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Studies on the Pathogenicity of *Listeria monocytogenes*

II. Influence of substances isolated from cells of *Listeria monocytogenes* on experimental listeriosis in white mice

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With 5 figures

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In a previous report (1) a substance of protein nature which markedly enhances listeric infection in white mice was described. This substance isolated from bacterial cells of *Listeria monocytogenes* was partially purified, its chemical and biologic properties were described. So far as has been determined we are dealing with a complex of proteins, polysaccharides, nucleoproteins and lipids. Since it hitherto has not been possible to further isolate the individual components, it was necessary to implement other methods in isolating some of these components and determine to what degree they also enhance listeric infection.

Material & Methods

Cultivation and preparation of bacterial cells of *L. monocytogenes* strain ŠE was carried out following the same technic as described earlier (1).

Preparation of protein from L. m. (L protein):
According to the method used in isolating streptococcal M-protein (2), a protein substance, appearing to be a chemical individual, was extracted with 0.1 n HCl at 37° C from glycine lysate (GL) of *L. m.* (1). After a thrice repeated 24 hour extraction a soluble protein was reprecipitated by adapting the pH to 4.0–5,0 with 1 n NaOH, and again dissolved in M/15 phosphate buffer of pH = 7.2. Reprecipitation with 1 n HCl was repeated 3–7 times. pH was measured with glass electrode in supernatants, following centrifugation of precipitated protein. We centrifuged at 3,500 r. p. m. After repeated dissolution of the precipitate in phosphate buffer (pH = 7.2) the protein was lyophilized.

Preparation of polysaccharides:
A soluble polysaccharide was obtained from microbial cells by extraction with NaCl at 100° C and precipitation with methanol (3).

Preparation of nucleoproteins:
According to the method of JONES (4) nucleoproteins were precipitated from mechanically disrupted cells of *L. m.*, following extraction with 0.001 M sodium arsenate. A 2% solution of cetyl-pyridinium-ammonium bromide was used in the precipitation. The

nucleoprotein complex with quaternary base, following thrice repeated reprecipitation by diluting the 1n solution of NaCl (in which the complex is soluble) with 2 volumes of distilled water, was dissociated by precipitating 1 volume of the complex in 1n NaCl with 9 volumes of 96% ethanol. The obtained white flaky precipitate, following storage for 24 hours at 4°C, was centrifuged and dissolved in saline. Precipitation was then repeated once more and the resulting solution was tested for the presence of detergents by growth inhibition of *L. m.* on blood agar. The result was negative.

Preparation of a protein-free extract:

An attempt has been made, employing Boivin's method modified by WEBSTER et al. (5), to extract with 0.5% TCA and by cold precipitation with ethanol to obtain from washed cells of listeria a substance analogous with the so called endotoxin of Gram-negative bacteria. Further purification of the thus obtained preparation was not successful (precipitation with 0.3M ethanol in a saturated solution of NaCl) because its properties differed from those of the antigenic complex of the majority of Gram-negative bacilli.

Analyses:

Proteins were determined by the biuret reaction in WEICHSELBAUM's modification (6), and in nucleoproteins with phenol reagent (7). Polysaccharides were determined with alfa-naphtol according to DISCHE (8), DNA with diphenylamine according to SEVAG et al. (9) and with cystein sulphuric acid according to DISCHE (10). Absorption spectrum of L protein and nucleoproteins was measured with Zeiss' universal spectrofotometr (1). Determination of rhamnose was made employing DISCHE and SHETTLES' reaction (11) with cystein in sulphuric acid, measurements of characteristic maximums in the UV range using 10mg% of rhamnose p.a. (MERCK) as a standard, and then by chromatography. In paper chromatography Whattman I paper was used in systems of n-butanol – acetic acid – water (4:1:5), and phenol – ethanol – water (2:1:1) with 0.1% 8-oxychinolin according to KOLOUŠEK (12). Ninhydrin and isatin were used in detecting aminoacids, and anilin-phtalate in the case of sugars. Hydrolysis for the chromatographic analysis was made with 6n HCl, and 2n H_2SO_4, respectively. Electroforesis was made to control the homogenicity of the isolated protein under the same conditions as in our previous report (1).

Activity was determined as described earlier (1) and expressed by an index and its logarithm. Solutions, unless otherwise specified, were adapted to correspond to 0.1% of undialysable substances.

Attempt of preparing antisera:

Three rabbits weighing at an average 2500g were immunized with an initial dose of 2ml/of 0.1% solution of L protein intravenously. Simultaneously the same was injected intramusculary with lanolin-mineral oil as adjuvant in three separate doses (14). The animals were bled by cardiac puncture after a three-week interval. Every experiment was terminated on the 14th day.

Results

L protein.

The extracted and precipitated protein readily dissolves at pH 7.2, giving a clear, foaming, brownish solution reminiscent of blood serum. Change of pH to 5.0 produces turbidity and rapid flocculation. In this way by repeated precipitation we obtained a substance which furnished in electrophoresis a high gradient of a mobility close to albumins (9.4×10^{-5}). A less mobile gradient found in other preparations, prepared in the same way at various times, most probably represents impurities adsorbed during precipitation (Fig. 1). Impurities were also found during analysis of polysaccharides and DNA as outlined in Table 2.

The protein is biuret positive and results of analysis correspond to the dry solids determined by usual methods. By two-dimensional chromatography in the hydrolysate the following amino-acids were found: lysin, histidine, ornitine, arginine, serine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, valine, tryptophan, phenylalanine and isoleucine. Some of these amino-acids may be component parts of accompanying substances. The UV spectrum

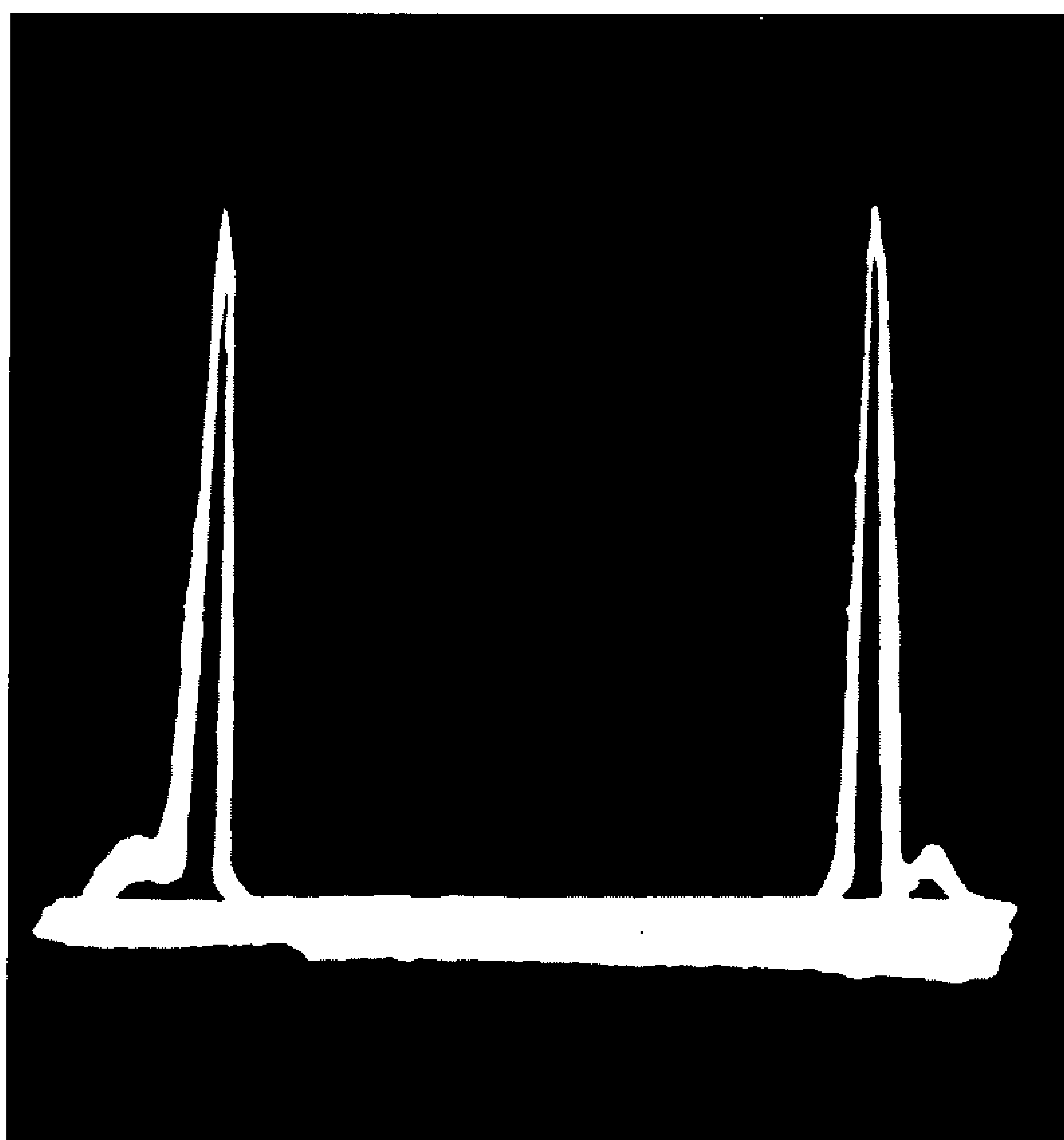

Fig. 1. Electrophoresis of L protein (batch E).

also discloses a small amount of nucleic acid (Fig. 2). The spectral zone of aromatic amino-acids of the protein, however, is much more prominent than in the complex preparation MA_2 (1). Table 2 and chromatograms of various preparations show that we are dealing with an inconsistent admixture of polysaccharides and nucleoproteins, of which the former is extracted in the second, and especially in the third step. Therefore we discarded the third yield in some batches. In biological experiments we considered the substance, after eliminating the activity of polysaccharides and nucleoproteins, as practically homogenous. Further purification and detailed studies on the chemical properties and composition of this hitherto undescribed protein, reminiscent of M protein, will be the subject of another paper.

This protein is not lethal on intravenous administration to mice and rabbits. A mild inflammatory reaction is produced on intradermal injection. In repeated experiments on enhancement of experimental listeriosis in mice we found the protein's activity to be in the limits of significance determined by us. We obtained positive results with certain not very active preparations only after raising the dose 6 and 10 times, leading to a rise of the index by 2 logarithms (Table 1).

Results of repeated experiments with various preparations prepared in a period of one year demonstrated on the average low activity of L protein. A certain variation in the results lies either in the nature of the agent, i.e. its

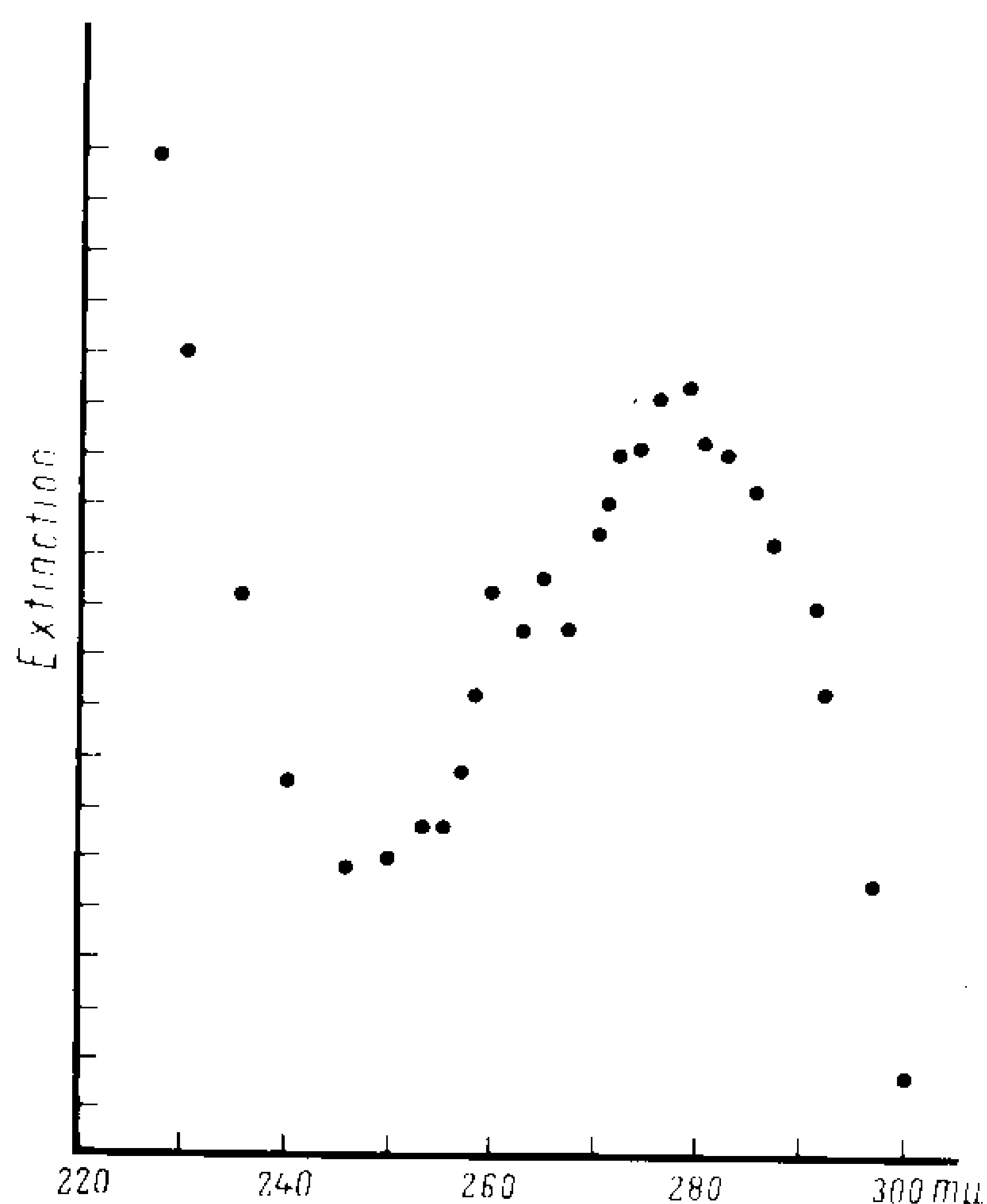

Fig. 2. Ultra violet spectrum of L protein (batch E) dissolved in phosphate buffer (pH= 7.2).

Table 1

Relationship of activity to dose of L protein

Batch	dose in μg	Index	Log
A	250	3	0.477
A	1500	197	2.295
A	2500	215	2.339

production which depends on varying metabolic conditions during cultivation (quality of nutrient medium, especially meat water), or in the seasonal changes in the sensitivity of experimental animals (14).

Table 2

Chemical composition of various batches of L protein and their activity

Batch	Proteins %	Polysaccharides %	DNA %	Index	Log
A	95.0	5.0	—	3	0.477
B	94.0	6.0	—	772	2.893
C	92.0	1.5	—	318	2.502
D	—	—	—	18	1.248
E	90.0	8.0	2.0	59	1.770

Doses of 250 μg dry weight were used.

L protein may also be extracted from a partially purified protein preparation MA_2 described earlier (1). This substance designated as LMA_2, has the same chemical properties as L protein extracted from glycine lysate in so far as isoelectric point, amino-acid content and the capacity to be precipitated with ammonium sulfate are concerned. (L protein is salted out in almost 90 % with ammonium sulfate of 50% saturation, as will be described in detail elsewhere.) Most probably we are dealing with an identical substance of greater purity in regard to polysaccharide content. (The amount of polysaccharides was decreased by precipitation with methanol and ammonium sulfate (1)). The effect of preparation LMA_2, similarly as other preparations of L protein, on enhancement of infection is small, even lesser than of MA_2 from which it was derived. The remainder of the protein complex MA_2 after extraction

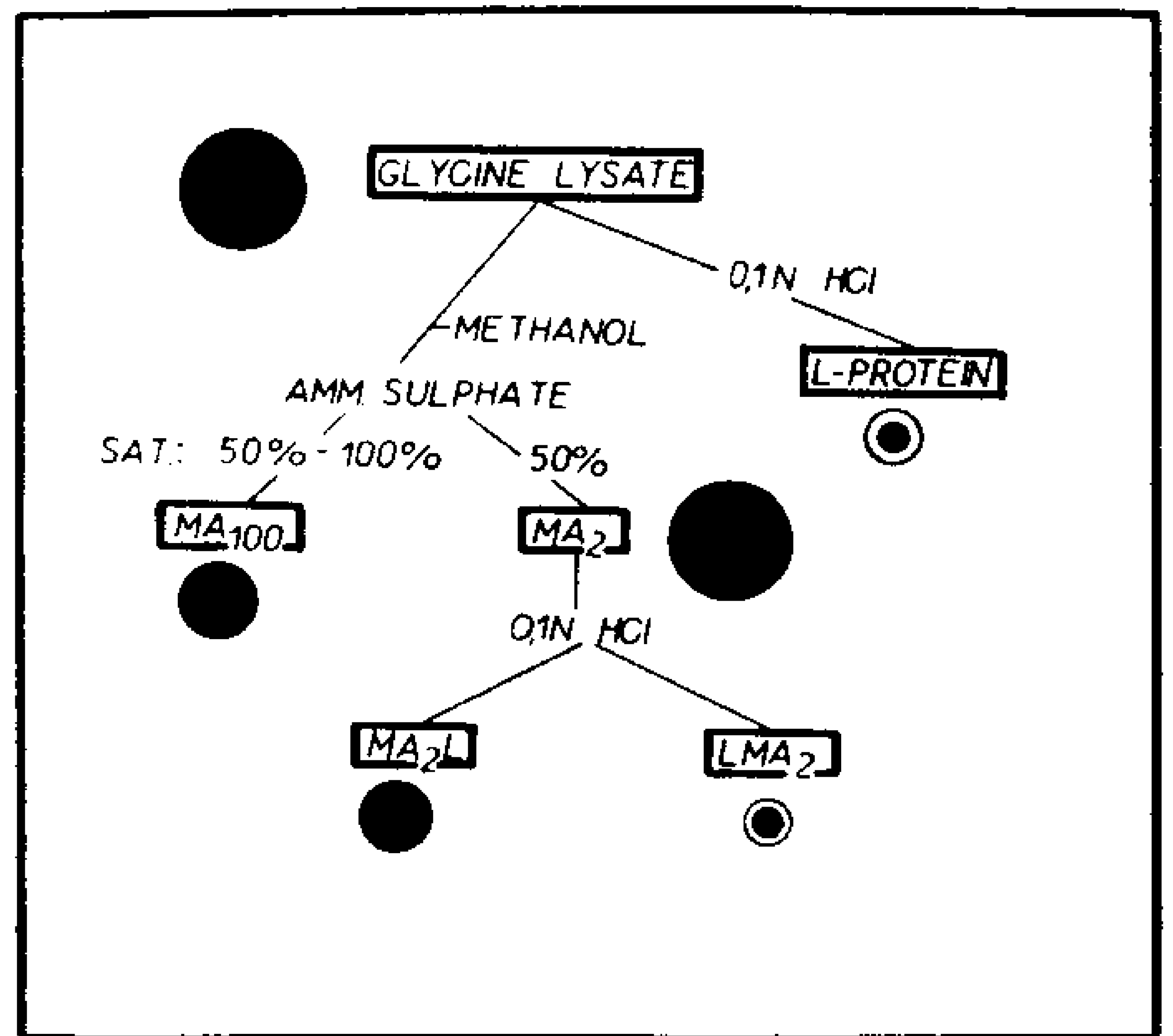

Fig. 3. Outline of preparations derived from glycine lysate. Full circles represent relative activity expressed by $\log_{index}$. Encircled disks represent relative activity below the limit of significance.

with 0.1 n HCl is less active than the original MA_2. This is probably due to partial denaturation of a not readily soluble mixture of proteins damaged by prolonged action of hydrochloric acid. This also may point to the individuality of MA_2. Table 3 and Fig. 3 illustrate results of these experiments.

L protein induces mild production of antibodies in rabbits. After intramuscular injection of L protein, followed by immunization with adjuvants, precipitins appeared in 1:10 antigen dilution. Agglutinins O and H were not demonstrated in serums of immunized rabbits.

Table 3

Comparison of activity and chemical composition of various preparations with L protein

Preparation	Proteins %	Polysaccharides %	DNA %	Index	Log
GL	58.0	17.8	6.5	16,980	4.231
MA_2	82.5	2,5	4.6	15,870	4.198
L protein	90.0	8.0	2.0	59	1.770
LMA_2	95.0	0.0	5.0	23	1.346
MA_2 -L*)	86.9	3.9	6.1	228	2.357

*) MA_2-L is a residue after extraction of L protein from MA_2.

Polysaccharides.

On isolation a colorless, clear and viscous solution containing 580 %mg of polysaccharides was obtained and then diluted to a concentration of 0.1 %. Following hydrolysis of a sample with 6n HCl of a biuret negative preparation amino-acids were demonstrated on chromatography confirming the presence of proteins. With Dische's cystein test methylpentoses were found to be present in the amount of almost 10 % of the total polysaccharides (Fig. 4). Paper chromatography disclosed the presence of rhamnose, an obligatory component in Gram-negative microbes, in the hydrolysate. Activity is outlined in Table 4.

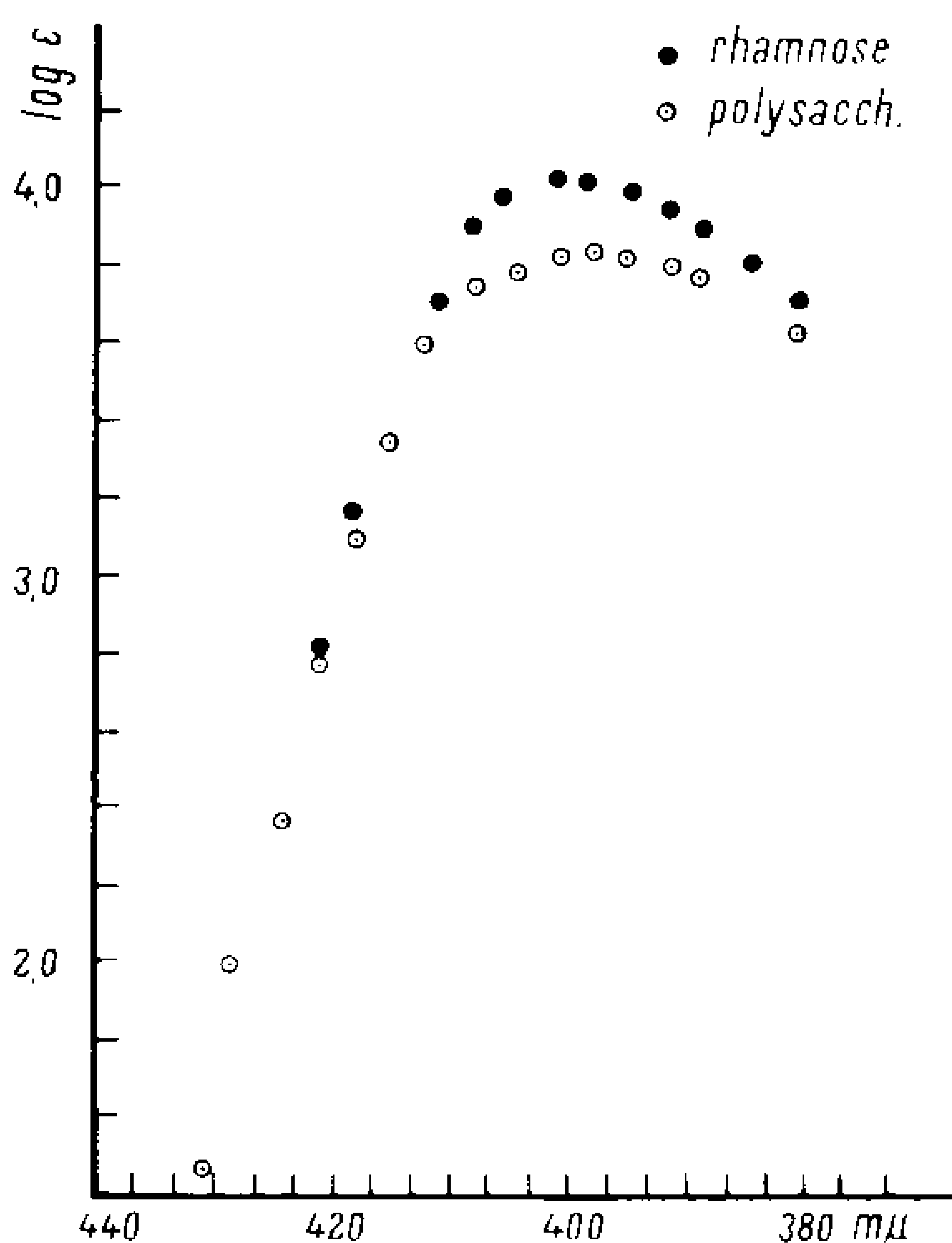

Fig. 4. Characteristic maximums of solution polysaccharides treated with cystein-sulphuric acid according to Dische & Shettles.

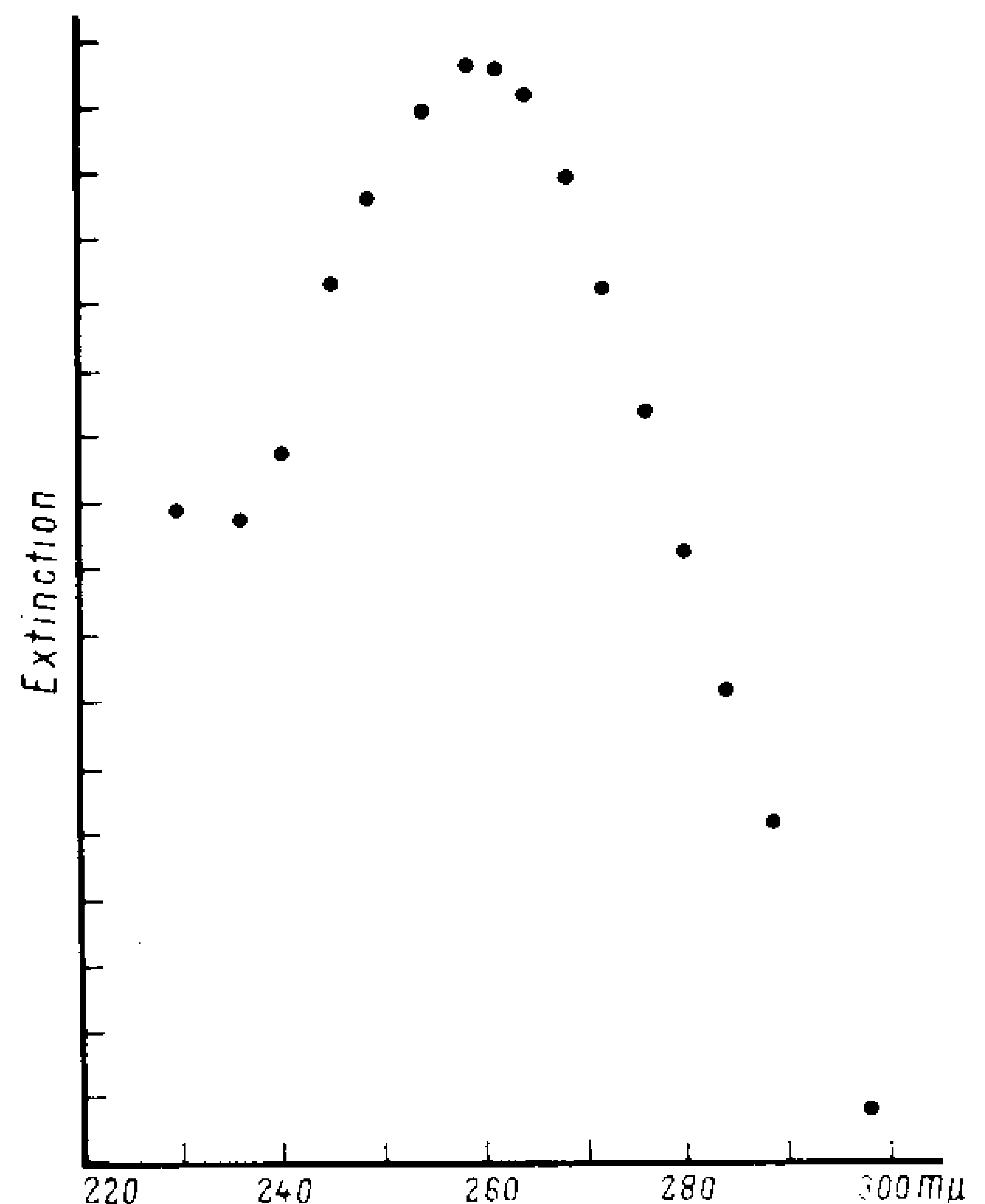

Fig. 5. UV spectrum of nucleoproteins extracted from cells of *L. monocytogenes*.

Boivin's extract.

This preparation, because of the identical method used in its preparation, was designated as Boivin's extract. It does not enhance listeric infection on administration of usual doses (Table 4).

Nucleoproteins.

An opalescent solution of nucleoproteins containing 36 mg % of desoxyribonucleic acid, 175 mg % proteins (phenol reagent) and 3 mg % of phosphorus, still gave positive reactions for DNA with cystein-sulphuric acid, and precipitated with sulphosalicylic acid and TCA. UV spectrum of this preparation is given in Fig. 5. Enhancement of experimental listeriosis was not demonstrated although a much higher dose was employed.

Table 4

Slightly active and inactive components in bacterial bodies of *L. monocytogenes*

Preparation	Dose (μg)	LD_{50} controls	LD_{50} test	Index	Log
Polysaccharides	250	8,043,000	168,700	47	1.669
Boivin's extract	250	1,349,000	46,420	29	1.420
Nucleoproteins	1000	179,500	158,500	1.1	0.054

Discussion

This report supplements a previous paper in which a protein or protein-like agent, isolated from bacterial bodies of a virulent strain of *Listeria monocytogenes* and capable of enhancing listeric infection in white mice on simultaneous administration with infecting dose, was described. The activity of a partial purificate of this agent first of all depends on its protein component other than nucleoproteins. It has not been hitherto possible to obtain a homogenous substance. The most active purificate still contained 2.5 % polysaccharides and 4.6 % nucleic acids, beside lipids. The activity of lipids has not been so far fully studied. Moreover, it is evident that repeated purification, according to the method employed by us, partially denatured the active agent. Therefore, an attempt has been made to prove, *per exclusionem*, that certain other more readily isolated and homogenous components of listerial cells do not influence experimental infection. Nucleoproteins proved to be inactive. An attempt to isolate from Gram-positive L. m., a substance similar to and analogous in its activity with the polypeptide-glycolipid endotoxic substance of Gram-negative enterobacteriaceae, according to Boivin, was unsuccessful. An almost pure polysaccharide was isolated by us from listeriae according to FULLER. This compound did not prove to be significant in its action. On the other hand a similar compound isolated by a different method is claimed by STANLEY to be highly toxic. The investigation of a practically homogenous protein substance designated as L-protein, isolated from listerial bodies following the rather harsh technic originally employed by HIRST and LANCEFIELD in obtaining the important factor of virulence of pyogenic streptococci – M protein, was prompted by certain analogies between these two microbes as studied by us. L protein proved to be active in a small degree. Therefore it will be proper to further investigate its activity using preparations isolated by more delicate methods. The exclusion and elimination of all other components of the bacterial

cell in respect to their activity clearly demonstrates that the agent influencing listeric infection necessarily has to be sought for in the complex protein substance of listeriae designated as MA_2.

Thanks are due to PhMr M. Štastný for carrying out electrophoresis, to Prof. Dr. J. Koštíř for reviewing the chemical data in the report, and to Dr. M. Chýle for the translation and assistance in the preparation of the manuscript.

Summary

An attempt has been made to isolate some components from bacterial cells of *Listeria monocytogenes*. Tests were made on white mice. Chemical properties and activity of the components are described.

Employing the method of acid extraction of M protein according to Hirst and Lancefield, a similar protein substance designated as L protein was obtained. Its chemical homogenicity was sufficient in determining its activity. Individual batches of L-protein varied in their activity but did not even equal the activity of the original substances from which they were derived.

The activity of the polysaccharide fraction was not prominent.

Nucleoproteins, isolated according to a modified method of Jones, proved to be inactive.

Isolation of a substance analogous to Boivin's extract was unsuccessful.

Zusammenfassung

Es wurde ein Versuch zur Isolierung von Bestandteilen der Bakterienzellen von Listeria monocytogenes gemacht und Versuche an weißen Mäusen angestellt. Die chemischen Eigenschaften und die Aktivität der Bestandteile werden beschrieben. Bei Anwendung der Säure-Extraktion von M-Protein nach Hirst und Lancefield wurde ein ähnliches Protein erhalten und als L-Protein bezeichnet. Seine chemische Homogenität genügte, um die Aktivität zu bestimmen. Anteile von L-Protein wechselten in ihrer Aktivität, erreichten aber nicht annähernd die Aktivität der Originalsubstanz, aus der sie gewonnen waren. Die Aktivität der Polysaccharid-Fraktion war nicht erheblich. Nukleoproteine, nach Jones Methode isoliert, schienen inaktiv zu sein. Isolierungen einer Substanz analog einem Boivin-Extrakt waren erfolglos.

Résumé

Etudes sur la pathogénité de Listeria monocytogenes IIe rapport

F. Patocka et collab.

Les auteurs ont procédé à une expérience visant l'isolement de constituants des cellules bactériennes de Listeria monocytogenes ainsi qu'à des expériences sur des souris blanches. Ils décrivent les propriétés chimiques et l'activité des constituants. L'application de l'extraction de protéine M par l'acide selon Hirst et Lancefield fournit une protéine analogue, dénommée protéine L. Son homogénéité chimique suffit pour déterminer l'activité. Des portions de protéine L varièrent par rapport à leur activité, mais n'atteignirent de loin pas l'activité de la substance d'origine, à partir de laquelle elles étaient obtenues. L'activité de la fraction polysaccharide n'était pas considérable. Des nucléoprotéines, isolées selon la méthode de Jones, semblèrent être inactives. Des isolements d'une substance, analogue à un extrait bovin, furent inopérants.

Resumen

Estudios sobre la patogenicidad de la Listeria monocytogenes

II. Comunicación

F. Patocka y otros

Se hizo un ensayo para el aislamiento de partes integrantes de las células bacterianas de Listeria monocytogenes y ensayos en ratones blancos. Son descritas las propiedades químicas y la actividad de las partes integrantes. Por aplicacíon de la extracción ácida de proteína-M según Hirst y Lancefield fué obtenida una proteína parecida y denominada proteína-L. Su homogeneidad química bastó para determinar la actividad. Algunas

fracciones de la proteína-L cambiaron en su actividad, no obstante no alcanzaron ni siquiera aproximadamente la actividad de la substancia original de la que fueron obtenidas. La actividad de la fracción polisacárida no fué considerable. Nucleoproteínas aisladas según el método de JONES parecieron ser inactivas. Aislamientos de una substancia análoga a un extracto-Boivin fueron infructuosos.

Изучение патогенности Listeria monocytogenes.

Сообщение 2-е

Ф. Патоцка и др.

Выводы

Авторы попытались изолировать составные части бактерийных клеток Listeria monocytogenes и для этой цели поставили опыты на белых мышах. Химические свойства и активность составных частей описываются. Применением кислотной экстракции М-протеина по Hirst и Lancefield ими получен аналогичный протеин, называемый L-протеином. Химическая гомогенность его достаточна для определения активности. Части L-протеина колебались в активности, но далеко не достигали активности оригинального вещества, из которого они получены. Активность полисахаридной фракции была незначительной. Изолированные по методу нуклеопротеины казались неактивными. Изоляция вещества аналогично вытяжке Boivin осталась безуспешной.

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