ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA

VOL. XVII, 14 SECTIO C 1962

Z Katedry Fizjologii Roślin Wydziału Biologii i Nauk o Ziemi UMCS
Kierownik: prof. dr Adam Paszewski

Barbara DUDZIAK, Zbigniew JÓŹWIK
Adam PASZEWSKI

Experiments on the Activity of Several Extracts from the Larvae of *Galleria mellonella L.* on *Mycobacterium tuberculosis 607*

Badanie aktywności różnych wyciągów z larw *Galleria mellonella L.* na *Mycobacterium tuberculosis 607*

Исследование активности различных экстрактов из личинок *Galleria mellonella L.* на *Mycobacterium tuberculosis 607*

In 1959 Paszewski demonstrated that an enzyme extract from the larvae of *Galleria mellonella* L. sensitized the bacteria to the action of penicillin and sulphatiazole.

In this investigation were examined the influence of glycerine-, buffer-, and water-extracts from the larvae of *Galleria mellonella* L. on the bacteria in various combinations with and without penicillin. The bacteria examined were the strain *Mycobacterium tuberculosis 607* growing on Sauton's medium.

The extracts from the larvae of *Galleria mellonella* L. were obtained by Willstaetter's method as modified by A. Lassota (cyt. Paszewski 1959) or Elimer-Stotz. The acetone powder was then extracted as follows:

a) with glycerine — 1 g acetone powder per 15 ml 60% glycerine,
b) with the buffer — 1 g acetone powder per 15 ml phosphatic buffer (pH = 7.2, 0.25 M.),
c) with water — 1 g acetone powder per 15 ml distilled water.

The glycerine, buffer, or water were kept in a refrigerator with the acetone powder for 12 hours, then filtered through Büchner's filter and Schott's Nr. 5, and finally the activity of the extracts in combinations with and without penicillin was tested on *Mycobacterium tuber-*

culosis 607. Experiments were also carried out on the enzymatic-esterase activity of the water-extracts obtained in both the aforementioned ways, by means of titration using ethyl butyrate or acetylcholine as substrate. Since the results of these experiments were negative we supposed that the active substance is not enzymatic. Hence all the extracts were heated at 100° for 15 minutes.

The tubercle bacteria were treated with glycerine-extract, buffer-extract and water-extract in the following combinations:

I. 0.5 ml bacterial suspension in saline solution (0.85%) + 2 ml glycerine-extract of the larvae of *Galleria mellonella* L., the extract being heated at 100°C for 15 minutes.

II. 0.5 ml bacterial suspension in saline solution + 2 ml glycerine extract from the larvae of *Galleria mellonella* L. — unheated extract.

III. 0.5 ml bacterial suspension in saline solution + 2 ml buffer-extract from the larvae of *Galleria mellonella* L., the extract being heated at 100°C for 15 minutes.

IV. 0.5 ml bacterial suspension in saline solution + 2 ml buffer-extract from the larvae of *Galeria mellonella* L. — unheated extract.

V. 0.5 ml bacterial suspension in saline solution + 2 ml water-extract from the larvae of *Galleria mellonella* L., the extract being heated at 100°C for 15 minutes.

Table 1. The action of the glycerine-extract from the larvae of *Galleria mellonella* L. heated at 100°C for 15 minutes and unheated, with penicillin and without, on *Mycobacterium tuberculosis 607*

Time of incubation	Glycerine—extract				Control	
	Heated for 15′ at 100° C		Unheated			
	with penicillin	without penicillin	with penicillin	without penicillin	with peniciilin	without penicillin
24 hrs.	—	+	—	+	+	+
72 hrs.	—	+	—	+	+	+

Table 2. The action of the buffer-extract (phosphatic buffer of pH = 72) from the larvae of *Galleria mellonella* L. heated at 100°C for 15 mins. and unheated, with and without penicillin, on *Mycobacterium tuberculosis 607*

Time of incubation	Buffer—extract				Control	
	Heated for 15′ at 100° C		Unheated			
	with peniciilin	without penicillin	with penicillin	without penicillin	with penicillin	without penicillin
24 hrs.	—	+	—	+	+	+
72 hrs.	—	+	—	+	+	+

Tables 3. The action of the water-extracts from the larvae of *Galleria mellonella* L. heated for 15 mins. at 100°C and unheated, with and without penicillin on *Mycobacterium tuberculosis 607*

Time of incubation	Water—extract				Control	
	Heated for 15′ at 100° C		Unheated			
	with penicillin	without penicillin	with penicillin	without penicillin	with penicillin	without penicillin
24 hrs.	—	+	—	+	+	+
72 hrs.	—	+	—	+	+	+

Table 4. The action of the protein fraction obtained from the buffer-extract from the larvae of *Galleria mellonella* L. heated for 15 minutes at 100°C, and unheated, with and without penicillin, on *Mycobacterium tuberculosis 607*

Time of incubation	Protein fraction				Control	
	Heated for 15′ at 100° C		Unheated			
	with penicillin	without penicillin	with penicillin	without penicillin	with penicillin	without penicillin
24 hrs.	—	+	—	+	+	+
72 hrs.	—	+	—	+	+	+

Table 5. The action of the protein — free fraction obtained after the removal of the protein from the buffer-extract, heated for 15 minutes at 100°C and unheated, with and without penicillin, on *Mycobacterium tuberculosis 607*

Time of incubation	Protein—free fraction				Control	
	Heated for 15′ at 100° C		Unheated			
	with penicillin	without penicillin	with penicillin	without penicillin	with penicillin	without penicillin
24 hrs.	—	+	—	+	+	+
72 hrs.	—	+	—	+	+	+

Key to Tables: — = inhibition of growth, + = growth, control = Sauton's medium + *Mycobacterium tuberculosis 607*.

VI. 0.5 ml bacterial suspension in saline solution + 2 ml of water-extract from the larvae of *Galleria mellonella* L. — unheated extract.

VII. For each combination the following control was used: 0.5 ml bacterial suspension in saline solution.

Test-tubes with cultures II, IV, VI were kept at a temperature of 4°C. Test-tubes in which the extracts were heated at 100°C for 15 minutes were kept at room temperature. After 24 and 72 hours the bacteria were transferred to: a) Sauton's medium, b) Sauton's medium with penicillin at a concentration of 10 000 units/1 ml.

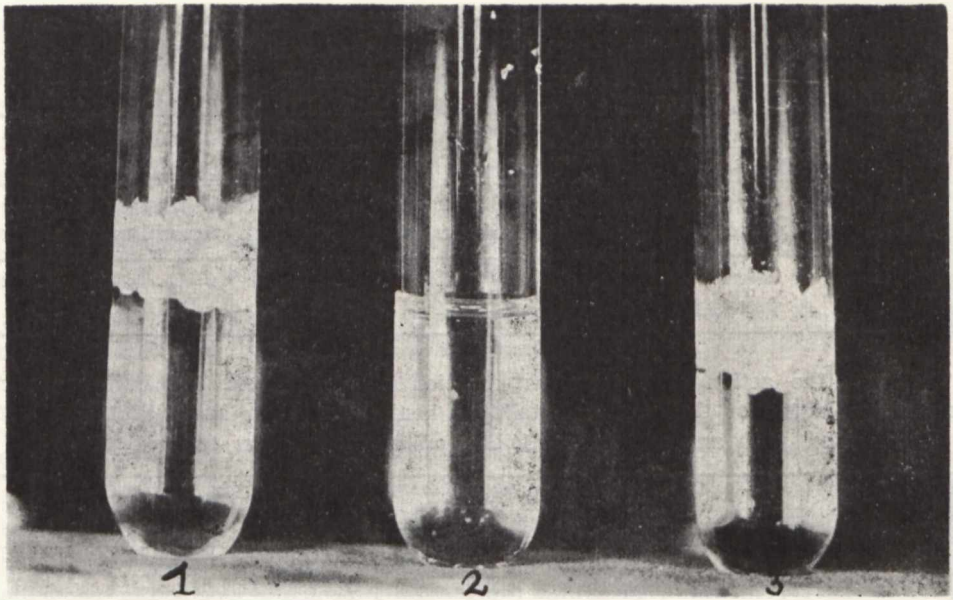

Fig. 1. Action of the buffer extract from the larvae of *Galleria mellonella* L. heated and unheated, in combinations with and without penicillin, on *Mycobacterium tuberculosis 607*

1 — Buffer extract heated (15′ at 100°C.) + Sauton's medium + *Mycobacterium tuberculosis 607*,
2 — Buffer extract, heated, + Sauton's medium + *Mycobacterium tuberculosis 607* + penicillin,
3 — Control.

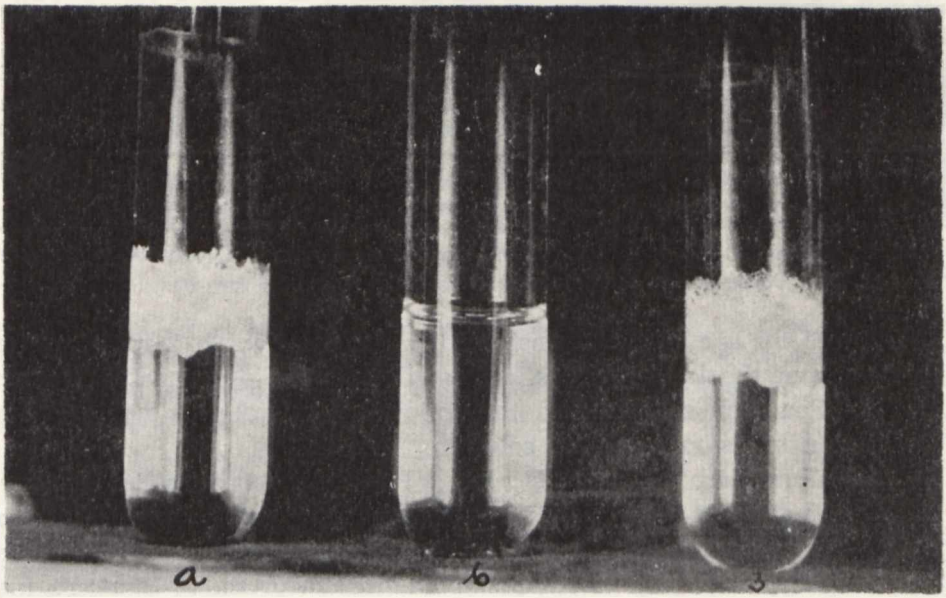

Fig. 2

1 — Unheated buffer-extract + Sauton's medium + *Mycobacterium tuberculosis 607*,
2 — Unheated buffer-extract + Sauton's medium + *Mycobacterium tuberculosis 607* + penicillin,
3 — Control

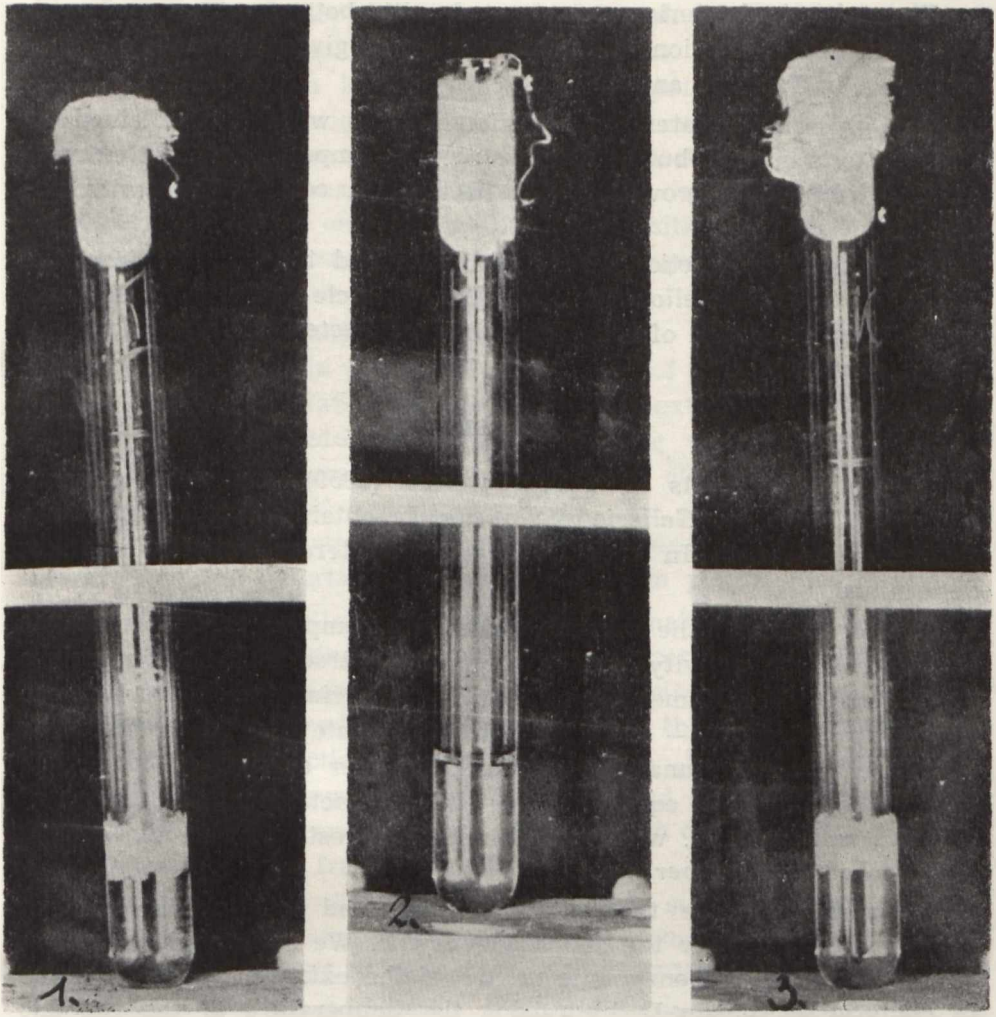

Fig. 3. Action of the water-extract, from the larvae of *Galleria mellonella* L., heated, with penicillin and without, on *Mycobacterium tuberculosis 607*;
1 — Water-extract, heated (15′ at 100°C.) + Sauton's medium + *Mycobacterium tuberculosis 607*,
2 — Water-extract, heated (15′ at 100°C.) + Sauton's medium + *Mycobacterium tuberculosis 607* + penicillin,
3 — Control.

The experiments were repeated thrice. Five days after the transference, the cultures of bacteria were examined. The results are given in Tables 1, 2, and 3 and in Figures 1, 2, and 3.

In order to define the active substance, we divided the buffer-extract into two fractions, one containing protein and the other not.

The tubercle bacteria were treated with both fractions and with penicillin in combinations identical with those given above. The results are given in Tables 4 and 5.

The glycerine-, water-, and buffer-extracts were also obtained by Willstaetter's method but without using low temperatures. The extracts did not inhibit the growth of the bacteria in combinations with penicillin.

After the direct action of the penicillin and the extracts from the larvae of *Galleria mellonella* L. on the tubercle bacteria in Sauton's medium, no inhibition of the growth of *Mycobacterium tuberculosis 607* was observed.

DISCUSSION

In the experiments of Paszewski (1959), enzymatic extracts from the larvae of *Galleria mellonella* L. obtained by Willstaetter's method were used. In them, the enzyme-esterase activity was not determined.

In the course of the present work we attempted to determine the enzyme-esterase activity in the ezymatic extracts. Basing ourselves on the method of Elimer Stotz (1955) — experiments on the esterase activity by titration — and using ethyl butyrate and acetylcholine as substrates, we were unable to obtain a greater acidity. Although the enzyme activity was equal to zero, the extracts from the larvae of *Galleria mellonella* L. with penicillin caused restriction in the growth of *Mycobacterium tuberculosis 607*.

It appears possible that ethyl butyrate and acetylcholine are not suitable substrates for the supposed enzyme. We attempted to resolve our doubts as to the enzymatic nature of the extracts from the larvae of *Galleria mellonella* L. by raising the temperature of the extracts to 100°C and maintaining them at this temperature for 15 minutes. If the active substance in the extracts obtained were an enzyme, then after such heating it would very probably give negative results in tests of its activity upon the tubercle bacilli.

Tables 1, 2, and 3, as also the Figures indicate that the extracts remained active after heating and gave positive results with *Mycobacterium tuberculosis 607*. These results agree with those given by T. Vályi-Nagy and co-workers (1954) and by Olivier (1947) who suppose the biological activity of the extract to be due to the presence of an antibiotic. In view of this, the results obtained in the present work may be explained by the synergetic action of the supposed antibiotic from the larvae of *Galleria mellonella* L. and penicillin.

The thermostability of the active principle in the extract from the larvae of *Galleria mellonella* L. leads us to suppose that it is pointless to maintain low temperatures (essential in the case of an enzyme) in a preparation of the extract. The extract obtained in conditions of room temperature was, however, inactive. This does not so much indicate the enzymatic nature of the factor as the supposed activity of a certain enzyme or the decomposition of the active substance we are discussing. Therefore, we prepared the extracts maintaining the temperature at about 0°C.

Since the proto-antibiotic obtained by T. Vályi-Nagy and co-workers dissolves in water and in view also of the use of a phosphatic buffer in the preparation of the enzymatic extract by Willstaetter's method, we investigated the influence of the water-extract and the buffer-extract on *Mycobacterium tuberculosis 607*. Both extracts were active in various different temperatures. The presence of the active factor in the phosphatic buffer may be the starting point for further research on the determination of the optimum pH for this substance.

We began some attempts to establish the nature of the active substance. In the protein-free fraction we observed the presence of sugars. A lipid fraction was not considered, since fats were removed when the acetone powder was obtained. Both the protein fraction and the protein-free fraction showed activity on *Mycobacterium tuberculosis 607;* and because of insufficiently exact methods of separating out the protein, we cannot ascribe the presence of the active substance to either one of the fractions exclusively.

It is very likely that part of the active substance was adsorbed into the protein removed. The action of this factor lies in the sensitizing of the tubercle bacilli to the action of penicillin. The results of our experiments lead us to assume that the active substance is not an enzyme. The final determination of the nature and mechanism of action of this active substance requires further research.

CONCLUSIONS

1. An active substance to be found in extracts from the larvae of *Galleria mellonella* L. is soluble in phosphatic buffer (pH = 72) in water, and in glycerine (60%).

2. Extracts from the larvae of *Galleria mellonella* L. sensitize *Mycobacterium tuberculosis 607* to the action of penicillin. Our results agree with those of A. Paszewski (1959).

3. The active substance in these extracts is thermostable.

4. The water-extracts from the larvae of *Galleria mellonella* L. do not give a positive reaction to enzyme-esterase activity.

STRESZCZENIE

Celem pracy było zbadanie wpływu wyciągów glicerynowego, buforowego i wodnego z larw *Galleria mellonella* L. na *Mycobacterium tuberculosis 607*, w kombinacjach z penicyliną i bez penicyliny.

Wyciągi otrzymywano metodą Willstaettera i Elimer-Stotza. Na bakterie działano wyciągami ogrzewanymi przez 15 minut w 100°C oraz wyciągami nieogrzewanymi. Poza tym wyciąg buforowy rozdzielano na frakcje białkową i nie zawierającą białka.

Mycobacterium tuberculosis 607 poddawano działaniu obu frakcji, zarówno w kombinacjach z penicyliną jak i bez penicyliny.

Stwierdzono, że wyciągi ogrzewane jak i nieogrzewane, oraz frakcja białkowa i nie zawierająca białek wyciągu buforowego, uczulają prątki na działanie penicyliny (Tab. 1, 2, 3, 4 i 5, i fotografie).

Czynnik aktywny jest termostabilny, rozpuszczalny w wodzie, buforze i glicerynie.

REFERENCES

1. Olivier H. R.: Antibiotic Action of an Extract of *Galleria mellonella*. Nature, 159, No 4046, p. 685, 1947.
2. Paszewski A.: Influence of an Enzyme Extract from the Larvae of *Galleria mellonella* together with Penicillin or Sulphatiazole on the Growth of *Mycobacterium tuberculosis 607*. Ann. Univ. Marie Curie-Skłodowska, sectio C, vol. XIV, 20, 435—438, 1959.
3. Stotz E.: Methods in Enzymology. Edited by Sidney P. Colowick and Nathan o. Kaplan, McCollum-Pratt Institute. The Johns Hopkins University, Baltimore, Maryland, New York, I, 657—659, 1955.
4. Vályi-Nagy T., Úsi J., Szilágyi I.: Primycyn a New Antibiotic. Nature, 174, No 4441, 1105—1106, 1954.

РЕЗЮМЕ

Целью работы было изучение влияния глицеринового, буферного и водного экстрактов из личинок *Galleria mellonella L.* на туберкулезные палочки (*Mycobacterium tuberculosis 607*) в комбинациях с пенициллином и без пенициллина.

Экстракты получались по Вильштеттеру и Элимер Штотца. На бактерии действовали нагретыми экстрактами в течение 15 минут

при температуре t—100°C, и экстрактами не нагретыми. Кроме того буферный экстракт разделялся на фракции — белковую и не содержащую белка. Туберкулезные палочки *(Mycobacterium tuberculosis 607)* подвергались действию обоих фракций, как и комбинациях с пенициллином, так и без него.

Установлено, что как экстракты нагретые, так и не нагретые, а также фракция белковая и не содержащая белков, полученные из буферного экстракта так влияют на палочки, что они становятся чувствительными на действие пенициллина табл. 1, 2, 3, 4 и 5, а также фото.

Действующий фактор является термостабильным, растворяется в воде, буфере и глицерине.

Papier druk. sat. III kl. 80 g. Format 70×100 Druku 9 str.
Annales UMCS Lublin 1961 Lub. Druk. Pras. Lublin, Unicka 4 Zam. 4089. 6.X.62
1100+125 egz. N-4 Manuskrypt otrzymano 6.X.62 Druk ukończono 19.III 63