae of the clothes moth (*Tineola bisselliella* Humm.) convert heavy metals, ingested with the food, to insoluble sulphides and excrete them in the faeces. These larvae also have a low oxidation-reduction potential in their midgut which is thought to cause the initial stages of digestion of wool (Linderstrøm-Lang and Duspiva 1936). The presence of a reducing substance such as hydrogen sulphide in the *Tineola* gut is therefore of importance in studying the mechanism of wool digestion and metal detoxification by these insects.

The production of H_2S from certain sulphur compounds by enzymes, the desulphydrases, has been observed in bacteria and mammalian tissues (Desnuelle 1939; Fromageot, Wookey, and Chaix 1941; Smythe 1942; Azarkh and Gladkova 1952) and, in view of the presence of cystine in the excreta of clothes moth and carpet beetle larvae (Powning 1953), these and other insects have been examined for desulphydrase activity. Some properties of such an enzyme from *Tineola* larvae have been examined and compared with those of other enzymes from other sources.

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II. Methods and Materials

Cysteine desulphydrase activity was measured by estimating H_2S production from *l*-cysteine (L. Light and Co.) in Thunberg tubes which had been modified as shown in Figure 1. One arm of the apparatus contained 2·4 ml of the enzyme-substrate mixture (see text below for composition), the other

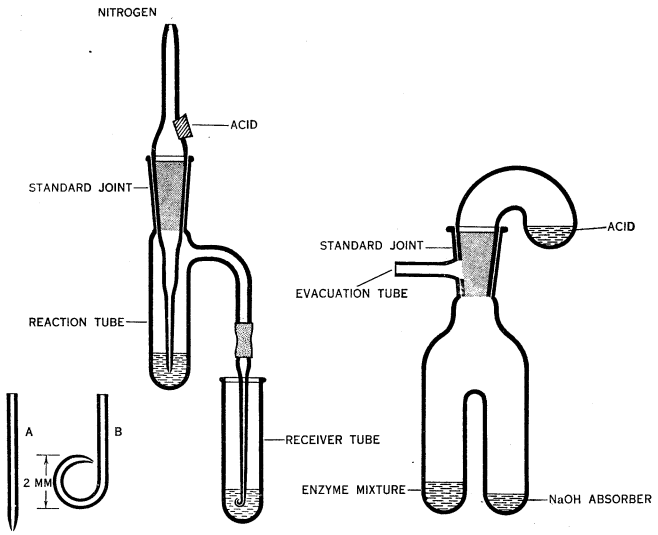

Fig. 1.—Apparatus for estimation of cysteine desulphydrase activity by vacuum diffusion of the hydrogen sulphide produced.

0·5 ml 0·5N NaOH; the stopper contained 0·5 ml 10 per cent. H_2SO_4. The freshly prepared solutions of cysteine and other reagents were adjusted to the desired pH and stored temporarily under anaerobic conditions in Thunberg tubes. The prepared modified Thunberg tubes were evacuated, filled with O_2-free nitrogen, then again evacuated and completely immersed in a water-bath at 37°C. After 2 hr the reaction was stopped by tipping the H_2SO_4 into the enzyme mixture. The H_2S evolved on gently rocking the tube was absorbed in the NaOH, which was then transferred to a 25-ml volumetric flask and the sulphide content estimated by the formation of methylene blue after a slight modification of the method of Fogo and Popowsky (1949). The relationship between optical density at 670 mμ and amount of H_2S appeared to be linear up to about 25 μg H_2S (Fig. 2). In estimations of enzyme activity variations of about ± 5 per cent. from the mean were observed.

The insects used in this work were taken from laboratory cultures of *Tineola bisselliella* (Humm.) larvae, *Ephestia kühniella* Zell. larvae, *Tribolium confusum* Duv. larvae and adults, *Rhizopertha dominica* (F.) adults, *Calandra granaria* (L.) adults, *Oryzaephilus surinamensis* (L.) adults, *Musca domestica* (L.) larvae, and *Anthrenus flavipes* Le Conte (= *A. vorax* Waterh.) larvae. Some *Coptotermes acinaciformis* (Froggatt) workers and adults of *Iridomyrmex*

detectus (F. Sm.) were obtained from the field. Rats used in this work were white laboratory-bred rats from the Australian National University.

All insect enzyme solutions were prepared by grinding the fresh tissue, generally whole insects, with sand and distilled water in a mortar. After centrifugation and removal of fat, all solutions were held at about −20°C, usually in small portions which were used once, then discarded. Samples of $CHCl_3$-treated desulphydrase were prepared according to the method of Smythe (1942).

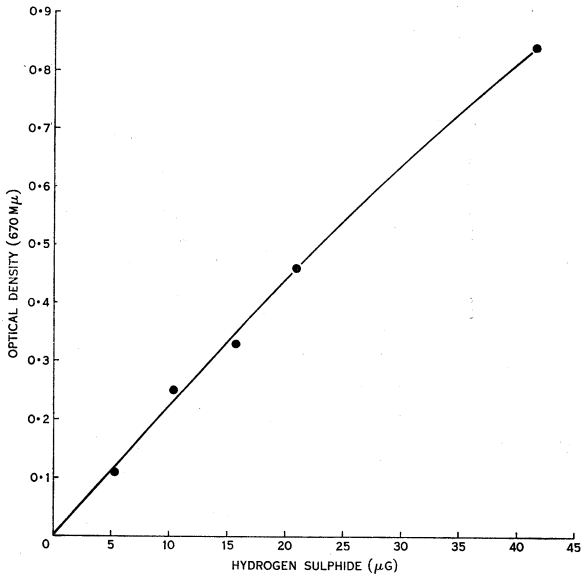

Fig. 2.—Relationship between optical density at 670 mμ and amount of hydrogen sulphide in 25-ml volume.

III. Experimental

(*a*) *General Properties of the Insect Enzymes*

The effects of reaction time and enzyme concentration on the yield of H_2S are shown in Figure 3*A*, *B*. Figure 3*C* represents the variation in the initial reaction rate with substrate concentration and these values are plotted in the reciprocal form of Lineweaver and Burk (1934) in Figure 3*D*. The Michaelis constant (K_m) derived from the slope and intercept of this line is equal to $3{\cdot}8 \times 10^{-2}$M.

Crude or chloroform-treated preparations of *Tineola* desulphydrase lost activity when stored at room temperature or even in the refrigerator, but acetone powders and solutions stored at sub-zero temperatures were relatively stable. High temperatures destroyed the enzyme very rapidly (Table 1).

The effect of pH on the activity of insect enzymes was studied by using 0·2M *l*-cysteine as substrate. In preliminary tests with lower concentrations of cysteine (0·0083M) buffered with phosphate, borate, glycine, histidine, or veronal considerable variations in enzyme activity were observed, depending

upon the buffer used. When tested for 2 hr at 37°C on a final concentration of 0·2M *l*-cysteine the insect enzymes studied were found to have the following optima: *T. bisselliella* pH 8·9, *E. kühniella* pH 8·7, *C. granaria* pH 8·6, *A. flavipes* pH 8·3, *M. domestica* pH 7·7 (Fig. 4).

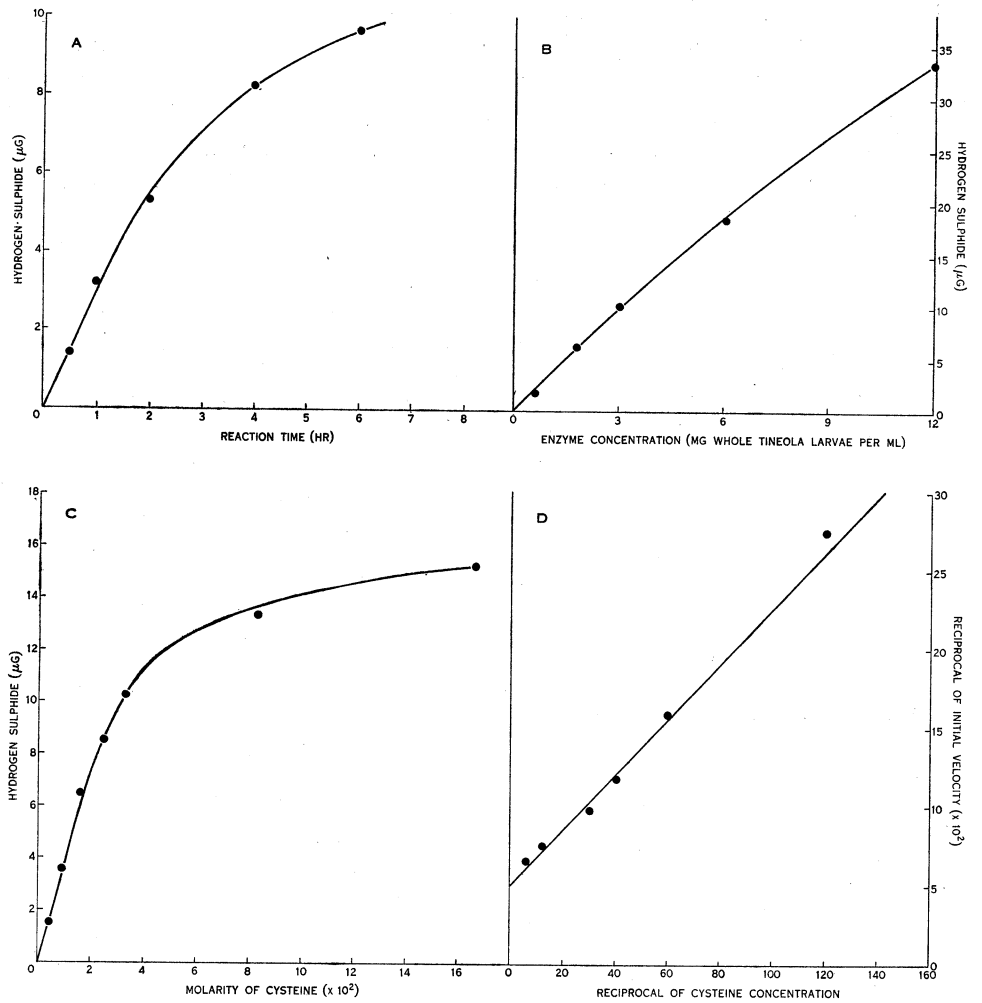

Fig. 3.—Effect of enzyme and substrate concentration and reaction time on the activity of *Tineola* desulphydrase. *A*, enzymic breakdown of cysteine at 37°C and varying times: 0·2M *l*-cysteine at pH 8·9. Buffered enzyme extract from 1·8 mg whole larval tissue. Final volume 2·4 ml. *B*, enzymic breakdown of cysteine in 2 hr at 37°C and varying enzyme concentrations: 0·2M *l*-cysteine, pH 8·9. Final volume 2·4 ml. *C*, enzymic breakdown of cysteine in 2 hr at 37°C and varying cysteine concentrations. Aqueous enzyme extract from 33 mg whole larval tissue. Final volume 2·4 ml. *D*, transposition of Figure 3*C*.

Estimations of desulphydrase in *Tineola* larval extracts showed that the activity varied considerably with the extraction technique. The more usual figure was about 400-600 μg H_2S per g tissue wet weight, although a figure of

4250 μg H_2S per g tissue was obtained for one ice-cold buffered extract. Most of the activity was to be found in the gut, one test yielding an activity of 1250 μg H_2S per g in the gut and only 78 μg H_2S per g in the remainder of the larval body. Comparative tests, using the same technique throughout, showed that *Tineola* larvae had a rather higher desulphydrase activity than a variety of other insects, including *A. flavipes* (see Fig. 4). Adults of *O. surinamensis*, workers of *C. acinaciformis*, larvae and adults of *T. confusum*, and adults of *I. detectus* had little or no desulphydrase activity.

Methionine, homocystine, homocysteine, and thioglycollate did not function as substrates with insect preparations and glutathione yielded an extremely small amount of H_2S. Neither H_2S nor cysteine (nitroprusside test) were obtained from cystine.

TABLE 1

STABILITY OF *TINEOLA* DESULPHYDRASE

All reaction mixtures contained 0·1-1·0 ml enzyme solution, depending upon the activity, a final concentration of 0·2M *l*-cysteine at pH 8·9 in total vol. 2·4 ml held at 37°C for 2 hr. The dialyses were carried out at 0°C for 18 hr with 500 volumes of distilled water, or of 0·01M glycine-NaOH buffer at pH 8·9

Preparation	Treatment	Activity (μg H_2S/ml enzyme)		Decrease in Activity (%)
		Original	*Final*	
Crude *A* (in water) ..	Deep freeze 18 hr	62	61	2
Crude *B* (in buffer pH 8·9)	Deep freeze 18 hr	176	174	0
Crude *A* (in water) ..	Refrigerator 18 hr	62	53	15
Crude *B* (in buffer pH 8·9)	Refrigerator 18 hr	176	158	10
Crude *A* (in water) ..	Dialysed against water	62	17	72
Crude *B* (in buffer pH 8·9)	Dialysed against buffer pH 8·9	176	141	20
Crude *C* (in water) ..	Heated 5 min at 50°C	8·8	3·3	63
Crude *C* (in water) ..	Heated 5 min at 60°C	8·8	0	100
Crude *C* (in water) ..	Refrigerator 9 days	80	30	62
$CHCl_3$-treated *D*	Refrigerator 9 days	23	3·3	85
Acetone-dried powder *E*	Extracted after 9 days at 0°C	10·7	9·8	10
Extract of powder *E* ..	Refrigerator 9 days	10·7	3·4	68

(*b*) *Effects of Various Activators and Inhibitors*

A group of substances, including some carbonyl reagents, was tested on insect preparations (Table 2). All the insect preparations were inhibited strongly by 10^{-2}M As_2O_3 and 10^{-2}M phenylhydrazine, and *Tineola* enzyme was also strongly inhibited by 10^{-2}M hydroxylamine but not by 10^{-2}M semicarbazide. Preparations of *M. domestica*, *C. granaria*, and *E. kühniella* were strongly inhibited by 10^{-2}M cyanide but *A. flavipes* was not affected and *T.*

bisselliella was strongly activated. Other experiments showed that the activation of *Tineola* enzyme occurred also with lower concentrations of cysteine as substrate irrespective of whether the buffer was composed of glycine, veronal, phosphate, borax, or histidine.

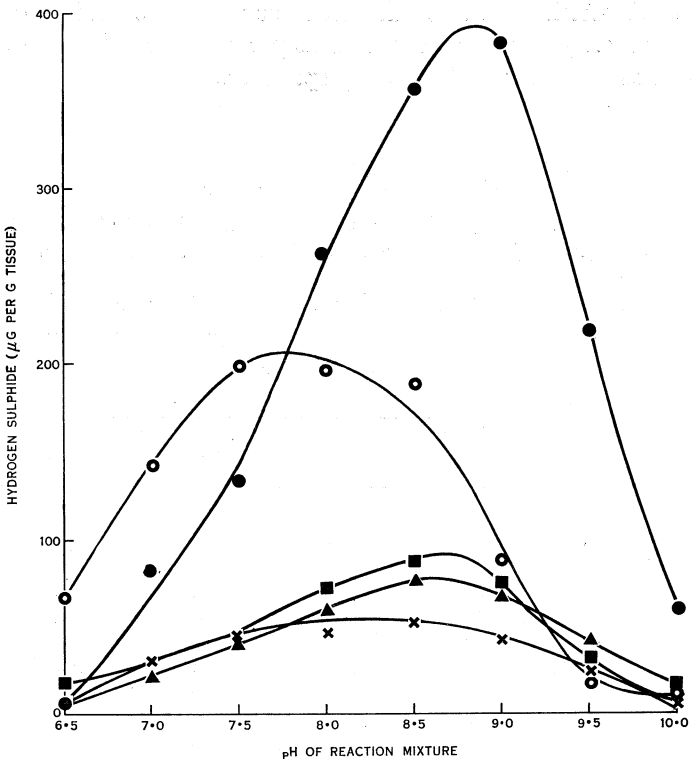

Fig. 4.—Effect of change of pH on desulphydrase in various insects. Reaction mixtures: 0·5 ml enzyme solution, 0·2M *l*-cysteine in final volume 2·4 ml, 2 hr reaction period at 37°C.
●–●, *Tineola bisselliella,* larvae.
O–O, *Musca domestica,* larvae.
■–■, *Ephestia kühniella,* larvae.
▲–▲, *Calandra granaria,* adults.
X–X, *Anthrenus flavipes,* larvae.

Relatively concentrated solutions of inorganic salts, i.e. 0·42M sodium chloride or potassium nitrate, inhibited all the insect enzymes tested (Table 2), but this inhibition was not as marked when a lower concentration of substrate buffered with glycine was used. Sodium fluoride (10^{-2}M) affected the insect enzymes only slightly, the maximum effect being 14 per cent. inhibition on *Anthrenus* desulphydrase. Sodium bisulphite (10^{-2}M), which is in the form of sulphite at the pH used, inhibited the *Tineola* enzyme strongly (Table 2). Two compounds known to combine with metallic ions, i.e. thiourea and sodium pyrophosphate (10^{-2}M), had little effect on the insect preparations.

The effect of sulphur-containing compounds on desulphydrase activity is shown in Table 2. Glutathione (10^{-1}M) and methionine (5×10^{-2}M) did not inhibit the *Tineola* preparation but slightly inhibited the other enzymes. When a lower concentration of substrate (0·0083M cysteine) was used 0·0083M methionine had no effect on *Tineola* enzyme but 0·0083M glutathione produced a 67 per cent. inhibition. Under the same conditions 0·0083M thioglycollate inhibited *Tineola* enzyme to the extent of 32 per cent. Table 2 shows also that 10^{-2}M serine has no effect on *Tineola* preparation.

TABLE 2

EFFECTS OF VARIOUS COMPOUNDS ON INSECT DESULPHYDRASE

Reaction mixture: 0·5 ml enzyme, 0·5 ml reagent solution, total volume 2·4 ml, final concentration *l*-cysteine 0·2M. All solutions adjusted to optimum pH for enzyme action and held at 37°C for 2 hr. The enzyme solutions had the following activities in μg H_2S per ml liberated under the above conditions. *Tineola* 16; *Anthrenus* 11; *Musca* 29; *Calandra* 32; *Ephestia* 18

Compound	Change in Activity of Enzymes (%)				
	Tineola	*Anthrenus*	*Musca*	*Calandra*	*Ephestia*
KCN 10^{-3}M	+33	+3	−6	−1	−4
KCN 10^{-2}M	+63	0	−38	−31	−33
As_2O_3 10^{-2}M	−47	−65	−71	−54	−38
Phenylhydrazine 10^{-2}M	−24	−52	−30	−51	−69
NaCl 0·42M	−43	−55	−63	−43	−54
KNO_3 0·42M	−73	−75	−88	−75	−74
Thiourea 10^{-2}M	+5	+4	+1	+3	0
$Na_4P_2O_7$ 10^{-2}M	−6	−19	0	−6	−10
NaF 10^{-2}M	−6	−14	−10	−12	−4
Glutathione 10^{-1}M	+4	−23	−30	−14	0
Methionine 5×10^{-2}M	0	−2	−4	−17	−19
Semicarbazide 10^{-2}M	−6				
Hydroxylamine 10^{-2}M	−52				
$NaHSO_3$ 10^{-2}M	−84				
Serine 10^{-2}M	−7				

(*c*) *Effect of Possible Coenzymes*

An aqueous extract of *Tineola* larvae lost 72 per cent. of its desulphydrase activity on dialysis against distilled water overnight in the refrigerator. Thirteen per cent. of activity was lost in an undialysed control, and the net effect of dialysis was therefore about 60 per cent. loss of activity (Table 1). An extract of *Tineola* larvae in 0·01M glycine-NaOH buffer, pH 8·9, lost only a net 10 per cent. of activity when dialysed against the same buffer overnight in the refrigerator. No reactivation of dialysed *Tineola* desulphydrase was observed on addition of a boiled undialysed extract, or of low concentrations of Mg, Mn, or Zn salts. In fact, Mg and Zn at 2×10^{-2}M were inhibitory.

TABLE 3

ATTEMPTED REACTIVATION OF DIALYSED *TINEOLA* EXTRACTS

Reaction mixture: 0·5 ml dialysed *Tineola* extract, 0·5 ml solution of Mn^{++}, Mg^{++}, or Zn^{++}, or boiled undialysed enzyme, 0·2M *l*-cysteine in final volume of 2·4 ml, pH 8·9. Held at 37°C for 2 hr. Activity of the *Tineola* enzyme solution under the above conditions equals 18·2 μg H_2S per ml

Additive	H_2S (μg)	Change in Activity (%)
0	9·1	
$MnSO_4$ 2×10^{-2}M	4·1	−55
$MgSO_4$ 2×10^{-2}M	9·0	0
$ZnSO_4$ 2×10^{-2}M	2·7	−70
Boiled undialysed enzyme	9·1	0

A dialysed extract of rat liver was activated on addition of pyridoxal phosphate or of ATP and either pyridoxal or pyridoxamine (Table 4). When dialysed *Tineola* extract was tested in the same type of experiment, it was found that pyridoxal or pyridoxamine alone inhibited the enzyme; however, when the vitamin B_6 derivatives and ATP were mixed no inhibition or activation was observed. The addition of pyridoxal phosphates to dialysed *Tineola* preparations also had no effect on their desulphydrase activity (Table 4).

TABLE 4

EFFECTS OF VITAMINS AND THEIR DERIVATIVES ON DESULPHYDRASE

Reaction mixture: 0·5 ml enzyme solution (dialysed against water 18 hr at 0°C), buffer and substrate as under, additives as shown; total volume 2·4 ml. Held at 37°C for 2 hr. The reaction mixture for the rat-liver and the *Tineola A* experiments contained 1 ml M/10 veronal-HCl buffer at pH 7·4 and 8·9 respectively and a final concentration of $8·6 \times 10^{-2}$M *l*-cysteine. The *Tineola B* experiment contained only a final *l*-cysteine concentration of 0·2M at pH 8·9. Original activity of enzymes in μg H_2S/ml was: rat liver = 5·4; *Tineola* expt. *A* = 21; expt. *B* = 20

Additive	Change in Activity (%)		
	Rat Liver	*Tineola*	
		Expt. *A*	Expt. *B*
ATP alone (2 mg)			0
ATP (2 mg) + 0·2 mg pyridoxal	+90	+5	−2
ATP (2 mg) + 0·2 mg pyridoxamine	+59	−9	−2
Pyridoxal alone (0·2 mg)	0	−79	−55
Pyridoxamine alone (0·2 mg)	−8	−88	−70
Pyridoxal phosphate (0·002 mg)	+39		+2
Folic acid (0·2 mg)			−2

IV. Discussion

Tineola larvae have a very active cysteine desulphydrase which is concentrated mostly in the gut. Extracts of some other insects and of rat liver appeared to be less active than those of *Tineola,* and some insects had no desulphydrase activity. It has been shown that the desulphydrases from the various insects tested have their pH optima in the alkaline range between pH 7·7 and 8·9, this being higher than that for the rat liver desulphydrase (about pH 7·4-7·8, Smythe 1942).

Although a Michaelis constant has not been published for other desulphydrases, Lawrence and Smythe (1943) have illustrated the relationship between rat liver desulphydrase and cysteine concentration under their conditions. Calculations from their published graph yields a figure of approximately $4{\cdot}6 \times 10^{-2}$M. It appears that there is a close similarity between rat liver and *Tineola* larval enzymes in this respect.

Waterhouse (1952*a,* 1952*b*) has shown that heavy metals such as mercury, copper, cobalt, etc., when given in the food, are excreted as sulphides by the clothes moth (*Tineola*) but not by the carpet beetle (*Attagenus*). It is possible that the desulphydrase in the *Tineola* larva may play a part in this detoxification mechanism. The relatively weak desulphydrase in carpet beetles is interesting in view of the lack of sulphide formation in these insects. The difference in desulphydrase activity between these two groups of keratin-digesting insects may also be correlated with the low cystine excretion in *Tineola* compared with that of *Attagenus piceus* (Powning 1953).

Laskowski and Fromageot (1941) found that crude dog liver desulphydrase was rather unstable since it lost activity rapidly on heating at 50°C and pH 6, and even on storage at 4°C. They also found that an acetone-dried powder was stable. These properties are matched closely by *Tineola* preparations (Table 1).

Neither Smythe (1942) nor Fromageot, Wookey, and Chaix (1941) found any large decrease of activity after dialysis of rat or dog liver desulphydrase; however, Binkley (1943) found a strong inhibition on dialysing preparations from microorganisms or mouse liver. A crude aqueous extract of *Tineola* desulphydrase lost considerably more activity on dialysis against water than when dialysed against buffer at pH 8·9. It appears likely that the loss of activity in water is due to denaturation of the enzyme. Vitamin B_6 derivatives have been shown by other workers to function as coenzymes in the desulphydrase systems of rat liver and microorganisms (Braunstein and Azarkh 1950; Kallio 1951; Azarkh and Gladkova 1952). However, the desulphydrase of *Tineola* larvae, after dialysis against water, is not activated by these compounds (see Table 4). It is possible that specificity exists for certain vitamin B_6 derivatives, i.e. pyridoxamine phosphate may be required instead of pyridoxal phosphate. The reverse of this has been shown to be true of coracemase activity in *Streptococcus faecalis* (Wood and Gunsalus 1951) in which pyridoxamine phosphate is inactive. No pyridoxamine phosphate was available; however, the test with pyridoxamine and ATP was negative.

It appears that even the 10 per cent. activity lost on dialysis against buffer is probably not due to the loss of a dialysable B_6 derivative. It is likely that if a coenzyme is required for this enzyme it is firmly attached to the apoenzyme and is not removed by dialysis. Mouse liver preparations suffered inhibition on dialysis but this effect was reversed on addition of 10^{-3}M Mn, Zn, or Mg ions; further inactivation followed addition of 10^{-3}M sodium fluoride (Binkley 1943). Slightly higher concentrations of Mn and Zn ions (2×10^{-2}M) than those used by Binkley were found to further inhibit dialysed *Tineola* preparations, although the same concentrations of Mg ions and sodium fluoride had no effect. In view of these results it is interesting that Smythe (1942) has discussed the possible role of Mg as a component part of rat liver desulphydrase.

An activation of desulphydrase by folic acid was described by Binkley, Christensen, and Jensen (1952). No activation was found with liver or bacterial preparations (Azarkh and Gladkova 1952), nor in the present work in dialysed rat or *Tineola* preparations.

Low concentrations of various compounds known to react with carbonyl groups cause a strong inhibition of rat liver desulphydrase (Lawrence and Smythe 1943). This was found to be true in general also of five insect preparations using 10^{-2}M phenylhydrazine, but *Tineola* extract was little affected by 10^{-2}M semicarbazide, also a carbonyl reagent. No explanation can be offered at this stage for the behaviour of the enzymes with 10^{-2}M potassium cyanide, which strongly activated *Tineola* desulphydrase but inhibited other insect enzymes as well as rat liver preparations. The effects of compounds forming metal complexes, inorganic salts in high concentration, and the possibility of a competitive inhibition by glutathione and thioglycollate show similarities between insect and rat liver preparations (see Lawrence and Smythe 1943).

Rat liver extracts have been shown to liberate H_2S from cystine at the same rate as from cysteine, and it was suggested that cysteine and sulphur could be end-products of this reaction (Smythe 1942). It was thought that if this reaction occurred in keratin-digesting insects it might be of importance in the primary breakdown of the disulphide bonds of keratin. It has been shown, however, that insects do not liberate H_2S from methionine, thioglycollate, or homocysteine and that neither H_2S nor cysteine are obtained from cystine. Possibly there was some reducing material present in the rat liver extracts which did not occur in the insect extracts.

V. Acknowledgments

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