Miscellanea : Demonstration of Trehalose in the vector of African Trypanosomiasis : the tsetse fly

Autor(en): **Geigy, R. / Huber, M. / Weinman, D.**

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Demonstration of Trehalose in the Vector of African Trypanosomiasis: the Tsetse Fly.

By

R. GEIGY and M. HUBER, Swiss Tropical Institute, Basle.
D. WEINMAN and G. R. WYATT, Departments of Microbiology and Biochemistry, Yale University, New Haven, Connecticut. [1]

Introduction.

One of the major problems in microbiology is to define the factors which distinguish a pathogenic microorganism from a non-pathogenic one. The question is highly complex, and involves a number of subsidiary problems. An essential point to be determined is why some organisms can infect man and other mammals, while others can not.

Studies on the mechanisms which control infectivity can be carried on particularly well with the African Sleeping Sickness trypanosomes. *Trypanosoma gambiense* and *Trypanosoma rhodesiense* can be readily isolated from patients or animals on blood agar slants (WEINMAN 1957, 1959). Growth is abundant and the cultures can be propagated indefinitely by serial transfer. However, these cultures have one striking characteristic: they are never infectious for mammals. This is a sudden loss of infectivity which takes place on the first transfer of the blood trypanosomes to medium, sometimes within 24 hours (HENRARD & PEEL 1950).

When the culture trypanosomes are stained and examined, they show the structural organization of the types which are found in *Glossina,* not those which occur in the blood of mammals (WEINMAN 1953). However, in *Glossina* these very same types evolve further, and eventually do become infectious for mammals. It is obvious that *Glossina* provides something for the trypanosomes which is lacking in cultures.

In 1956 and 1957, it was reported that trehalose is a major blood sugar of a variety of insects (WYATT & KALF 1956/1957). Accordingly, it was decided to investigate the effect of trehalose on the restoration of infectivity to cultured trypanosomes, although it was not known prior to the present report whether trehalose does occur in *Glossina.*

Initial observations showed that *T. rhodesiense* maintained in cultures for periods up to 69 days after isolation became infectious when grown with added trehalose. Control cultures consisting of the same strain of trypanosome grown upon the same batch of medium for the same period and maintained under the same conditions, but without trehalose, were non-infectious (WEINMAN, 1957).

The response to trehalose has now been found in another strain of *T. rhodesiense* and one of *T. gambiense.* While all strains do not act precisely alike, and there are other variables concerned, it has become very clear that cultures containing added trehalose may be infectious, whereas cultures on the same medium without additions never are. Now under study is the effect of other carbohydrates and a formal demonstration that it is indeed the trehalose in the commercial preparation of the sugar and not, as seems very unlikely, some impurity, which is the active substance. Meanwhile, it became of the highest interest to determine whether trehalose is to be found in *Glossina,* and if

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possible to ascertain the distribution of this sugar in different parts of the body of the insect.

Supply and Fixation of Tsetse Material.

Our studies required fresh tsetse material from several thousands of flies, that is more than could be obtained from any laboratory colony. Two of us (G. and H.) therefore went to Ifakara in the Ulanga-District of Tanganyika to the Tropical Institute's field laboratory. In addition to the equipment required for dissecting and fixing tsetse organs this laboratory offers also various other technical facilities, such as breeding rooms for insects and small mammals, optical equipment, cold storage for the dissected organs, and others. At a distance of about 5 to 10 km. from Ifakara is a fly belt in the so-called "miombo" (grassland with *Brachystegia* trees), which is the specific habitat of *Glossina morsitans*. Besides this main species *G. brevipalpis* is found also, though less frequently, in certain riverine thickets and *G. pallidipes* in certain moist ground depressions. The colonies of *G. pallidipes* were too small to satisfy our requirements. From time to time the very big species *G. brevipalpis* was used, the organs of which are three to four times heavier than those of *G. morsitans,* so that a considerably smaller number of flies of this species had to be dissected in order to obtain a given quantity of fixed organs. However, preference was deliberately given to *G. morsitans,* as this species is one of the most important carriers of sleeping sickness. Near Sofi, south of Ifakara, there is a well-known infected area of *Trypanosoma rhodesiense.*

For the capture of *G. morsitans* it was best to drive the car at moderate speed through the tsetse area and to stop every 50 to 100 m. at shady places. The flies were attracted by the passing landrover, they settled on the canvas hood and could easily be caught with fly-nets. The best time for catching was the early morning and the evening at sunset, preferably with slightly clouded sky, while towards midday, during the heat and very clear sky, the number of flies attracted was smaller. On the homeward journey it was necessary to protect the fly cages with banana leaves from the deadly heat of the sun and dessication. Three persons, a European and two natives trained as fly boys, were able in this way to collect 150 to 200 specimens of *G. morsitans* in 1½ to 2 hours under favourable conditions.

G. brevipalpis was not attracted by the car. In the time between 17.30 and 18.15—the best for catching—the males were sitting on leaves in the bush or on the ground, shortly before dark also on the sandy road where they had to be caught singly. No females were obtained, as these rest either in the breeding haunts or high up in the tree tops (for the biology of *G. brevipalpis* cf. BRUCE, 1914; BURTT, 1950; GEIGY, 1950; VAN DEN BERGHE, 1955). Each outing yielded about 40 flies, in particularly successful cases up to 60 flies.

Fixation was done only with fresh flies still alive, caught the same day or the previous evening. Generally only hungry flies bite; so practically all the collected ones had empty intestinal tracts (designed as "unfed" in the tables). Some of them, used for whole fly-preparations, were fed on a goat. The following material was fixed: whole flies, as well as the organs involved in the transmission of trypanosomes, i.e. the whole intestinal tract and the salivary glands (MURGATROYD and YORKE, 1937). Examination of the hemolymph had to be omitted as it is present in these flies in quantities too small to be collected.

The flies were dissected unnarcotisized. After removal of legs and wings the salivary glands and intestines were extracted under ice cold Ringer solution with very fine tweezers; a binocular magnifying glass ×15 was used. With

practice it was found sufficient to open the anterior part of the abdomen between two tergites. First the slightly winding crystal clear salivary glands, lying dorsally under a thin layer of fat body, were carefully extracted from the abdomen and thorax in their full length and placed in the fixing solution. However, it was never possible to obtain the two glands joined together, but the common duct was always present. Then the anterior gut section with the proventriculus and the anterior part of the midgut were removed from the thorax. Usually the crop was also included and the whole winding midgut and the hindgut with the rectal ampulla carefully separated from the adherent tracheae, fat body and Malpighian tubes. In order to obtain easily the rectal ampulla, the hypopygium of the male and the posterior end of the abdomen of the female were removed before the gut was prepared. Rupture of the intestine occurred only exceptionally, so that the organ could be fixed with its whole intact content.

Dissection of a pair of salivary glands seldom required more than 30 seconds from the opening of the fly in Ringer solution to their fixation. The time required for the intestinal tract was about 45 seconds.

Fixation was done with absolutely pure ethyl alcohol 70%. At the beginning (preparations of *G. morsitans* of 26.9 and 23.10; of *G. brevipalpis* of 26.9 and 18.10) we tried to determine the fresh weight in addition to the weight after fixation (Table I). In order to obtain a ponderable quantity it was necessary to collect the day's output in a refrigerator on ice. After weighing the intestines were fixed in alcohol 70% at 70°C. As for the salivary glands the size of these organs is so small that even a day's yield is too insignificant to make any weighing attempt worth while [2]. The glands were therefore collected in cold alcohol 70% and each evening the whole day's output was heated to 70°C. (Shipments of 18.10. = *G. morsitans;* 23.10. = *G. brevipalpis*). Whole flies were fixed undissected in cold alcohol 70% (*G. morsitans* 8.10. and 18.10.).

Analysis of these organ preparations and of whole flies showed, however, that fixation had to be done very quickly in heated alcohol in order to avoid degradation of sugars through certain enzymes still active in cold alcohol. Therefore, all the subsequent material was fixed in alcohol 70%, heated to 70°C. Salivary glands and intestines were fixed in this way immediately after preparation, without the fresh weight being determined. Experiments made at Yale University in the meantime with *Sarcophaga bullata* showed that whole flies have to be cut up before fixation, as even hot alcohol penetrates chitinous substance very slowly, so that enzymatic degradation processes may still occur inside whole flies. In order to examine the results obtained with whole flies three tests were made (11. 12., 9. 1., 31. 1.) in which 20 *G. morsitans* on one side were fixed in 10 ml. hot alcohol 70%, on the other side 20 *G. morsitans* in 10 ml. hot alcohol with an addition of 10 mg. glucose and 10 mg. trehalose per litre. As explained below these tests should show conclusively whether the whole amount of sugars present at the beginning could be found again by chemical analysis or whether fixing and chemical preparation would lead to certain losses.

In order to obtain 100 mg. salivary glands of *G. morsitans* approximately 1300 flies had to be dissected. The same weight was obtained from 320 specimens of the much bigger *G. brevipalpis*. Approximately 70 *G. morsitans* yielded 100 mg. intestinal tracts, while 25 *G. brevipalpis* only were necessary

[2] Mean weight of one *G. morsitans* intestinal tract = 1.43 mg. (after fixation); mean weight of one *G. morsitans* pair of salivary glands = 0.077 mg. (after fixation). The weight of an intestinal tract is approximately 20× that of a pair of salivary glands.

TABLE I

Preparations of Whole Flies and Tsetse Organs Supplied from Ifakara.

The sample numbers indicate the preparations which gave reliable results (see Table II).

Dispatched Ifakara	Arrived Yale	GLOSSINA MORSITANS											GLOSSINA BREVIPALPIS						
		whole flies			*intestines*				*salivary glands*				*intestines*				*salivary glands*		
		number of flies	fresh weight mg	sample No.	number of flies	weight in mg fresh	weight in mg fixed	sample No.	number of flies	weight in mg fresh	weight in mg fixed	sample No.	number of flies	weight in mg fresh	weight in mg fixed	sample No.	number of flies	weight in mg fresh	weight in mg fixed
26. 9. 58	2. 10. 58				84[1] u	125	115						27[1] u	125	115				
8. 10. 58	13. 10. 58	150[2] u	3490*																
18. 10. 58	27. 10. 58	100[2] f	2325*						647[5] u	60+	50		38[1] u	170	140				
23. 10. 58	30. 10. 58				80[1] u	125	110										160[5] u	60+	50
8. 11. 58	18. 11. 58	100[3] u	2325*	6	82[6] u	144+	120	8					25[6] u	132+	110	4			
		100[3] f	2325*	7															
4. 12. 58	11. 12. 58								1336[6] u	120+	100	9							
11. 12. 58	22. 12. 58	20[3] u	465*	10															
		20[4] u	465*																
29. 12. 58	5. 1. 59								1352[6] u	120+	100	12							
9. 1. 59	15. 1. 59	20[3] u	465*	14	80[6] u	144+	120	16					22[6] u	106+	90	5			
		20[4] u	465*																
31. 1. 59		20[3] u	465*	17															
		20[4] u	465*																
Total of flies used and of organ weights		570	13255		326	538	465		3335	300	250		102	533	450		160	60	50

[1] Fixed in alcohol 70% after storage on ice.
[2] Whole flies fixed in cold alcohol 70%.
[3] Cut up in hot alcohol 70%.
[4] Cut up in hot alcohol 70% + sugar.
[5] Fixed in cold alcohol 70%.
[6] Fixed in hot alcohol 70%.

* weight calculated on the basis that 1 fly weighs 23.25 mg.
\+ weight calculated on the basis that fresh weight = fixed weight × 1.20.
u unfed.
f fed.

for the same quantity. Table I shows the supply of preparations of organs and of whole flies.

Preparations were sent by air mail, packed in small glass tubes with wax sealed corks. Close contact with Yale University was constantly maintained and the preparations were examined within the shortest time possible so that often after only about 3 weeks an answer either by letter or by telegram was received. This was of the greatest value especially with regard to possible alterations of the fixing methods. It should be mentioned also that the preparations were collected between middle of September 1958 and beginning of March 1959, i. e. before the heavy rain period, which started in Ifakara at the end of February and put an end—at least temporarily—to tsetse capture.

Preparation of Extract for Analysis.

The tissues were ground and extracted 3 times with hot 70% ethanol, using first the alcohol in which they were fixed and shipped and then two fresh portions. The combined extracts were evaporated to dryness in early experiments under a stream of air at 70°C, and in later experiments under nitrogen or *in vacuo* at 55°C or lower. The residue from evaporation was extracted with water, insoluble material being discarded. To remove substances which interfered with the oxidation of the dye in the assay, the solution was deionized by passage through a small ion exchange resin column containing a bed of Dowex 1 formate layered upon a bed of Dowex 50 hydrogen. Neutral substances were washed through the column with water and the effluent was evaporated to dryness and redissolved in a measured small volume of water.

Enzymic Assay of Glucose and Trehalose.

Two identical series of tubes were prepared containing unknowns, standards (0.1 μmole glucose and 0.05 μmole trehalose) and blanks, each in total volume of 0.05 ml. To one series was added a further 0.05 ml. of water or buffer, to the other was added 0.05 ml. of trehalase solution (KALF und RIEDER, 1958) containing 80 units per ml. in 0.1 M sodium acetate buffer, pH 5.4. Both series were incubated at 37° for 40 minutes, then chilled, 0.7 ml. of glucose oxidase reagent was added, and the tubes were returned to 37° for a further 10 minutes. The reagent was purchased as "Glucostat" from Worthington Biochemical Corp., Freehold, N. J., and contains glucose oxidase, peroxidase, and o-dianisidine (HUGGETT and NIXON, 1957). Finally, the tubes were again chilled, 0.1 ml. of 4 N HCl was added to each, and after centrifuging them the color was read in a spectrophotometer at 420 mμ. Blank values were subtracted, and the amount of glucose in the unknowns was calculated from the readings of the tubes incubated without trehalase. Trehalose was calculated from the additional yield of color in the tubes containing trehalase. Color yields from standard solutions of both sugars were linearly related to the amounts used.

Precision of the Method.

When known amounts of glucose and trehalose were added to the deionized concentrated insect extract, or to the crude alcoholic extract immediately before treatment, they were recovered in the assay to the extent of at least 90%. When the sugars were added to the alcohol used for fixation of the flies at the field laboratory, however, recoveries in the analysis were less satisfactory: glucose, 39% and 78% (2 experiments); trehalose, 82%, 74%, 56% (3 experiments). This indicates that some loss of sugars, especially glucose,

was occurring in the preserved material; oxidation catalyzed by the iron of hemoglobin in the flies' intestines is a possible cause. The results must therefore not be regarded as highly accurate; nevertheless, it is felt that their general order of magnitude is significant. The procedure finally adopted was the outcome of many experiments directed toward improving precision, and it is concluded that the accurate determination of sugars in the low concentrations which occur in this material is a matter of considerable difficulty.

TABLE II.

Glucose and Trehalose in Tissue of Tsetse Flies.

	Sample No.	*Mean analysis* Glucose mg./100g.	Trehalose mg./100 g.
Whole flies:			
G. morsitans	6	3	3
	7	6	0.3
	10	—	0.1
	14	2	3
	17	2	4
Sarcophaga bullata (included for comparison)	2a	—	88
Intestines:			
G. morsitans	8	—	12
	16	3	16
G. brevipalpis	4	—	10
	5	3	19
Salivary glands:			
G. morsitans	9	—	4
	12	4	0

Results.

The results of the analyses are shown in Table II. With samples treated by the final procedure, results are given for both sugars. With some earlier samples, the extracts were evaporated in air: this caused loss of glucose, presumably by catalyzed oxidation, so that results are given only for trehalose, which was not affected.

It is necessary to comment on the results obtained for salivary glands, which differ between the two samples. Sample 9 gave an anomalously high value (over 100 mg./100 g.) for glucose. We cannot explain this, but suppose that it may have resulted from some artifact causing breakdown of glycogen. However, it did contribute to experimental error in the determination of trehalose (as the difference of two relatively large numbers), and 3 replications of this analysis gave values of 0, 1, and 12 mg./100 g., the value given in the Table being the mean. In addition, we analyzed one sample of salivary glands from *G. morsitans* and one from *G. brevipalpis* which were fixed at room temperature and the extracts were evaporated under air. In these, the values for glucose were comparable to that for sample 12 (Table II), and no trehalose was detected in either. In intestinal samples prepared and analyzed under the same conditions, trehalose was always found, although only 20 to 40% of the

amount found in samples fixed at 70°. It is therefore probable that the result for sample 12 is more accurate than that for sample 9, and there is exceedingly little, if any, trehalose in *Glossina* salivary glands.

Discussion and Conclusions.

As yet, no other determinations of the content of trehalose in the cellular tissues of insects seem to have been published. The blood of various species, however, contains trehalose at concentrations of 100 up to 3000 mg./100 ml. (WYATT & KALF, 1957; HOWDEN & KILBY, 1956; EVANS & DETHIER, 1957). It was therefore of some surprise to us to find such small amounts of trehalose in whole tsetse flies. Although, as discussed above, there is reason to expect some error in our results, there is no reason to suspect major error in order of magnitude. It is significant that a sample of sarcophagid flies analyzed by the same procedure showed many times the concentrations of trehalose found in the tsetse flies, and that two samples of *Hyalophora cecropia* blood (not shown in Table II) gave values similar to those found previously by a different method. The content of glucose in tsetse flies also appears to be remarkably small. It would be of interest to examine these insects for the possible presence of other sugars, as well as to obtain quantitative estimations of glycogen, which is presumably their main carbohydrate reserve.

As already stated in the introduction, it is possible for avirulent trypanosomes maintained in blood-agar cultures, to become infective after addition of minimal amounts of trehalose to the culture medium. From the morphological point of view, no significant difference between avirulent culture-trypanosomes and those treated with trehalose has been found. On the other hand, trehalose is known to occur in considerable amounts in the blood of insects while mammalian blood is free of this sugar. Consequently this led us to investigate the presence of trehalose not only in whole tsetse flies, but also in those organs where trypanosomes evolve to become infectious for mammals. As the infective forms are present only in the salivary glands, an important concentration of this sugar could have been expected in these organs. Chemical analyses, however, revealed extremely small and variable amounts of trehalose in the salivary glands, whereas in the intestine greater amounts were found. A comparison with our analyses of whole flies show that the intestine contains a higher concentration of trehalose than other tsetse tissues [3]. It is possible therefore that trehalose does not influence the completely developed metacyclic trypanosomes in the salivary glands, but acts at an earlier stage of development. In the intestine of the fly trehalose might act upon the trypanosomes taken up with the blood meal in such a way that their evolution is completed morphologically as well as physiologically. MURGATROYD and YORKE showed that in only 20% of the flies, approximately, do trypanosomes reach the salivary glands and that they frequently go only so far as the extra-peritrophic space or the proventriculus. Whether this fact is related to different trehalose concentrations in the intestine or in the salivary glands would have to be examined. The possible role of the intestine as a site of trehalose synthesis would be of great interest in this connection.

Summary.

1) The finding that trehalose *in vitro* can control the infectivity of *T. rhodesiense* and *T. gambiense*, made it of the highest interest to determine whether this sugar occurs in *Glossina*.

[3] Mean weight of the intestine relative to the mean weight of the whole fly for *Glossina morsitans* = 1 : 16.3.

2) Field catches of *G. morsitans* and *G. brevipalpis* provided the material for chemical analysis. This consisted of 570 whole flies, 428 intestines and 3,495 salivary glands.

3) These samples were analyzed for glucose and trehalose by a specific enzymic method. The trehalose levels found were: whole flies 0.1 to 4.0 mg. per 100 g. fresh weight, intestines 10 to 19 mg. per 100 g., and salivary glands trace amounts of doubtful significance. The various tissues also contained glucose in small amounts.

4. The question arises: does a connection exist between the concentration of trehalose in the intestine and the beginning of the trypanosome cycle in the fly? Does trehalose induce the trypanosomes to start and complete their evolution into metacyclic infective forms?

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