Bee World

ISSN: 0005-772X (Print) (Online) Journal homepage: http://www.tandfonline.com/loi/tbee20

The Applicability of the Holst Milk Test for A.F.B. to Studies on Pollen, Bee Bread and Other Hive Materials

N. Patel & T. A. Gochnauer

has a number of advantages over the traditional methods of beekeeping. The quality of beeswax exported has been improved by the enforcement of new export rules, but a considerable wastage still occurs. The production of high-grade honey has been started, and an appreciation of the value of honeybees as pollinators is developing.

REFERENCES

HARRIS, W. V. (1931) A short note on the races of honey bees in Tanganyika Territory *Bee World* 12(8) : 90–91

—— (1940) Beeswax *Pamphl. Tanganyika Dep. Agric.* No. 23

Skaife, S. H. (1930) The South African honey-bee *S. Afr. Bee J.* 5(2) : 12–16

Smith, F. G. (1951*a*) Preliminary report on *Trigona* wax *E. Afr. agric. J.* 16(4) : 185–187

—— (1951*b*) Beekeeping observations in Tanganyika, 1950/51 *E. Afr. agric. J.* 17(2) : 84–87

—— (1952) Beekeeping observations in Tanganyika, 1951/52 *E. Afr. agric. J.* 18(2) : 59–61

—— (1953) Beekeeping in the tropics *Bee World* 34(12) : 233–245

—— (1954) Notes on the biology and waxes of four species of African *Trigona* bees *Proc. R. ent. Soc. Lond. Ser. A* 29 (4/6) : 62–70

—— (1955) Beeswax *Pamphl. Tanganyika Bee Div.* No. 1

—— (1956*a*) Honey *Pamphl. Tanganyika Bee Div.* No. 3

—— (1956*b*) Bee botany in Tanganyika *University of Aberdeen* : *D.Sc. Thesis*

—— (1957) Bee botany in East Africa *E. Afr. agric. J.* 23(2) : 119–126

THE APPLICABILITY OF THE HOLST MILK TEST FOR A.F.B. TO STUDIES ON POLLEN, BEE BREAD AND OTHER HIVE MATERIALS*

N. PATEL & T. A. GOCHNAUER

Dept. Entomology & Econ. Zoology, University of Minnesota, St. Paul, U.S.A.

The Holst milk test for American foul brood was based on the finding of Holst and Sturtevant (1940) that diseased brood showed a milk-curdling and digesting (peptonizing) activity. This peptonizing activity was proposed as a specific test for the disease by Holst (1946), on the basis of studies which showed that neither healthy larvae, nor larvae infected with other agents, nor various hive materials, showed any peptonizing activity. It was suggested by Holst and Sturtevant that the enzyme was intimately connected with the process of sporulation in the causative organism *Bacillus larvae*, since growing cultures produced acidity without peptonization in skimmed milk ; further, non-sporulating mutants liberated no enzyme when the cells were autolysed, indicating that the vegetative cell contained no such enzyme.

Hitchcock (1956) reviewed the literature on enzymes in stored pollen, and also reported his results which appeared to demonstrate a peptonizing enzyme in pollen stored in the comb (bee bread), the activity of which was described by use of the original Holst test for A.F.B. Such activity, he felt, should not interfere with the Holst test as a diagnostic agent, since the contamination of A.F.B. samples with pollen was not likely. We have found, however, that the samples of comb-stored pollen and dried pollen from foraging bees available to us have no peptonizing activity, but are sufficiently acid to curdle skimmed milk, and thus produce a false positive reaction in the Holst test as originally used. An explanation of the dif-

* Paper No. 3873 Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 1, Minnesota

ferences between our results and those of Hitchcock is given below, together with some general considerations on the Holst test reaction of A.F.B. larval scales.

METHODS AND RESULTS

Hitchcock used the Holst test as originally described for field use in the diagnosis of A.F.B. We have found it more convenient to prepare a 4% (w/v) suspension of Difco powdered skimmed milk in distilled water as our basic substrate. To this were added clarified water extracts of the various substances tested. This procedure avoids a possible masking of milk-peptonizing activity by the turbidity caused by release of spores, pollen grains, or other solids into the test medium from the solid materials (which are themselves used in the Holst test). The materials examined for enzymatic activity were weighed, suspended in distilled water, and blended by use of the Waring Blendor. Solid particles in suspension were removed by centrifuging and, where required, by filtering through a sintered-glass filter. The Seitz asbestos filter cannot be used, because the activity from the A.F.B. scale is removed by the filter.

If it was necessary to concentrate the extracts, this was done by vacuum distillation at room temperature, until a volume 0·6 of the original was obtained.

The following concentrations per 10 ml. distilled water were used to prepare the initial extracts used : A.F.B. scales, 60 scales or 1·38 g.; bee bread, the contents from 30 pollen cells or 4·2 g.; pollen pellets, 3·0 g.; dissected honeybee stomachs, 60. Final volumes were reduced to 6 ml. as described.

For testing, 0·3 ml. of the concentrated extracts were added to 5 ml. skimmed-milk substrate in tubes maintained at 50°C. in a water bath.

TABLE 1. Assay of hive materials for milk-digesting activity
T tube containing reaction mixture at 50°C.
H tube containing extract heated to 100°C.
+ positive milk digestion; — negative reaction
p casein precipitated without apparent digestion

time (min.)	*controls (no extracts)*		*midgut*		*A.F.B. scales*		*bee bread*		*pollen pellets*					
									5 *yr.*		10 *yr.*		20 *yr.*	
	T	H	T	H	T	H	T	H	T	H	T	H	T	H
15	—	—	—	—	—	—	—	—	—	—	—	—	—	—
30	—	—	±	—	+	—	p	p	—	—	—	—	—	—
45	—	—	+	—	+	—	p	—	—	—	—	—	—	—
60	—	—	+	—	+	—	p	p	—	—	—	—	—	—
90	—	—	+	—	+	—	p	p	—	—	—	—	—	—
120	—	—	+	—	+	—	p	p	—	—	—	—	p	—

The tubes were examined at intervals for evidence of turbidity reduction (indicative of digestion) ; results obtained are given in Table 1. A second set of tubes, with 0·3 ml. extract, was heated to 100°C. for 2 minutes, before mixing with substrate. The results suggest that the reaction brought about by bee-bread extract is heat-stable, and produces precipitation rather than digestion of the casein. It seemed likely that the results might be explained by a high level of acidity in the bee bread, and this was demonstrated by measuring the pH of the various extracts with a glass electrode (Beckman Model H2 pH meter) :

midgut	6·3
scale	4·8
bee bread	3·9
pollen 20 years old	5·1
bee bread + skimmed milk..	4·6
scale + skimmed milk ..	6·0

The results demonstrate the free acidity found in the extracts of bee bread, and the acid reaction — resulting in precipitation — which occurs when these extracts are mixed with skimmed milk. When the extract of bee bread was brought to neutrality with NaOH, no milk-digesting activity was found in the neutralized extract.

Enzymes are frequently characterized by their failure to pass through a membrane of appropriate effective pore size. In practice, cellophane dialysis tubing is used to remove diffusable extraneous substances from the crude enzyme, which is retained. Bee-bread extract was placed in dialysis tubing and dialysed overnight against twice the volume of cold distilled water. The dialysed portion and the dialysate reached virtual equilibrium at pH 4·0 for the dialysed portion remaining in the tubing, and 3·9 for the dialysate or diffusate.

Both fractions precipitated skimmed milk, when added 1 : 1 to a 4 per cent suspension. Neutralization of the extracts removed their ability to precipitate the milk. There was no evidence, after 4 hours incubation, of any digestion of the skimmed milk by neutralized extracts. Final reaction mixtures attained a pH of 5·3 (after precipitation) for the un-neutralized fractions, compared with 6·0 – 6·5 for the neutralized fraction - skimmed milk reaction mixture.

DISCUSSION

From the data presented, it seems likely that the ' active ' agent in bee bread is the free acidity of the extract. The precipitating activity of boiled extracts, together with the appearance of ' activity ' in dialysate from the extracts, indicates the probability of a non-enzymatic reaction. Svoboda (1935), in his study of the preservation of pollen stored in the comb, stated that bee bread undergoes a lactic-acid fermentation, and this would account in part for the acid encountered. Lactic fermentations may also result in the release of proteolytic enzymes by the micro-organisms involved, but this does not seem to have occurred under the conditions of the present experiments. The results of these assays do not preclude the presence of other proteolytic enzymes with activities and characteristics differing from those which would appear under the conditions imposed by the milk test. In fact there is some evidence (Patel, unpublished data 1957) of a rennin-like enzyme in bee bread. However, our interpretation is that a light precipitate was formed by the effect of acid on the dilute skimmed milk used by Hitchcock, and that this may very well have been obscured by the presence of pollen pellets, bee bread, *etc.*, which he used as an enzyme source. It has been our experience that the turbidity of dilute skimmed milk may be affected by a number of non-enzymic variables — including the salt concentration, pH level, and the presence of such oxidants as dilute peroxide—in such a way as to simulate the effect of enzymatic reduction of the turbidity. It is fortunate that A.F.B. scales fulfil the requirements of pH when in a suspension of skimmed milk, and that false reactions do not occur. This seems not to be so with other materials, and caution is needed when using them.

The activity of the scale enzyme is of continuing interest to us in this laboratory, and separation of other enzymes or enzyme activity from that of the scale enzyme assumes importance in this field. It has been observed (Gochnauer, 1957) that vegetative cells of *Bacillus larvae* liberated milk-digesting activity when they were ruptured by grinding with alumina. It was further observed that vegetative cultures grown on liver nutrient agar containing skimmed milk produced milk-digesting activity long before sporulation could be seen. It is not known whether the same or a similar enzyme is liberated during the growth of the organism in infected larvae, but it seems possible that this might occur, and play a role in the disease.

CONCLUSIONS

The application of the Holst milk test to studies of proteolytic enzymes from sources other than honeybee larvae infected with A.F.B. must be done with caution, to avoid false positive reactions. Our observations indicate that the reaction of bee bread with dilute skimmed milk arises from an acid precipitation of the casein rather than from true digestion.

ACKNOWLEDGEMENTS

We are indebted to Professor M. H. Haydak and Mr. J. D. Hitchcock for the samples of bee bread and aged pollen pellets used in these studies.

REFERENCES

GOCHNAUER, T. A. (1957) Proteolytic activity associated with *Bacillus larvae*. *Bact. Proc. Soc. Amer. Bact.* : 62

HITCHCOCK, J. D. (1956) A milk-digesting enzyme in pollen stored by honey bees *Amer. Bee J.* 96(12) : 487–489

HOLST, E. C. (1946) A simple field test for American foulbrood *Amer. Bee J.* 86(1) : 14, 34

HOLST, E. C. & STURTEVANT, A. P. (1940) Relation of proteolytic enzymes to phase of life cycle of *Bacillus larvae*, and two new culture media for this organism *J. Bact.* 40(5) : 723–731

SVOBODA, J. (1935) Konservování pylu *Čes. Včelař* 69(5) : 177–179

THE ROYAL JELLY BOOM

Publications on the components, properties and uses of royal jelly are abstracted in *Apicultural Abstracts*, but there is no intention to report there work which does not come up to normal scientific requirements. Unfortunately royal jelly is now of interest not only to those concerned with the properties it does possess ; it has also come to have a peculiar fascination for unscrupulous people in a number of countries, who see in it a possibility of making money easily and quickly.

There is now a flood of literature about royal jelly which is concerned more with describing properties which the authors wish their readers to believe it possesses, than with what has been proved by properly conducted experiments. Many Governments are taking action to protect the public from being misled by unwarranted claims ; some items of news from different countries are given below.

In **England** a firm trading as Apiserum has been fined £70 on four charges at Kettering, the charges arising out of misleading advertisements,