

OBSERVATIONS ON THE BIOLOGICAL PROPERTIES OF ATYPICAL HAEMOLYTIC CORYNEBACTERIA ISOLATED FROM MAN AS COMPARED WITH COR. HAEMOLYTICUM, COR. PYOGENES BOVIS AND COR. OVIS

II. In vitro investigations

A. SOUČEK, A. SOUČKOVÁ, M. MÁRA F. PATOČKA

Technical assistance: VĚRA SAHULOVÁ & MIROSLAVA ŠKVOROVÁ

Department for Medical Microbiology and Immunology, Charles University, Prague

The subject of this second communication is a detailed investigation of two demonstrable *in vitro* products of five strains of corynebacteria previously described.

The first substance is a haemolysin very well demonstrable on blood agar around colonies of strains K 29, 73/61, E 419 and ATCC 9345. In broth cultures only the dynamics of its production were studied and its thermolability tested. The second is a factor demonstrated by Záhlová and Kubelka [8] in human variants of pyogenic corynebacteria. This factor is so helpful in the detection of these microbes that it has made the isolation of almost 200 strains possible in the past two years, although they were sometimes present in small numbers and in mixed cultures (Plate I).

In two previous communications [13, 14] we have pointed out that the factor inhibiting staphylococcal alpha-beta haemolysis seems to us to be identical with the dermonecrotic toxic antigen of this microbe. Therefore, the greater part of this study deals with its production and quantitative determination *in vitro*.

MATERIALS

1. Inhibition of staphylolysin was tested on plates of blood agar with 10% citrated sheep blood. For *Cor. ovis*, in place of whole sheep blood, suspensions of thrice washed sheep RBC with calf serum were added to agar. Strains under investigation were inoculated simultaneously with test-staphylococcus and/or 24 hrs. prior to inoculation of the latter. The employed staphylococcus produced alpha and beta haemolysin.

2. *Filtrates* of each strain were prepared by the technique described in the previous paper.

3. *Diluents*: Todd-Hewitt's broth was used for two-fold dilutions of filtrate. T-H broth diluted with an equal volume of buffered physiological saline was used in the titration of haemolysins. Buffered physiological saline (NaCl 8.5 g, KH₂PO₄ 0.25 g, Na₂HPO₄ 0.75 g,

aq. dest. ad 1000 ml.) was also used to wash blood cells and for the dilution of staphylolysin.

4. *Erythrocytes*: Rabbit RBC were used in titrations of staphylolysin, haemolysins and inhibitors.

5. Staphylolysin (strain Wood) kindly supplied by Dr. Jitka Součková from the Serum & Vaccine Institute, Prague. Merthiolate [1:5000] was used as antiseptic. The titre of staphylolysin was found by determining 50% haemolysis as described in detail below.

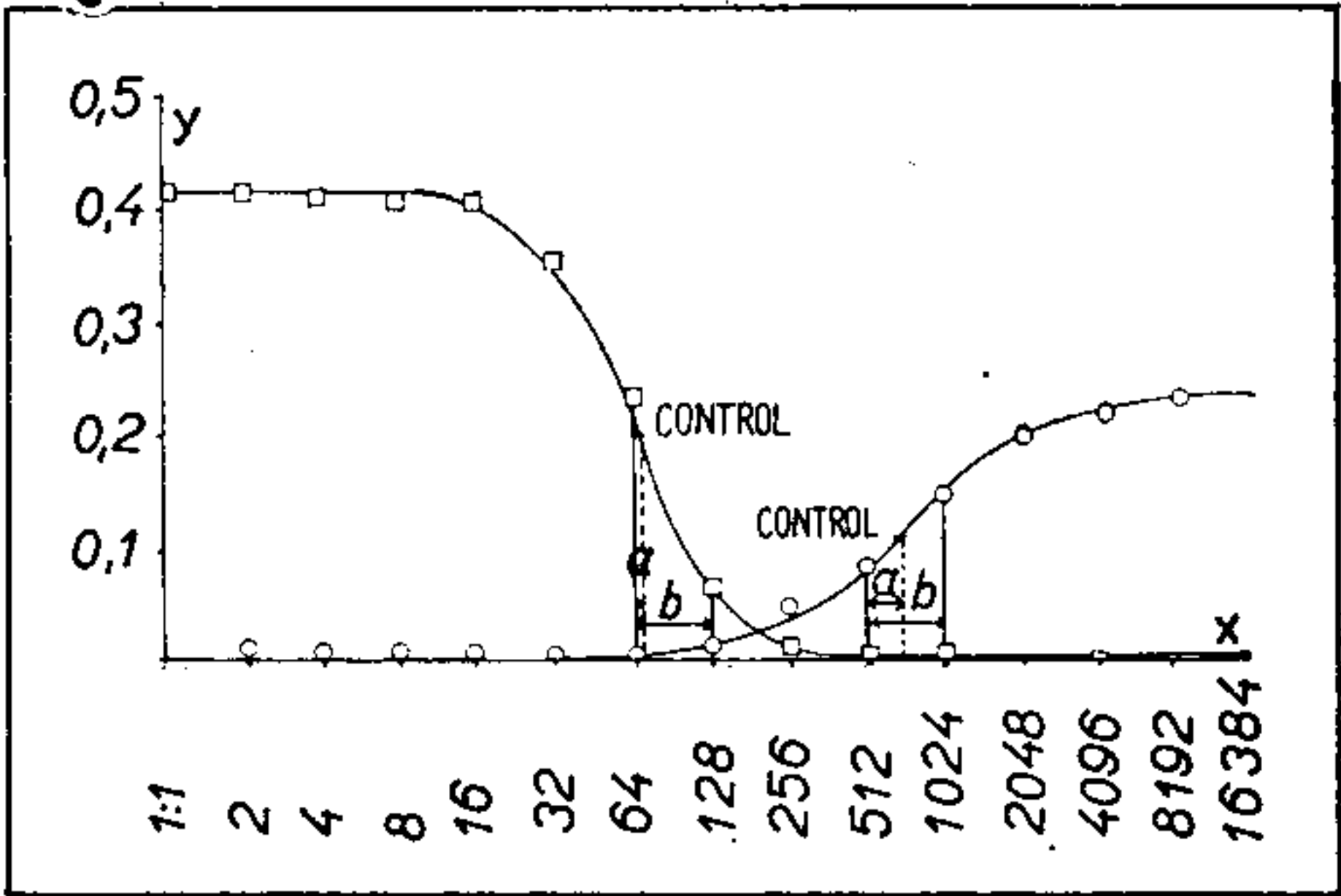


Fig. 1.

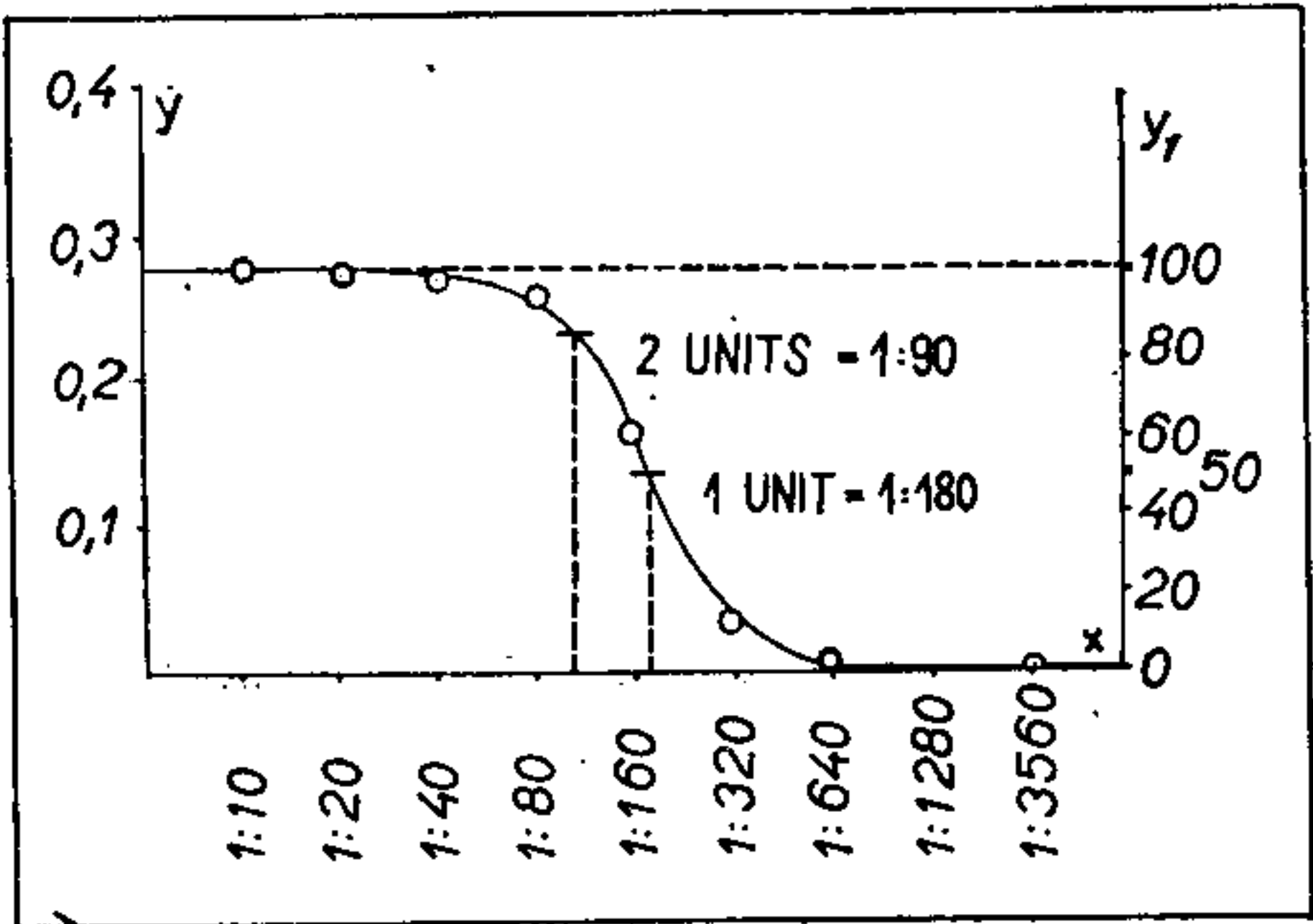


Fig. 2.

Fig. 1. Determination of 50% haemolysis in titration of haemolysin (□) and inhibitor (○). Optical density of control designates 50% haemolysis. a=log of proportional distance (in equation log p) ; b=log 2. y=optical density ; x=dilution. — Fig. 2. Titration of staphylolysin. Calculation of 1 unit by determination of optical density of control. 2 units obtained by diluting halfway to 1 unit. y=optical density ; x=dilution.

6. All measurements was made by the Zeiss' Universal Spectrophotometer, using a flint glass prism. Measurements were taken at 550 mμ, using a caesium-antimony photo-cell MSVB.

METHODS

1. The preparation of standard suspension of rabbit RBC : Blood collected into 3.8% sodium citrate (1 part citrate : 10 parts blood). RBC were washed three times in physiological saline. Sediment was prepared to make an approximately 1.5% suspension. 0.2 ml. of this suspension was lyzed by 1.8 ml. distilled water and after centrifugation, the optical density measured in a photometer; the calculation of the volume of physiological saline necessary to reach the optical density 0.100±0.005 was made according to Stein et al. (17). The resulting suspension (approximately 1 %) was used in titrations.

2. Determination of 50% haemolysis (Fig. 1).

50% haemolysis was found by measuring the optical density of the control, consisting of half the number of erythrocytes completely lyzed. The exact titre was determined by calculating the proportional distance between the nearest lower and nearest higher dilution (in relation to the optical density of the control). The number of units equals the reciprocal value of the filtrate titre multiplied by the proportional distance p. The proportional distance is calculated according to the formula :

$$\log p = \frac{OD_L - OD_{K1}}{(OD_L - OD_{K1}) + (OD_{K1} - OD_H)} \cdot \log 2$$

OD_L= optical density of nearest lower dilution than control K 1, giving 50 % haemolysis; OD_H= optical density of nearest higher dilution than control. This relationship is valid for the titration of haemolysis and staphylolysin. In titration of inhibitor the formula is :

$$\log p = \frac{OD_{K1} - OD_L}{(OD_{K1} - OD_L) + (OD_H - OD_K)} \cdot \log 2$$

3. Titration of haemolysin

Filtrate was diluted two-fold by 1.5 ml. T-H broth (diluted with an equal volume of physiological saline). 1.5 ml. of 1% suspension of standardized rabbit RBC was added and incubated for 60 min. at 37° C in a water bath. Centrifugation was carried out for 5 min. at 2000 r.p.m. after removal from the water bath and the supernatants were pooled. Optical density was measured in a spectrophotometer.

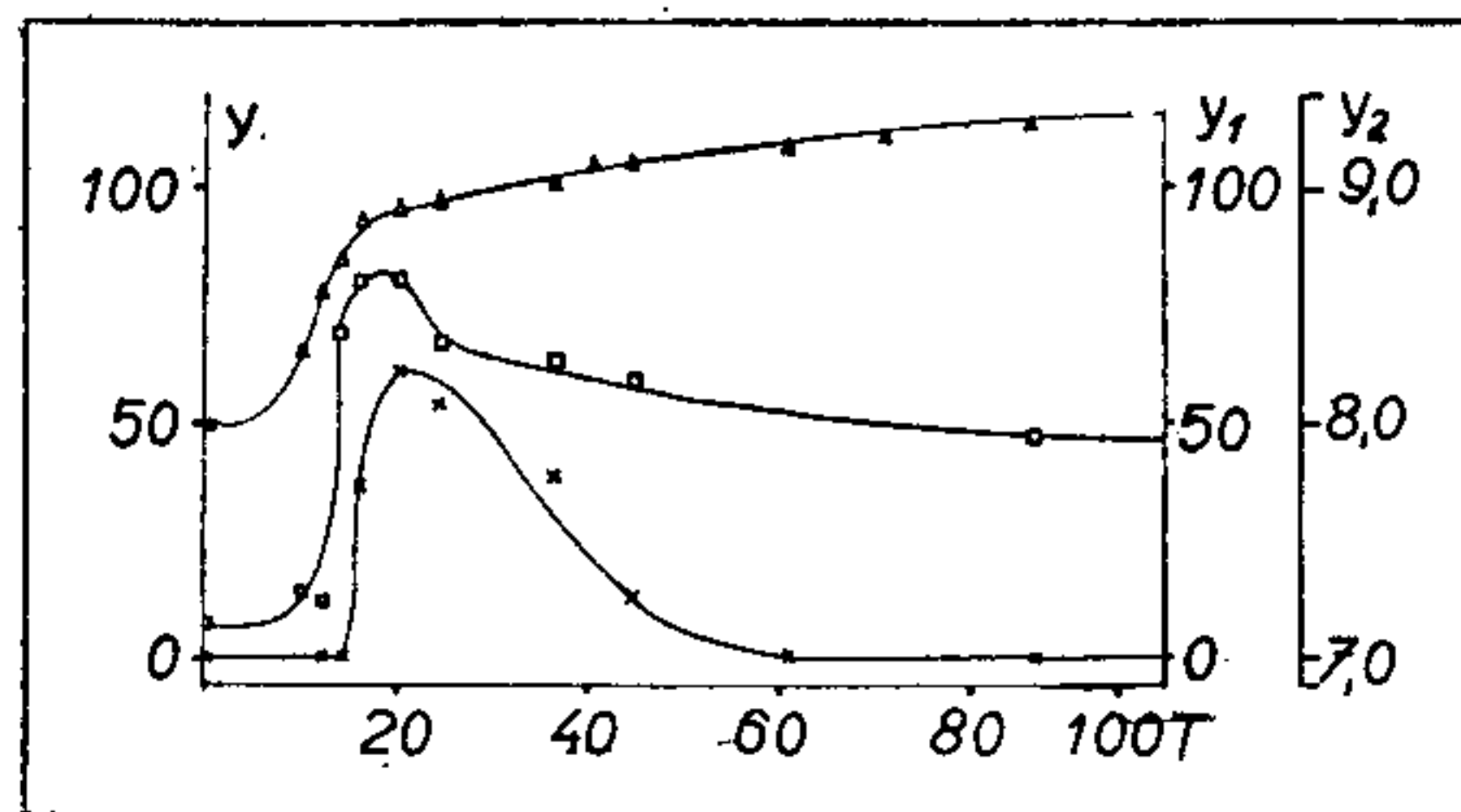


Fig. 3.

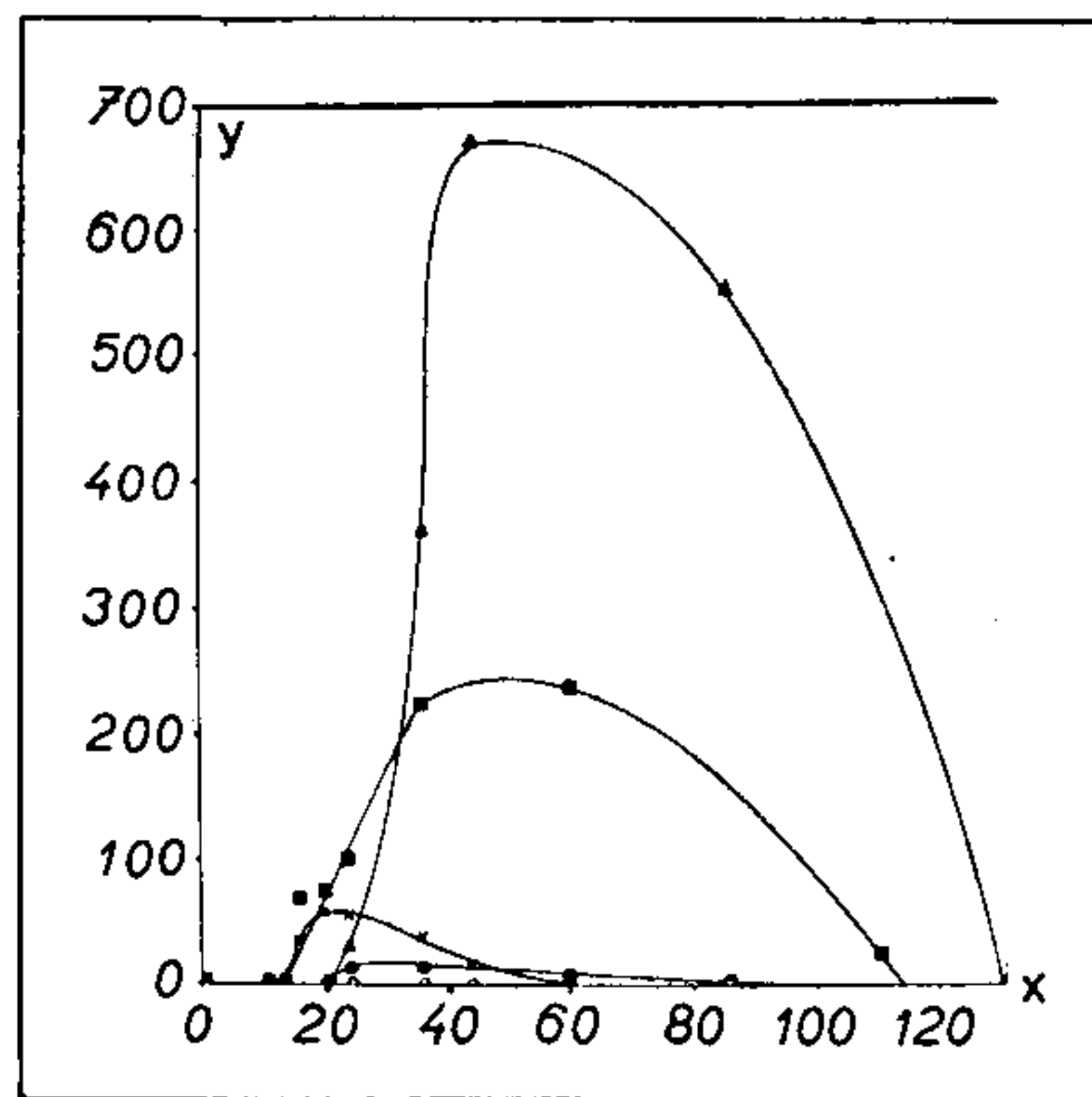


Fig. 4.

Fig. 3. Comparison of growth curves with production of haemolysin in strain ATCC 9345. Δ bacterial density; \square viable cels; \times haemolysin. y = units of haemolysin; x = time in hours; $y_1 = \log_2$ of viable cells mil./ml.; $y_2 = \log_2$ of bacterial density. — Fig. 4. Production curves of haemolysins. \bullet K 29; \blacksquare E 419; \triangle 73/61; \times ATCC 9345. y = units of haemolysin; x = time in hours. $y_1 = \log_2$ of viable cells mil./ml.; $y_2 = \log_2$ of bacterial density.

Controls: blank — 1.5 ml. diluted T-H broth
 1.5 ml. standardized rabbit RBC
 K 1 — 0.75 ml. concentrated T-H broth
 0.65 ml. physiological saline
 0.1 ml. concentrated staphylolysin
 0.75 ml. standardized rabbit RBC
 0.75 ml. supernatant of RBC

The number of units of haemolysin is calculated according to formula sub 2.

4. Titration of staphylolysin

1.8 ml. physiological saline and 0.2 ml. concentrated staphylolysin was serially diluted two-fold in 1 ml. physiological saline. To each tube 1 ml. T-H broth and 1 ml. standardized RBC was added and incubated 30 min. at 37° C in water bath, and then for 120 min. at room temperature. After centrifugation the optical density of supernatants was measured.

Controls: blank — 1.0 ml. physiological saline
 1.0 ml. T-H broth
 1.0 standardized RBC
 K 1 — 0.1 ml. concentrated staphylolysin
 0.9 ml. physiological saline
 1.0 ml. T-H broth
 0.5 ml. standardized RBC
 0.5 ml. supernatant of standardized RBC

The calculation of the titre of staphylolysin is the same as that of haemolysin. One unit of staphylolysin (Fig. 2) for titration of inhibitor is the amount of staphylolysin that causes 50% haemolysis. [This unit must not be confused with the unit of staphylolysin given by reciprocal titre.]

5. Titration of inhibitor

1.0 ml. of the specimen was serially diluted two-fold in 1 ml. T-H broth. 1 ml. of standardized rabbit RBC was then added to each tube and left to stand 30 min. at room temperature. Then 1 ml. staphylolysin diluted to contain 2 units in 1 ml. as defined above was added to each tube. Incubation for 30 min. at 37° C in a water bath

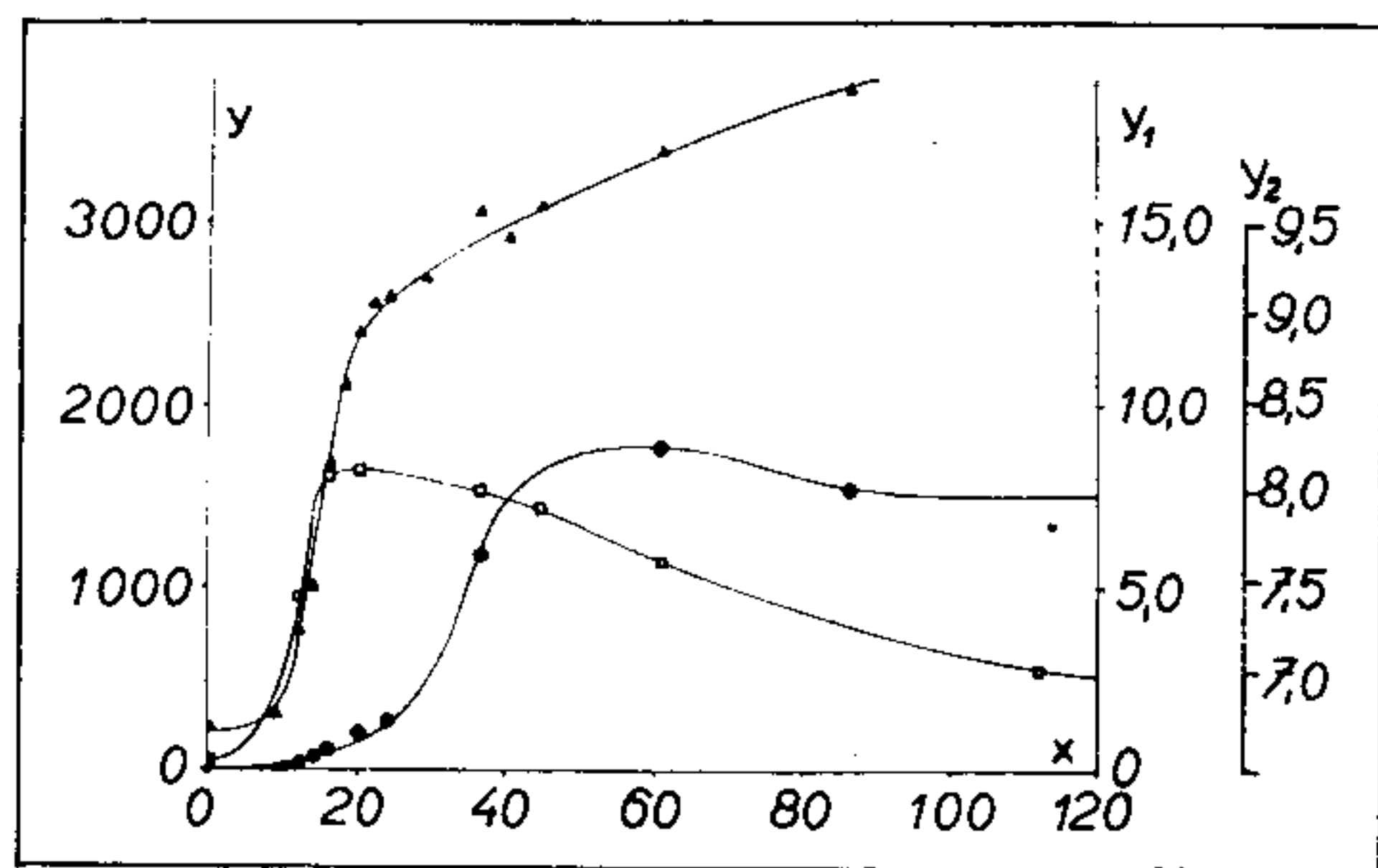


Fig. 5

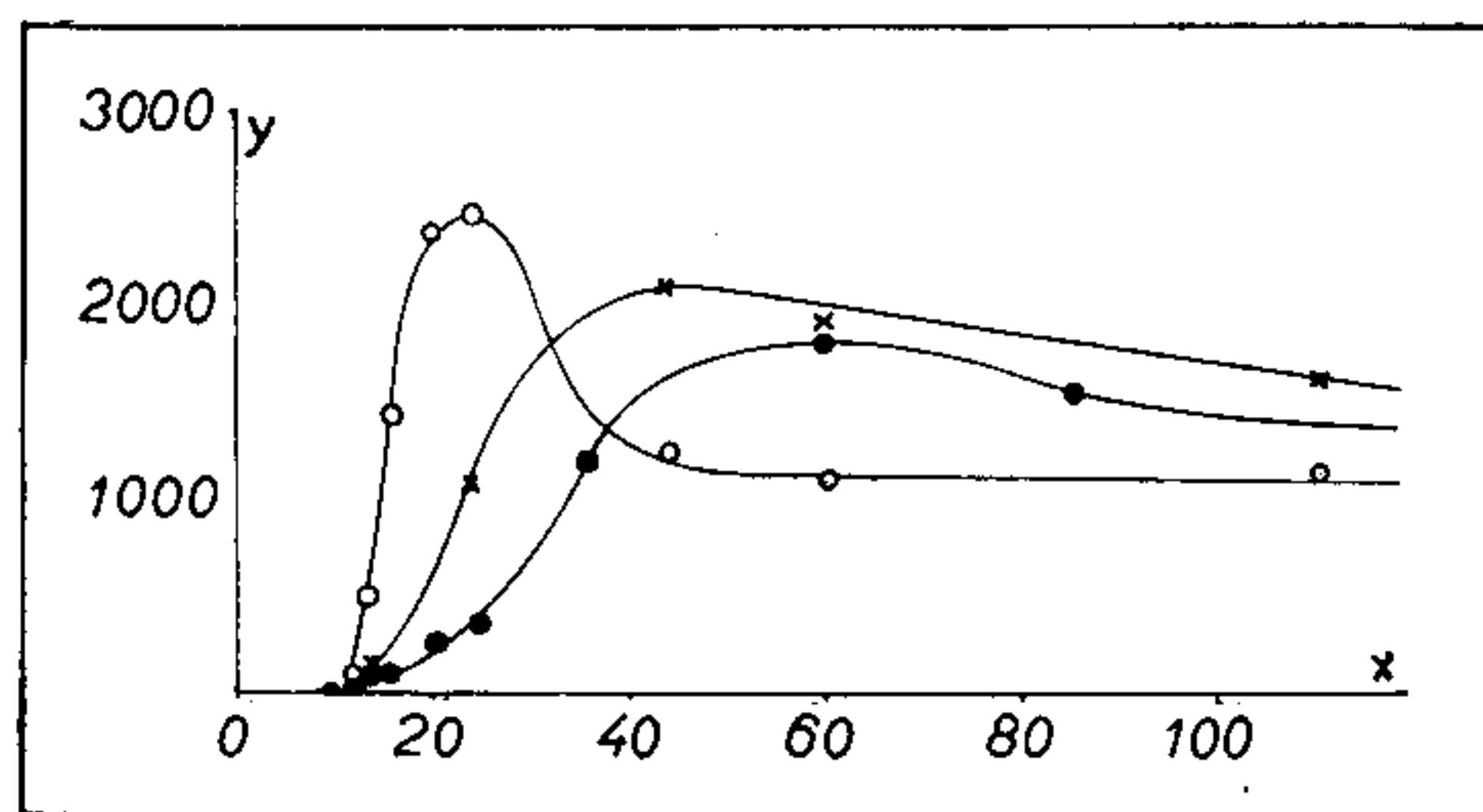


Fig. 6.

Fig. 5. Comparison of growth curves with production curve of inhibitor in strain K 29. Δ bacterial density; \square viable cells; \bullet inhibitor. y =units of inhibitor; x =time in hours. Fig. 6. Production curves of inhibitors. \bullet K 29; \circ KC 472; \times ATCC 9345. y =units of inhibitor; x =time in hours.

was followed by standing for 120 min. at room temperature. The rest of the procedure was the same as in the titration of haemolysin and staphylolysin.

Controls: blank and K 1 same as in titration of staphylolysin.

Adsorption and elution

The filtrate of a 48 hr. culture of strain K 29, heated for 5 min. at 56° C (to inactivate haemolysin) was subjected to the adsorption-elution procedure. Sheep, rabbit and human O RBC were used as adsorbent. Citrated blood was centrifuged for 15 min. at 2000 r.p.m. and RBC were washed three times in buffered physiological saline. The required concentrations were prepared by pipetting sedimented RBC and their concentration expressed in overall volume at 4° C, room temperature (given at 20° C in the Figures), and at 37° C. Adsorption was performed in Patočka's vessels at 4 C°, 20° C and 37° C. The quantity of adsorbed inhibitor was calculated after the titration of inhibitor in supernatant following centrifugation of RBC. Elution was performed after adsorption and three-fold washing of RBC in physiological saline and the addition of physiological saline or T-H broth to the original volume. The titre of inhibitor was determined in the supernatant after adsorption. In the final washing and in the eluate. No inhibitor was ever demonstrated in the final washing.

Production of haemolysins

The formation of haemolysin was investigated in the filtrate of the broth culture, specimens of which were taken at specified intervals. The commence-

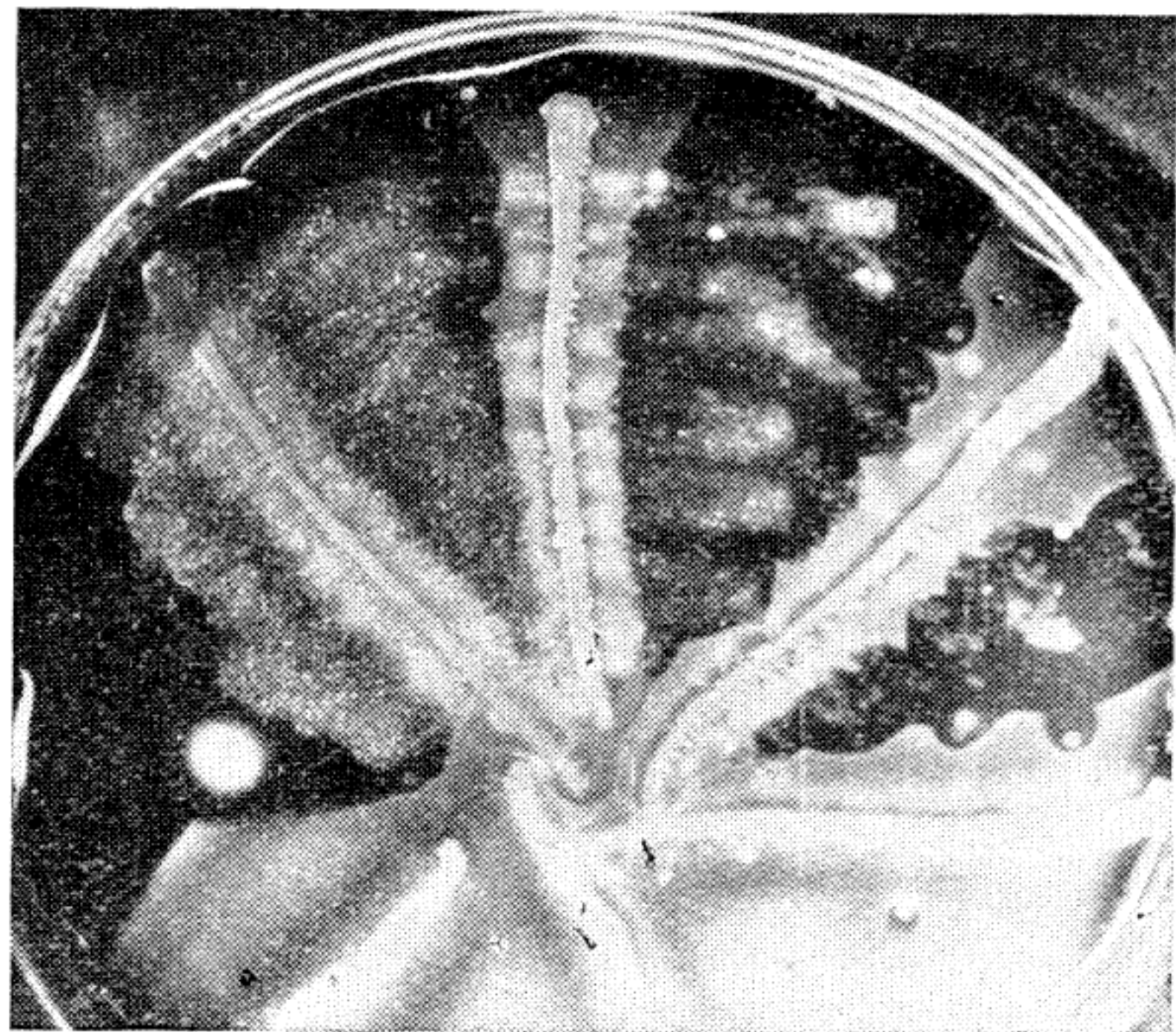


Photo 1.

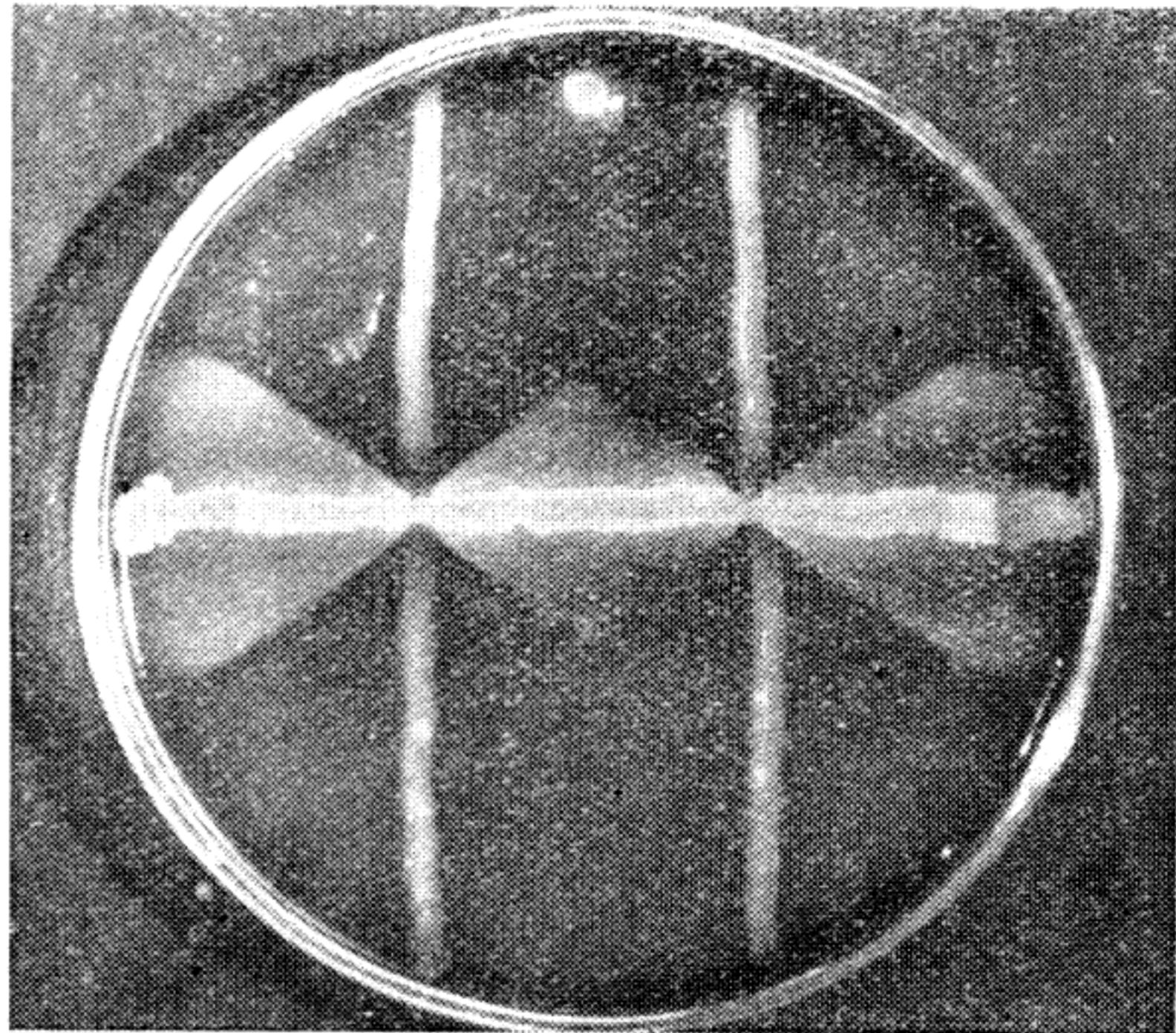


Photo 2.

Photo 1. Isolation of *Corynebacterium pyogenes* var. *hominis* from throat swab. Zone of inhibition of staphylococcal-haemolysis well defined around colonies of *Cor. pyogenes* var. *hominis*. — Photo 2. Inhibition of staphylococcal haemolysis by strain K 29.

ment of formation can be demonstrated after the beginning of the exponential phase of growth, and maximum of production coincides with the exponential phase (Fig. 3). The highest titres of haemolysin were found in strains 73/61 and E 419, lower titres in strains ATCC 9345 and K 29. We were not able to demonstrate soluble haemolysin in filtrates of strain KC 472 (*Cor. ovis*) by our methods. The activity of haemolysin rapidly diminished in broth culture and maximal values soon fell sharply towards zero (Fig. 4). Haemolysin from every strain was inactivated by heating to 56° C for 5 min.

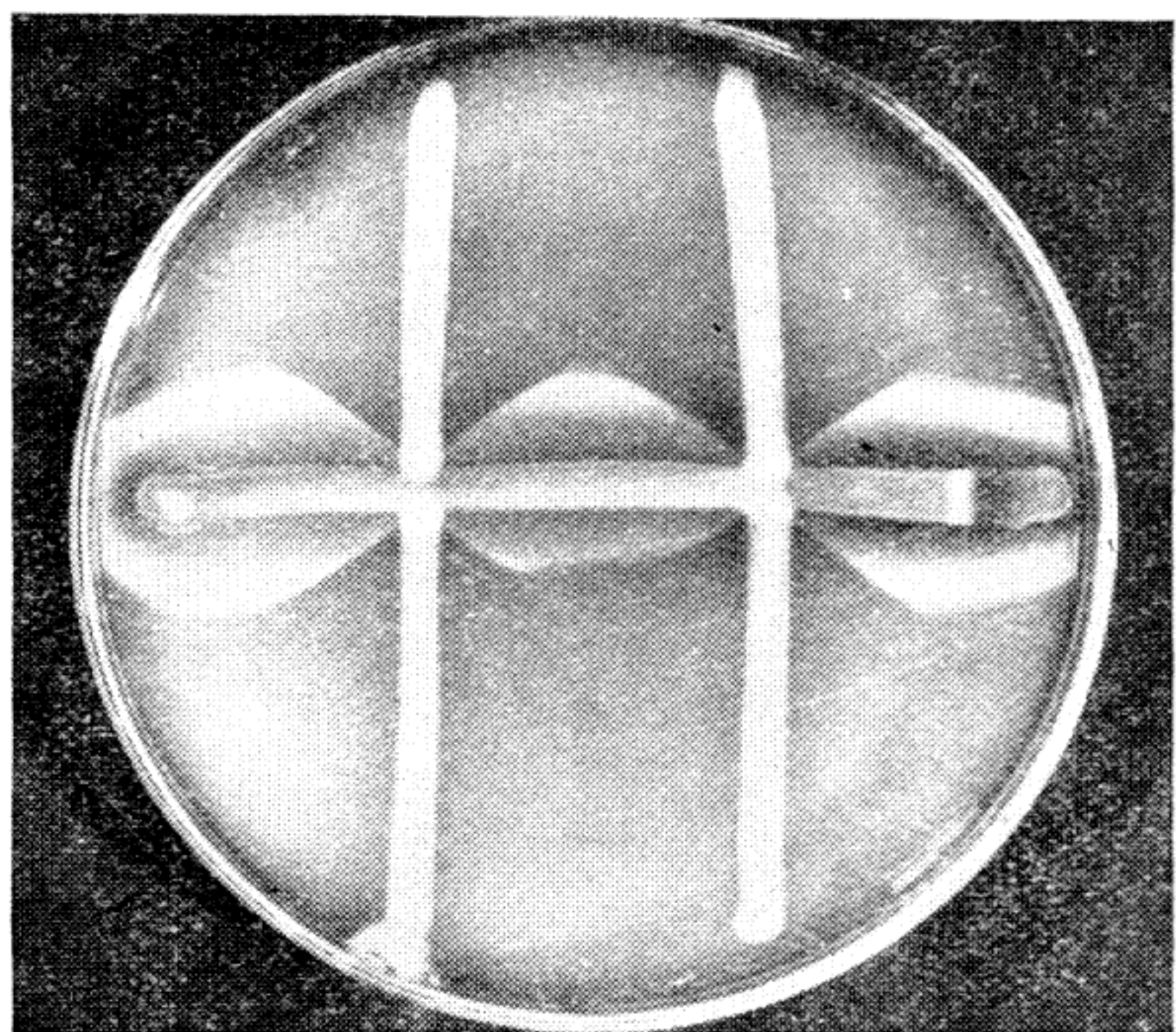


Photo 3.

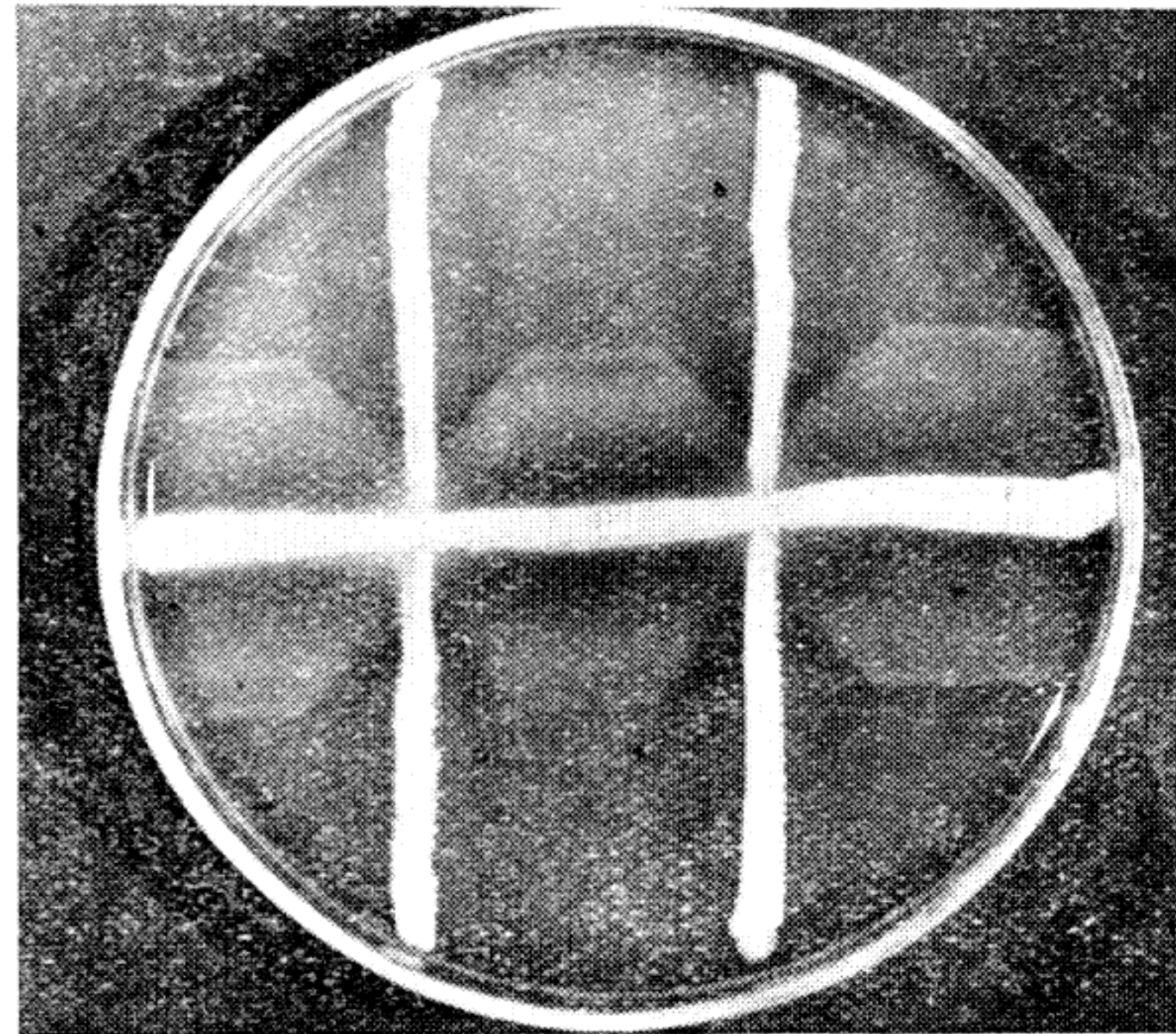


Photo 4.

Photo 3. Inhibition of staphylococcal haemolysis by strain ATCC 9345. — Photo 4. Inhibition of staphylococcal haemolysis by strain KC 472.

In Photos 1—4 both cultures were inoculated simultaneously.

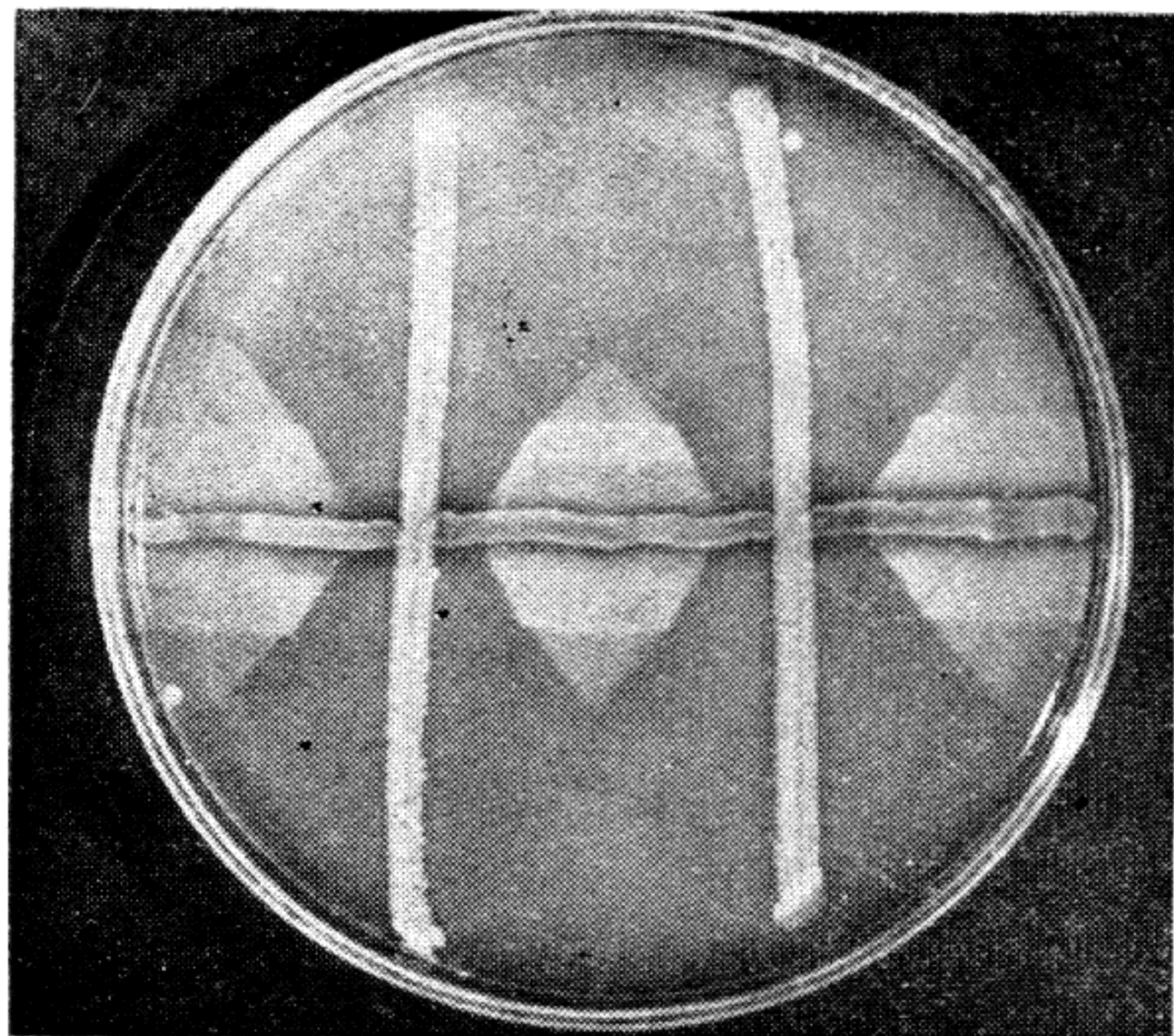


Photo 5.

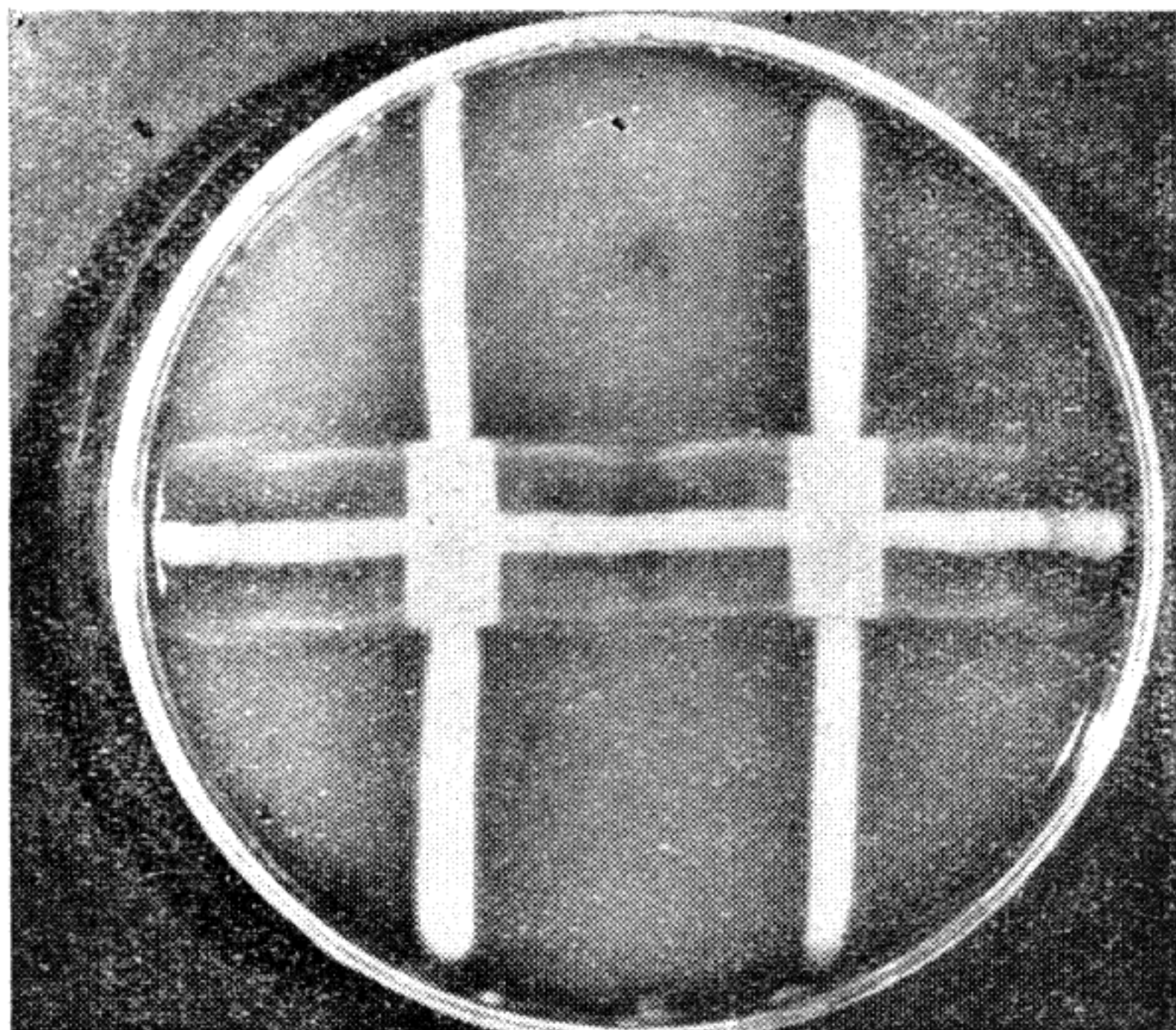


Photo 6.

Photo 5. Inhibition of staphylococcal haemolysis by strain KC 472. Staphylococcus inoculated 24 hrs after inoculation of strain K 472. — Photo 6. Enhancement of staphylococcal haemolysis by strain E 419.

Inhibitor of staphylococcal haemolysis

This factor was demonstrated according to Záhrová and Kubelka (18). Plates I—VII show that strain K 29 (Plate II), ATCC 9345 (Plate III) and KC 472 (plates IV, V) produced a substance which inhibited alpha-beta haemolysis of the employed strain of staphylococcus. When simultaneously inoculated, after 48 hours incubation at 37° C, well defined wedge-shaped areas of inhibition of staphylococcal haemolysis appeared at the site of streak crossings; the apex of the “wedge” points to streak of staphylococcus. When the staphylococcus was streaked across a 24-hr. culture of the human strains the zone of inhibition

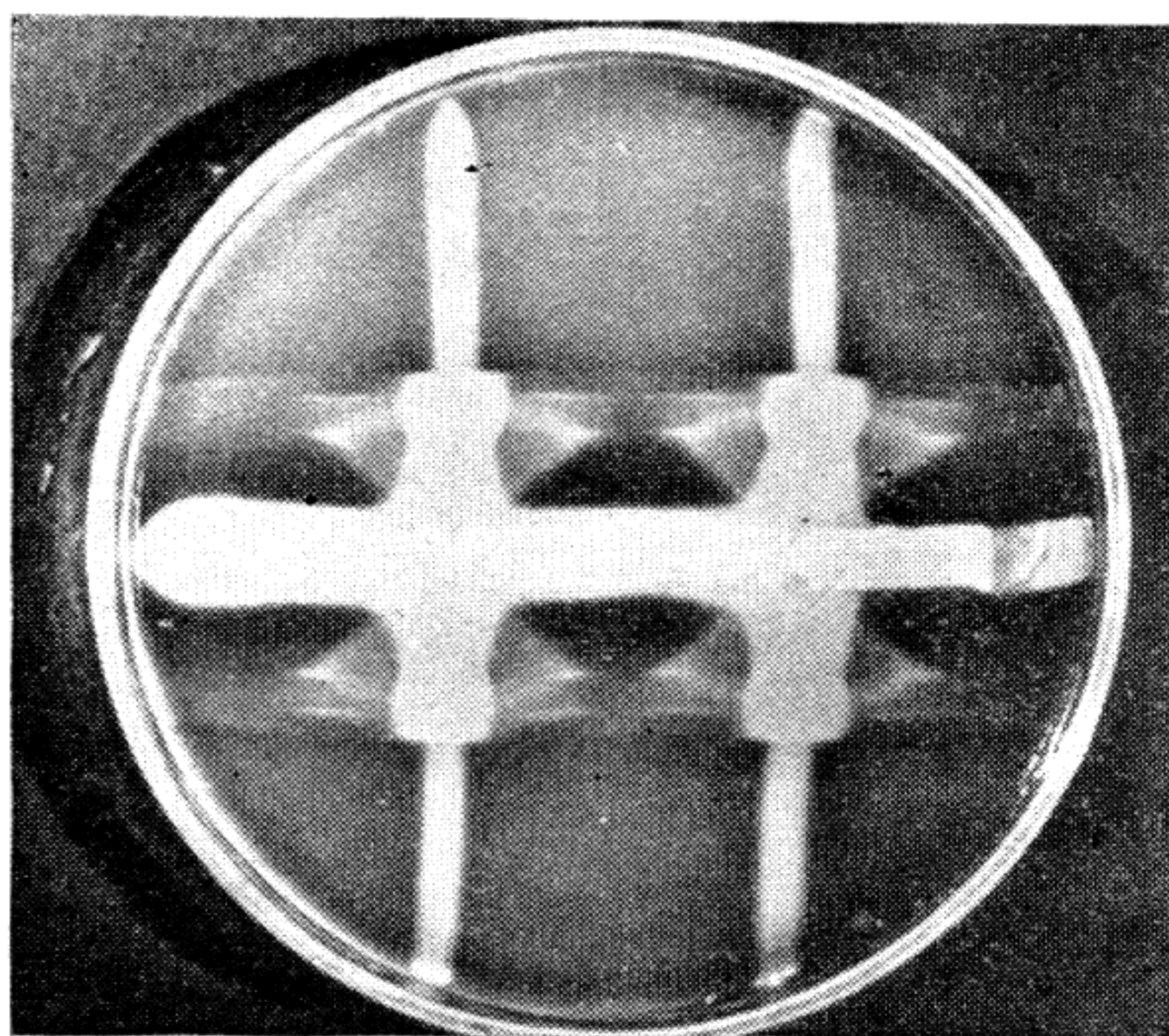


Photo 7. Enhancement of staphylococcal haemolysis by strain 73/61. In Photos 2—7 strain of *Staphylococcus pyogenes* inoculated horizontally, strains of corynebacteria inoculated perpendicularly in 2 streaks.

was greatly enlarged because of the already present inhibitor. In contrast, animal strains E 419 and 73/61 (Plates VI, VII) enhanced staphylococcal haemolysis: in strain E 419 the area of enhanced haemolysis was rectangular, in strain 73/61 in addition to this rectangle of complete haemolysis, a triangular area of enhanced haemolysis appeared in the beta-haemolysis zone.

Demonstration of inhibitor in the filtrate

Záhorová's method was modified (supra). The titre of inhibitor was investigated in the same specimens as haemolysin and toxin. Figure 5 illustrates that the production of inhibitor commenced during the initial stages of the exponential phase of growth. With increasing numbers of microbes the production of inhibitor increased greatly and after reaching the stationary phase remained at a constant level over a period of one month. The production curves of inhibitor in strains K 29 and ATCC 9345 are similar to the production curves of the toxic substances (previous communication). The shape of the curve in *Cor. ovis* differs from them in that the curve declines more steeply right at the beginning of the stationary phase. Even here we find agreement when comparing the amount of produced inhibitor with the formation of the toxic substance.

In strain E 419 no inhibitor was found in the filtrate and, as has been previously reported, neither was toxic activity demonstrated. There is a discrepancy only in strain 73/61, despite the fact that no inhibitor has so far been found in its filtrate, nevertheless, the injection of *F* produced the response previously described. The toxic effect of this strain on the skin of the rabbit was different from that of strains K 29, ATCC 9345 and KC 472 (Fig. 6).

Adsorption of inhibitor on sheep RBC

Záhorová and Kubelka (18) have already pointed out that a certain period of contact of filtrate with RBC prior to the addition of staphylolysin is necessary for inhibition of haemolysis. When RBC, filtrate and staphylolysin were mixed together simultaneously inhibition was greatly diminished. It can therefore be assumed that a substance (inhibitor) is adsorbed onto the surface of RBC. This finding was confirmed by us with the addition that adsorption was demonstrated in a large series of experiments qualitatively and quantitatively. The conditions under which adsorption of inhibitor to RBC takes place are shown below.

Dependence of adsorption on concentration of sheep RBC & temperature

Tab. 1.

Concentration of RBC	Percent of adsorption at		
	4° C	20° C	37° C
1%	45.6	10.9	—
10%	91.3	93.5	39.1
20%	93.5	93.5	93.5

Period of adsorption 30 min., 46 units inhibitor per 1 ml. of filtrate.

Table 1 shows that the amount of adsorbed inhibitor is dependent on the concentration of RBC. An increase in the concentration of RBC is followed by

a diminution of inhibitor in the filtrate. In addition to the concentration of RBC, adsorption depends on the temperature at which it takes place. The same table shows that at low concentrations of RBC temperature is an important factor.

Elution of inhibitor from sheep RBC

RBC with adsorbed inhibitor were centrifuged and after washing in physiological saline they were resuspended in either physiological saline or T-H broth. In both instances elution into the milieu took place, when using physiological saline elution was more rapid and more complete. Table 2 shows the temperature-time relationships for elution of inhibitor from RBC.

RBC adsorbed 30 min. at 20° C in 10 ml. amounts of filtrate, containing 237 units of inhibitor per 1 ml. Sheep RBC added to 20% concentration.

Elution was also influenced by temperature. The property of adsorption and elution of inhibitor was utilized in subsequent work. Similar results were obtained with rabbit and human RBC.

Tab. 2. Dependence of elution on temperature, time and milieu

Temperature	Time in hrs.	Percentage of elution in	
		saline	T-H broth
4 °C	1	4.6	...
20 °C	1	3.4	...
37 °C	1	6.3	...
4 °C	24	19.4	15.1
20 °C	24	39.9	24.9
37 °C	24	74.1	35.1

DISCUSSION

It follows from the data presented that all the studied strains of corynebacteria, with the exception of *Cor. ovis* strain KC 472, produce a soluble haemolysin. The antigenicity of this haemolysin has already been studied in animal strains of pyogenic corynebacteria by Lovell (8,9) who considers it to be a component part of the toxicity of this corynebacterium. We have investigated haemolysin in human strains, partially concentrated it (12), and demonstrated its thermolability. In the present communication we have shown, contrary to Barksdale et al. (1) and McLean et al. (10), that *Cor. haemolyticum* strain ATCC 9345 just as strain K 29, produces a soluble haemolysin. A detailed investigation of these factors will be the subject of a future report.

The production and activity of inhibitor of staphylococcal alpha-beta haemolysis has been studied in detail. The time of its production in strains K 29, ATCC 9345 and *Cor. ovis* (KC 472) coincides with the production of a toxic substance. This alone suggests that we may be dealing with identical agents. As an inhibitor it probably causes surface adsorption on RBC, thus obstructing selective haemolysis by staphylolysin.

Adsorption on RBC and elution of inhibitor has been used for the first time as an economical and rapid means of concentration and preliminary purification. The partially concentrated and purified product not only possesses greater in-

hibitory activity but also increased dermonecrotic activity. In view of the fact that antiserum, prepared by immunization of rabbits with toxic substance of strain K 29, neutralized not only the dermonecrotic and lethal effect of the toxic substance, but also in the same degree the inhibitory activity of the filtrate. it may be considered highly probable that these two agents are identical (in strains K 29 and ATCC 9345). On the basis of this finding a workable *in vitro* method has been elaborated by which inhibitor and toxin can be determined quantitatively in cultures of various strains. The final decision on whether the two agents are identical or not can be made only after complete immunological analysis of toxic substances and their purificates. Their chemical isolation and purification is the subject of investigations which are in progress.

Our finding that the phenomenon of inhibition of staphylococcal haemolysis is demonstrable not only in human strains of pyogenic corynebacteria but also in *Cor. ovis*, shows in our opinion, that it is possible to consider the formation of a subgroup of corynebacteria which are analogous in these characteristics. In view of Munch-Peterson's description of the same phenomenon in *Cor. striatum (flavidum)*, this group should include this species. It would also be advisable to review the findings of Fried (3) and to investigate the other corynebacteria described by Saxholm (16), Jebb (7), Henriksen (6), Barrat (2, 15), Gilbert and Stuart (5), etc.

S U M M A R Y

1. It has been found that *Corynebacterium haemolyticum* strain ATCC 9345 produces soluble haemolysin like human pyogenic corynebacteria and strains of *Cor. pyogenes bovis*.

2. Human strains of pyogenic corynebacteria, like *Cor. haemolyticum* strain ATCC 9345, produce a substance inhibiting staphylococcal alpha-beta haemolysis.

3. *Cor. ovis* strain KC 472 was found to produce an analogous substance.

4. Production curves of human pyogenic corynebacteria, *Cor. haemolyticum* and *Cor. ovis* show that inhibitor and toxic substance are produced simultaneously, and persist in cultures for the same length of time.

5. The toxic substance and inhibitor of strain K 29 can be partially concentrated and purified by adsorption/elution with sheep RBC. Both factors can be neutralized by immune serum in the same ratio. These circumstances, and further possibilities of purifying both agents suggest that they are closely related, or even identical.

6. An exact method for the determination of inhibitor *in vitro* has been elaborated.

7. It has been found that assay of staphylococcal haemolysis inhibition is also characteristic of other pathogenic species of corynebacteria.

R É S U M É

1. On a établi que *Corynebacterium haemolyticum* ATCC 9345 est le même producteur de la hemolysine soluble que les soi-disant corynebacteries pyogènes de l'homme et les souches du *Corynebacterium pyogenes bovis*.

2. Les souches humaines des corynebacteries pyogènes produisent de même que la souche ATCC 9345 du *Corynebacterium haemolyticum* une substance empêchant l'alpha-beta hemolyse staphylococcique.

3. Chez la souche du *Cor. ovis* KC 472 on a établi que la souche en question est la productrice d'un facteur analogue.

4. Des courbes de la production du *Corynebacterium pyogenes* d'homme, du *Corynebacterium haemolyticum* et du *Corynebacterium ovis* on peut, avec facilité, déduire que

l'inhibiteur et l'antigène toxique sont produits simultanément, survivant dans des cultures pour le même temps.

5. C'est par l'adsorption sur des globules du sang de mouton et par l'élution d'elles que l'on peut partiellement purifier et concentrer l'antigène toxique ainsi que l'inhibiteur chez la souche K 29. On peut neutraliser tous les deux facteurs par l'antisérum dans les mêmes proportions. Ces faits comme aussi la possibilité ultérieure de purifier tous les deux facteurs à la fois prouvent qu'ils sont très rapprochés ou bien, peut être, identiques.

6. On a élaboré une méthode exacte pour mesurer des inhibiteurs in vitro.

7. L'expérience a démontré que le test d'inhibition de l'hémolyse de staphylocoques est caractéristique aussi pour autres espèces pathogènes de *Corynebacteria*.

ZUSAMMENFASSUNG

1. Es wurde festgestellt, daß *Corynebacterium haemolyticum* ATCC 9345 derselbe Produzent des solubilen Hemolysins ist, wie die sogenannten pyogenen Korynebakterien des Menschen und die Stämme des *Cor. pyogenes bovis*.

2. Die menschlichen Stämme der pyogenen Korynebakterien sowie das *Cor. haemolyticum* Stamm ATCC 9345 produzieren eine Substanz, die die Staphylokokken-Alpha-Beta-Hemolyse hemmen.

3. Beim Stamm *Corynebacterium ovis* KC 472 wurde festgestellt, daß derselbe ein Produzent des analogen Faktors ist.

4. Aus den Produktionskurven der menschlichen pyogenen Korynebakterie, des *Corynebacterium haemolyticum* und *Cor. ovis* geht klar hervor, daß der Inhibitor und das toxische Antigen gleichzeitig produziert werden und gleich lange in Kulturen überleben.

5. Das toxische Antigen und der Inhibitor können bei dem Stamm K 29 durch Adsorption an Hammelerythrozyte gebunden und aus derselben durch Elution teilweise purifiziert und konzentriert werden. Beide Faktoren kann man im gleichen Verhältnis durch Antiserum neutralisieren. Diese Umstände sowie weitere Möglichkeiten der Purifikation beider Faktoren zu gleicher Zeit sprechen dafür, daß dieselben sehr ähnlich und vielleicht identisch sind.

6. Man hat eine exakte Methodik für das Messen von Inhibitoren in vitro ausgearbeitet.

7. Es wurde festgestellt, daß die Probe der Inhibition der Staphylokokken-Hemolyse auch für andere pathogene Arten von Korynebakterien charakteristisch sind.

RESUMEN

1. Los autores han establecido que el *Corynebacterium haemolyticum* ATCC 9345 es el mismo productor de la hemolisina soluble como las así llamadas corinebacterias piogénicas humanas y las cepas del *Cor. pyogenes bovis*.

2. Las cepas humanas de las corinebacterias piogénicas igualmente como el *Cor. haemolyticum* la cepa ATCC 9345 producen una sustancia inhibente la alpha-beta-hemólisis estafilocócica.

3. En lo que se refiere a la cepa del *Cor. ovis* KC 472 se ha establecido que es la productora de un factor analógico.

4. De las curvas productoras de las corinebacterias piogénicas humanas es evidente que el inhibidor así como el antígeno toxico se producen al mismo tiempo y sobreviven en los cultivos por el mismo tiempo.

5. El antígeno toxico y el inhibidor de la cepa K 29, obtenidos por adsorción sobre los globulos del sangre de carnero, se pueden parcialmente purificar de ellos por la elución y concentrar. Los dos factores se pueden neutralizar en la misma relación con ayuda del antisuero. Estas circunstancias y las posibilidades de purificar los dos factores a la vez testimonian de su proxima concordancia o quizá identidad.

6. Los autores han elaborado un metodo preciso para medir los inhibidores in vitro.

7. Resultó que la reacción de la inhibición de hemólisis estafilocócica es característica también para otros generos de las corinebacterias.

REFERENCES

1. Barksdale W. L., Cummings C. S., Harris H.: *J. gen. Microbiol.* 16, 749—758, 1957.
- 2. Barrat: *J. Path. Bact.* 36, 369, 1933. — 3. Fried R.: *Zbl. f. Bakt.* 165, 25—38, 1956. —
4. Fraser G.: *Nature* 189, 246, 1961. — 5. Gilbert R., Stewart F. C.: *J. lab. clin. Med.* 12, 756, 1927. — 6. Henriksen S., D.: *J. Path. Bact.* 74, 503—511, 1952. — 7. Jebb W. H. H.: *J. Path. Bact.* 60, 403—412, 1948. — 8. Lovell R.: *J. Path. Bact.* 52, 295—303, 1941. —
9. Lovell R.: *J. Path. Bact.* 45, 339—355, 1937. — 10. McLean P. D., Liebow A. A., Rosenberg A. A.: *J. Inf. Dis.* 79, 69—90, 1948. — 11. Munch-Peterson E.: *Austr. J. Exp. Biol. Med. Sci* 32, 361, 1954. — 12. Patočka F.: *Čas. lék. čes.* 94, 1323, 1955. — 13. Patočka F., Souček A., Mára M.: *Journal of Hygiene, Microbiology and Immunology* 4, 307—308, 1960. —
14. Patočka F., Souček A., Mára M., Jedličková A., Záhorová L.: *Čs. Epidemiol. Mikrobiol. Imunol.* 10, 184—191, 1961. — 15. Petrie G. F., McClean D.: *J. Path. Bact.* 39, 635, 1934. — 16. Saxholm: *J. Path. Bact.* 63, 303—313, 1951. — 17. Stein G. J., Dang Van Ngu: *J. Immunol.* 65, 12—39, 1950. — 18. Záhorová L., Kubelka V.: *Folia microbiologica* 5, 57—59, 1960.

Received November 16, 1961.

A. Souček, Ústav pro lékařskou mikrobiologii,
Studničkova 7, Praha 2, Czechoslovakia

Our acknowledgements are due to Dr. E. O. King of the Communicable Disease Center, Atlanta, to Dr. L. Záhorová and Dr. Kolář for supplying us with the strains used in this investigation, and to Dr. M. Chýle for help in the preparation of the manuscripts.