Sudan J. Vet. Res. Vol 5 1983

* EFFECT OF PH AND GLUCOSE ON GROWTH AND HASMOLYSIN PRODUCTION BY LIETERIA MONOCYTOGENES

G.E. Mohamed, A Seaman and M. Woodbine

INTRODUCTION

L. monocytogenes is a pathogen to a variety of other animals species as well as man. It has been reported from wide geographical areas. The report on the incidence of Listeriosis in man and animals is increasingly high during the past few years (Problem of Listeriosis, 1975, 1979, 1981 and Communicable Disease Report, 1980, 1979, 1981). This could be attributed to improved methods in the isolation and identification of the organsim.

L. monocytogenes has been known as being B. haemolytic ever since it was first described (Murray, Webb and Swann, 1926). The organism produces extracellular, soluble and filterable haemolysin which is capable of destroying the red cells (RBC) of man, horse, cattle and rabbit with no differences in susceptibility (Seeliger, 1961). The lethal and cytotoxic effects of listerial haemolysin have been demonstrated by a number of workers, among these Liu and Bates, 1961; Siddique and Walker, 1967: Siddique, 1969 and Kingdon and Sword 1970 a.b.

Strains vary, in quality (Njoku-Obi et al, 1963) and quantity (Jafari, 1980), in their haemolytic activity. The amount of haemolysin produced also depends upon the medium and the cultural conditions (see Njoku-Obi et al., 1963; Muenker and Roots, 1963 and Jafari, 1980). The increased haemolysin production in the nutrient broth at pH 6.5 obtained during the study of the effect of Nisin on haemolysin production (see Mohamed, 1982) prompted the detailed investigation on the optimum medium pH for Listerial haemolysin production, and this in turn led to the study on the effect of glucose with controlled and uncontrolled pH on the growth and haemolysin production.

* Extract from a Ph. D. the sis submitted to University of Nottingham, U.K. 1982

MATERIALS AND METHODS

Cultures:

Listeria monocytogenes strain 4379, 5214 m, 10357, C 52, 4121 and 4885.

Nutrient broth (Oxoid No. 2) was used as a culture medium. The inoculum was 1.5 X (10)5 viable cells per ml of the medium. Peptone water (Oxoid 0.1% Bacteriological peptone pH 7.2) was used as a diluent. Viable count was done by the spreader method. Samples for viable count and haemolysin assay were taken at appropriate intervals according to the rapidity of the growth. The pH of the medium was adjusted to 5.5 and 6.5 by 5 N HCL.

The effect of glucose on growth and haemolysin production with uncontrolled pH was examined in 200 ml nutrient broth containing 0.5% membrane filtered glucose (Fisons, Loughborough).

The effect of glucose with controlled pH was examined in 5L fermenter (Biotec-Ltd., Sweden) which was modified for batch culture fermentation. 2L sterile nutrient broth containing 0.5% glucose was added to the fermenter under aseptic conditions. Throughout the fermentation the automatic pumped titrant (5W NaOH) was used to maintain the desired pH (7.4).

Samples for haemolysin assay were centrifuged at 6000 r.p.m. for 20 min. at 5°C and measurement of haemolysin activity were carried out according to the known routine procedure (see Mohamed, 1982 and Girand and Sbarra, 1963). For haemolysin indicator plate (HI) a method similar to that proposed by Park and Mikolajcik (1977) was used.

For qualitative testing of the haemolytic activity of cultures grown at pH 6.5 and 7.4, haemolysin was extracted as above, diluted 1:4 in phosphate buffer saline containing a reducing agent (2% sodium hydrosulphite) and equal amounts were added to each well in the HI plates. After incubation the haemolytic zones were recorded.

RESULTS

These studies were done with five strains of L. monocytogenes. (Strain 10357 was also examined, the trace of soluble haemolysin which was observed at pH 7.4 (4 MHU) was not influenced by increasing hydrogen ion to 6.5 and was completely absent at pH 5.5, however, this strain had a clear haemolytic activity on blood agar.

Growth and haemolysin production in nutrient broth inoculated with L. monocytogenes 4379 (type 5) are shown in Fig. (1). In general the rise in haemolysin titre followed the increase in cell number. The outstanding feature was the dramatic increase in haemolysin production in the late log/early stationary growth phase at pH 6.5, even though the acid pH had little effect on the growth in numbers. A decline in haemolysin titre at 48 hr was observed in culture grown at pH 6.5 (from 320 - 256 HU) while that at pH 7.4 remained at constant level. The phenomenon of increased haemolysin production with increasing hydrogenion concentration to pH 6.5 was also observed with the

other four strains, these results are summarized in Fig. (2). The results obtained in the haemolysin indicator plate (HI) (not shown) although were qualitative ones, they clearly demonstrated the superiority of pH 6.5 over the pH 7.4 in supporting haemolysin production.

An experiment was carried out to examine whether the increased haemolytic activity observed at pH 6.5 was mainly due to the increase in biological activity or increased production. Here the haemolysin obtained from culture grown at pH 7.4, when adjusted to pH 6.5 and 5.5, yielded similar titre, as that of the original sample (at pH 7.4) and nutrient broth of pH 7.4 6.5 and 5.5 produced no lytic action on 2% horse RBC, (see table 1) Fig. (3) shows the result obtained from the experiment undertaken to investigate the stability of Listereal haemolysin under different temperatures and pH's, here, although haemolysin was not stable at the three pH's, (7.4 6.5 and 5.5) at 37 C, the inactivation increased with the increase in hydrogen ion concentration, haemolysin incubated at 22 C showed more stability than that at 37C.

Effect of glucose on growth and haemolysin production:

The presence of glucose markedly improved the growth of, and haemolysin production by, strain 4379 as compared with those obtained in glucose-free medium. This comparison is shown in Fig. (4). The stimulatory effect of glucose on growth was apparent from the increase in the maximum viable yield obtained. The effect of addittion of glucose was to extend the period of the logarithmic growth by some 2-4 hr. However, the increased maximum population was not sustained, the stationary phase of the basal medium being replaced by an immediate decline. The haemolysin production paralleled the growth in numbers, the sole difference being that the production became apparent by some 4hr. later and there was a sharp drop in the haemolysin titre after 16hr. of incubation when glucose was present. The growth of L. monocytogenes in nutrient broth produced no significant drop in the initial pH. In culture which contained 0.5% glucose, the initial pH 7.4 remained constant in the first 4 hr, then dropped to 6.5 at 8 hr. and to 5.2 at 12 hr. The decline in pH from 7.4 to 6.5 and 5.2 was concomittant with logarithmic growth and the rapid accumulation of haemolysin in the medium. When a pH of 5.2 was read, the population started to decline. At 16 hr. and to 72 hr of incubation the pH remained constant at 4.9, during this period the decrease in the haemolytic activity was observed.

The general effect of glucose on growth of , and haemolysin production by 5214 m was similar to 4379. The initial pH of 7.4 was also dropped to 7.0, 5.5 and sustained at 4.2

The effect of 0.5% glucose on growth and haemolysin production under controlled pH (7.4) was examined in strain 4379 (Fig. 5). Under these conditions growth reached the maximum within 12 hr. after inoculation of the organism.

The rate of growth was comparable to and the peak viable cells greater than those obtained when the organism was grown in the presence of glucose with uncontrolled pH. The decline after the peak number was also not so marked. The haemolysin production under controlled pH was slow to reach a titrable level. The maximum titre of 320 HU was reached within 16 hr. of incubation, this level of haemolytic activity was higher than that obtained with culture in glucose-free medium (128 HU) and lower than that obtained in the presence of glucose with uncontrolled pH (640 HU). The decline was also sharper than when the pH was not controlled.

DISCUSSION

It can be seen that the capacity of L. monocytogenes strains to produce haemolysin was markedly affected by the pH of the medium in which they had been grown, higher titres of haemolysin were obtained at pH 6.5; pH 7.4 was moderated whereas pH 5.5 gave low amounts. The increase in haemolysin titres of individual strain was proportional to its haemolytic activity at pH 7.4. It seems that the amount of toxin produced per cell grown at pH 6.5 is more than that formed per cell at pH 7.4 since the rate and extend of growth were the same at both pH's.

Jenkins et al (1966) found that the biological activity of Listerial haemolysin was at its peak at pH 6.5 and Kingdom and Sword (1970 a) found that pH 6.6 was the optimum pH for the lytic activity of haemolysin on lysosomes. In the present investigation the increase in haemolytic activity at pH seems mainly due to the increase in productivity for the following reasons: Firstly the supernatants taken from the cultures grown at pH 7.4 and 6.5 were titrated in the same phosphate buffered saline at 6.6. Secondly haemolysin produced by the culture grown at pH 7.4, when acidified to pH 6.5 and 5.5, yeilded the same haemolysin titres as the original sample at pH 7.4. Thirdly, nutrient broth at pH 7.4, 6.5 and 5.5 was not toxic to horse RBCS. The increase in haemolysin productivity at pH 6.5 was also demonstrated by the method of haemolysin indicator (HI) plate. In addition, when considering the effect of pH on stability of haemolysin, although it was carried on the supernatant after centrifugation which slightly changed the natural environment in which haemolysin had existed, may further indicate that the higher titres observed at pH 6.5 over that obtained at pH 7.4 were mainly due to increase in toxin release per cell, since haemolysin was found to be less stable at pH 6.5 that at pH 7.4.

The same explanation advanced for the differentiation between high productivity and greater biological activity of haemolysin of pH 6.5 can help to explain the lower productivity of haemolysin at pH 5.5 as distinct form its reduced biological activity of this pH. The little amount of haemolysin produced at this pH (5.5) can be partly related with some strains

(4121, 5214 m), to the relatively poor growth at this pH but, in general, the effect is more drastic on haemolysin production than on growth. However, good growth is not always accompanied by good toxin production. Girard and Sbarra (1963) found that a complete synthetic medium, despite supporting good growth of L. monocytogenes, yielded lower haemolysin titres.

From this it seems that when L. monocytogenes grows in nutrient broth of different pHs, stimulatory or inhibitory effects corresponding to the pH value occur at the site responsible for cellular multiplication.

The data in the present investigation indicate that the addition of 0.5% glucose to nutrient broth stimulate both the growth of, and haemolysin production by, L.monocytogenes 4379 and 5214 m. The assay for the toxigenesis should be determined just prior to the end of the retardation phase to avoid significant loss which happens thereafter. "The magnitude of toxigenesis is low in the early hours of growth (logarithmic phase) while the strong influence can be seen during the retardation phase". The findings of Njoku-Obi et al (1963) that brain-heart infusion broth (Difco) containing 0.5% glucose gave a higher concentration of haemolysin than liver infusion, veal infusion or tryptose phosphate broth (Difco), indicate the toxigenic effect produced by the presence of glucose.

When comparing the growth and haemolysin production in the presence of glucose with pH controlled at 7.4, it appears the toxigenic effenct of glucose is mainly due to its influence on cellular multiplication rather than the influece on the productivity per cell. The toxigenesis of glucose is even more pronounced when the initial pH of 7.4 is not controlled. This , together with the fact that the growth of the organism in the presence of glucose under uncontrolled pH has not yielded more viable cells than that obtained with controlled pH, strongly suggests that the reduction of pH during the growth of the organism under uncontrolled pH has its role in the higher productivity of haemolysin. This suggestion is further enforced by the fact that better haemolysin production was obtained in pH 6.5, lower than the neutral pH (7.4) and in glucose-containing media the titre of haemolysin began to rise after the pH had started to shift towards acidity.

In the presence of glucose, regardless of the pH, whether controlled or uncontrolled, a loss of haemolytic activity in the early hours of the stationary phase was observed. It appears a factor other than instability of haemolysin at acidic, pH, plays an important role in the observed rapid loss. The rapid loss of Listerial haemolysin in media containing glucose has also been shown to occur by Njoku-Obi et al (1963) and Jafari(1980).

Physiological states which follow from an increase in the available glucose or decrease in the pH from its neutral state may have

an impact on the progess of Listeriosis, since under these conditions the productivity as well as biological activity of haemolysin may be greatly enhanced and according to Kingdom and Sword (1970 a) the lytic effect of haemolysin on lysozome and phagocytic cells is proportional to its added concentration. Andrews and Eveleth (1963) studies the effect of blood glucose concentration on the response of sheep to intravenous injection of L. monocytogenes and whether hyperglycaemia acted as a stress factor. They concluded that intracellular fluid from a hyperglycaemic animal might provide a more favourable environmental for growth of L. monocytogenes than from a normal or hypoglycaemic animal. These authors also quoted the work done by Nordland in 1960 who had supported the possibility of an effect of blood glucose on the severity of Listeric infection and on the susceptibility of sheep to Listeriosis.

SUMMARY

The production of Listerial haemolysin under the effect of pH and glucose was evaluated. Cells at pH 6.5, as compared with pH 7.4 and 5.5, were profoundly toxigenic. The toxigenesis of cells in the presence of glucose was more pronounced with uncontrolled than with controlled pH. The stimulation of haemolysin production at pH 6.5 is not related to the cellular multiplication. The increase in haemolysin production at pH 6.5 is also confirmed by the study on the haemolysin indicator plate. Glucose was stimulatory to the cellular multiplication.

REFERENCES

ANDREWS,
M.F. and EVELETH, D.F. (1963). Blood glucose in Listeriosis. In Second Symposium on Listeric Infection pp. 114-115, M.L. Gray (Ed.) Bozeman, Montana.

Communicable Disease Report C.D.R. (1979, 1980 1981). Public Health Laboratory Service, Weekly Edition. U.K.

GIRARD,
K.F. and SBARRA, A.J. (1963). Some characteristics of the soluble haemolysin of Listeria monocytogenes. In the Second Symposium on Listeric Infection, pp. 198-209, M.L. Gray (Ed.), Bozeman, Montana.

JAFARI,
N.A. (1980). Ph. D. Thesis, Nottingham University.

JENKINS,
E.M., ADAMS, E.W., and WATSON, B..B. (1966). Further investigation on the production and nature of the soluble haemolysin of Listeria monocytogenes. In proceedings of 3rd International Symposium on Listeriosis, pp. 109-123. Bithoven.

KINGDON,
G.C. and SWORD, C.P. (1970 a). Effect of Listeria monocytogenes haemolysin on phagocytic cells and lysosomes. Infection and Immunity. 1, 356-362.

KINGDON,
G.C. and SWORD, C.P. (1970 b). Biochemical and Immunological effects of Listeria monocytogenes haemolysin. Infection and Immunity. 1, 363-372.

LIU, P.V.
and BATES, J.L. (1961). An extracellular haemorrhagic toxin produced by Listeria monocytogenes. Canadian Journal of Microbioloty. 7, 106-108.

MOHAMED,
G.E. (1982). Ph. D. thiseis, Nottingham University.

MUENKER,
W. and ROOTS, E. (1963). Influence of incubation temperature and Co 2 tension on motility, haemolysin production, virulence and monocytosis producing ability of Listeria monocytogenes. In the Second Symposium on Listeric Infection pp. 193-197, M.L. Gray (Ed.) Bozeman, Montana.

MURRAY,
E.G.D., WEBB, R.A. and SWANN, M.B.R. (1926). A disease of rabbits characterised by large nomonuclear leucocytosis caused by a hitherto undescribed bacillus Bacferium monocytogenes. Journal of Patholoty and Bacteriology. 29, 407-439.

NJOKU-OBI, a.N., JENKINS, E.M., NJOKU OBI, J.C.,
ADAMS, J.
and COVINGTON, V. (1963). Production and nature of Listeria monocytogenes haemolysin Journal of Bacteriology. 86, 1-8.

NORLAND,
O.S. (1960). Host-parasite relations in intiation of infection. II-Hyperglycaemia and stress in experimental infection with L. monocytogenes. Canadian journal of Compartive Medicine. 24, 57-74.

PARK, Y.
and MIKOLAJCIK, E.M. (1977). Factors affecting quantitification of Clostridium perfringens alpha toxin by haemolysin indicator plate test and other procedures. Journal of Food Protection. 40, 626-631.

PROBLEMS
OF LISTERIOSIS (1975, 1977, 1981). The proceedings of 6th, 7th and 8th. Symposia on the problems of Listeriosis.

SEELIGER,
H.P.R. (1961). Listeriosis. Basel, New York.

SIDDIQUE,
I.H. (1969). Cytotoxic activity of haemolysin from listeria monocytogenes on L.M. strain of mouse cells. Canadian Journal of Microbiology. 15, 955-957.

SIDDIQUE,
I.H. and WALKER, C.A. (1967). Effects of Listeria monocytogenes haemolysin on the isolated ileum of rabbit. Ammerican Journal of Veterinary Research 28, 1843-1849

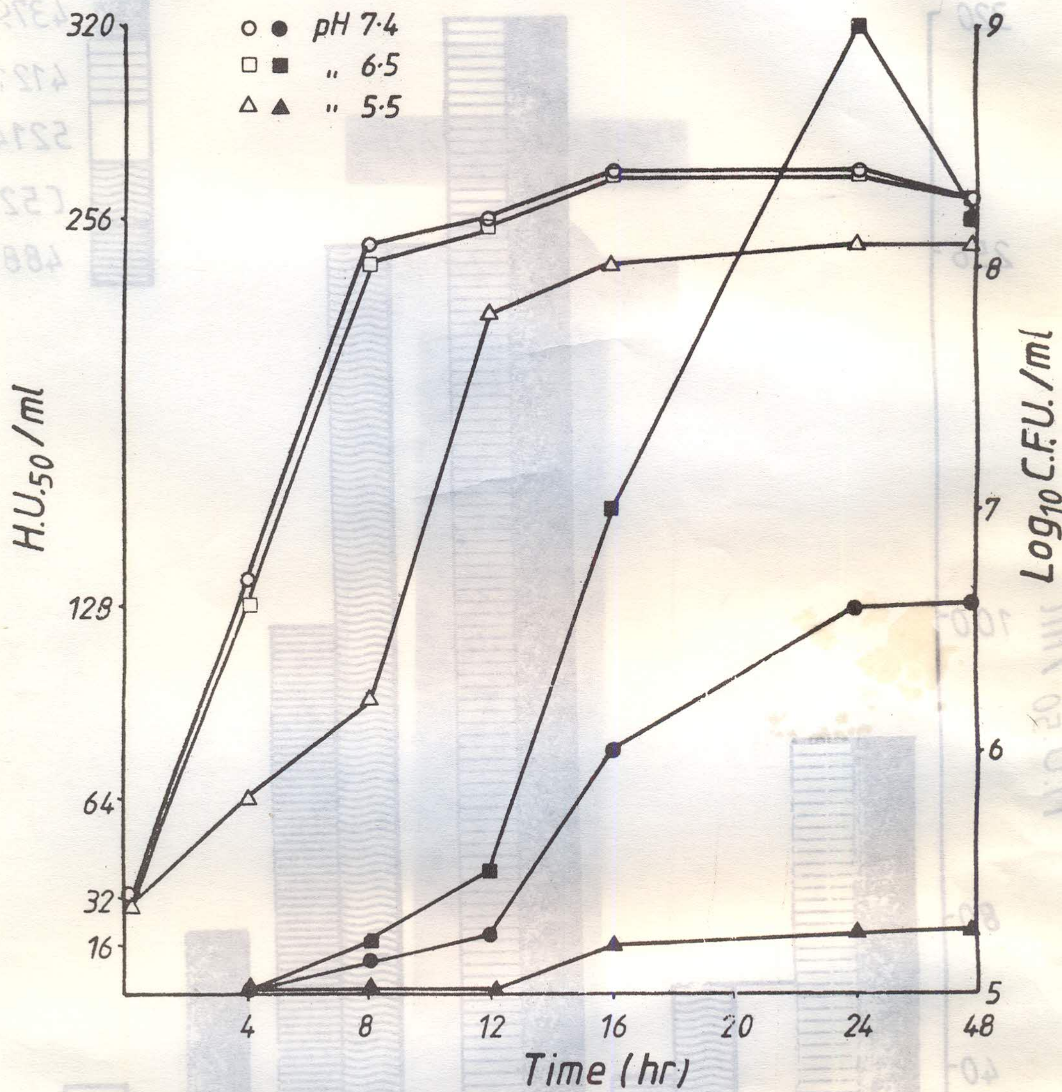


Fig. (1) Effect of pH on growth of, and haemolysin production by, L. monocytogenes 4379.

open symbols = growth
closed symbols = haemolysin

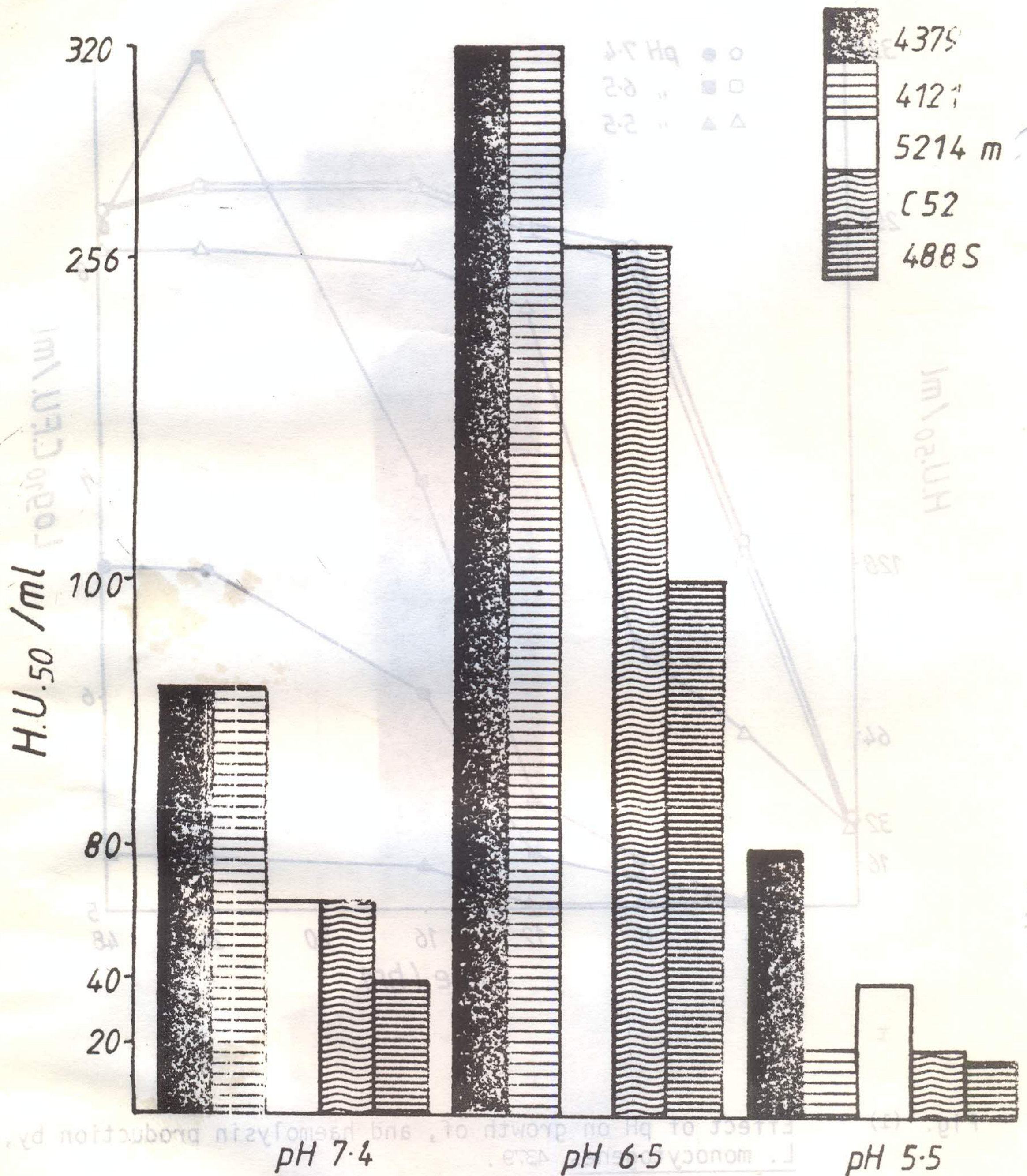


Fig. (2) The maximum haemolysin titres produced by different strains of L. monocytogenes at different pHs at 37C.

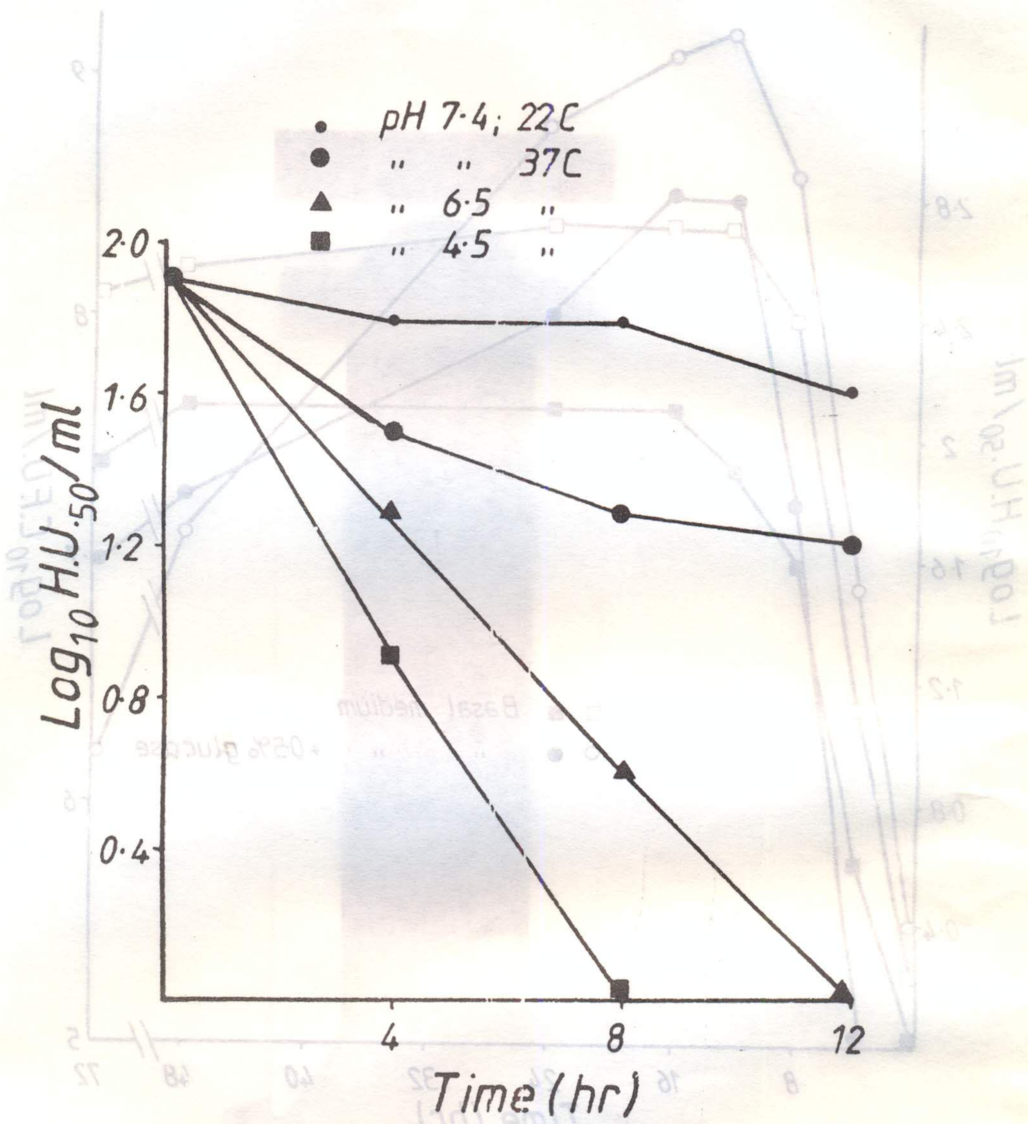


Fig. (3) Effect of temperature and pH on haemolysin stability.

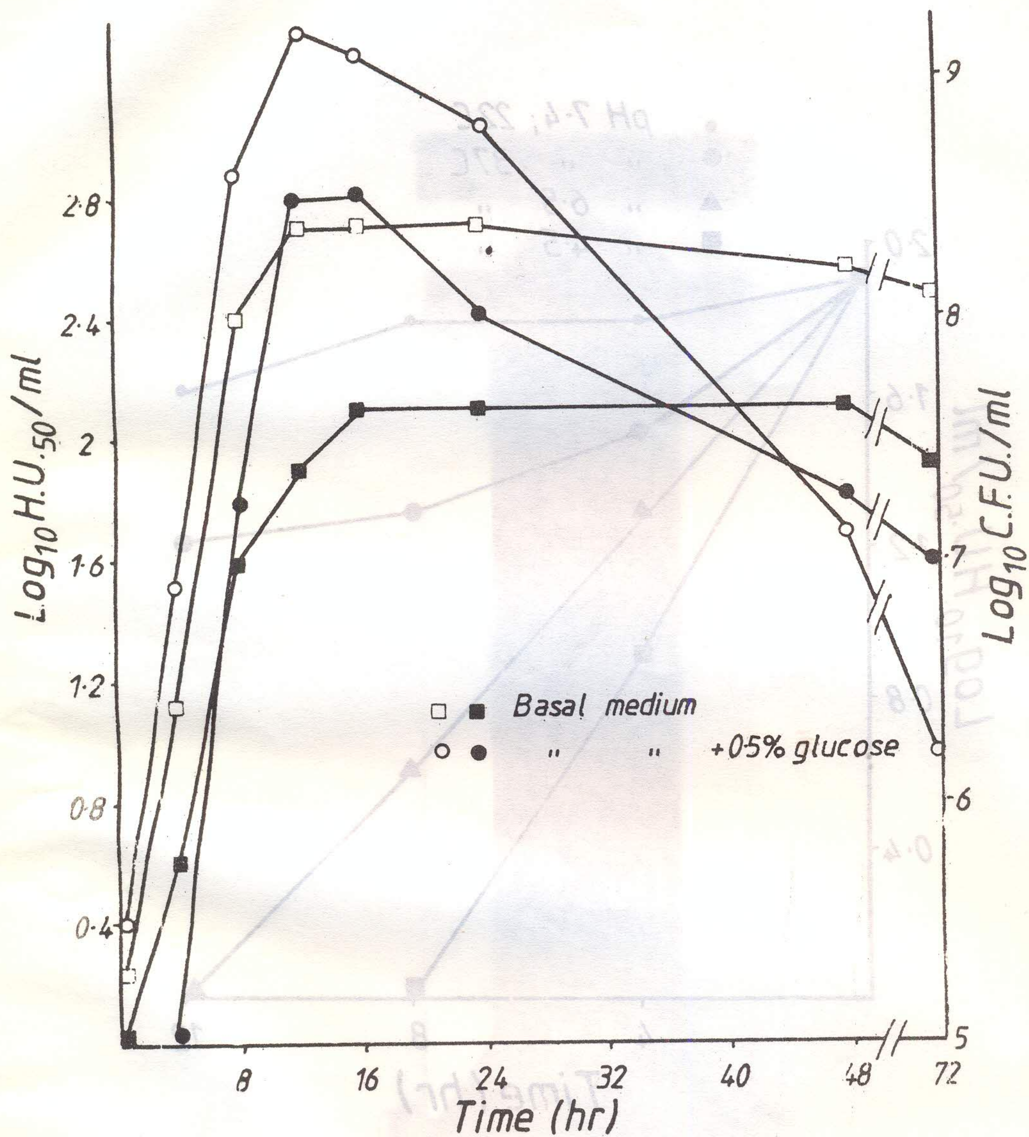


Fig. (4)

Effect of glucose on growth of, and haemolysin production by, L. monocytogenes 4379 with uncontrolled initial pH of 7.4 at 37C.

open symbols = growth
closed symbols = haemolysin

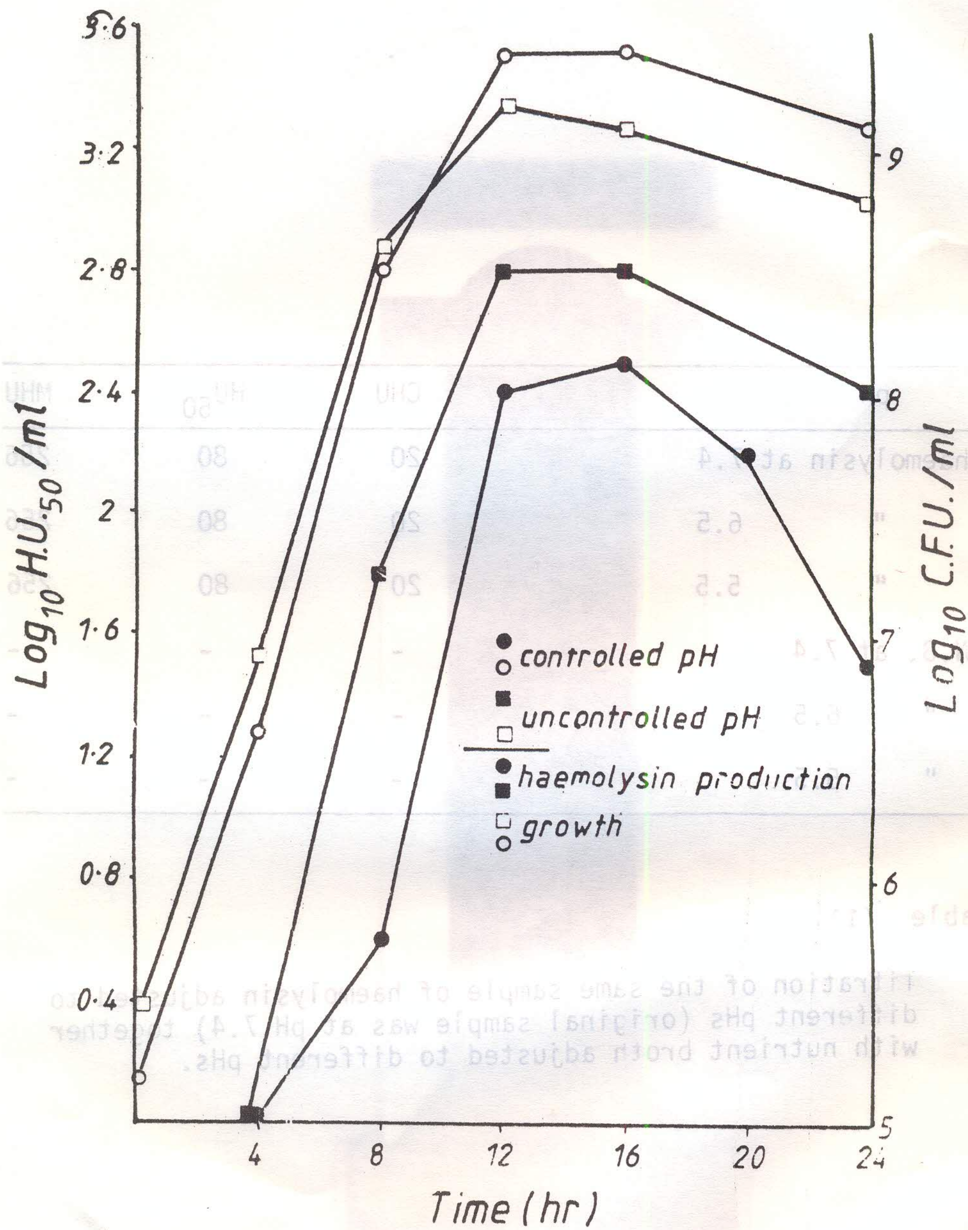
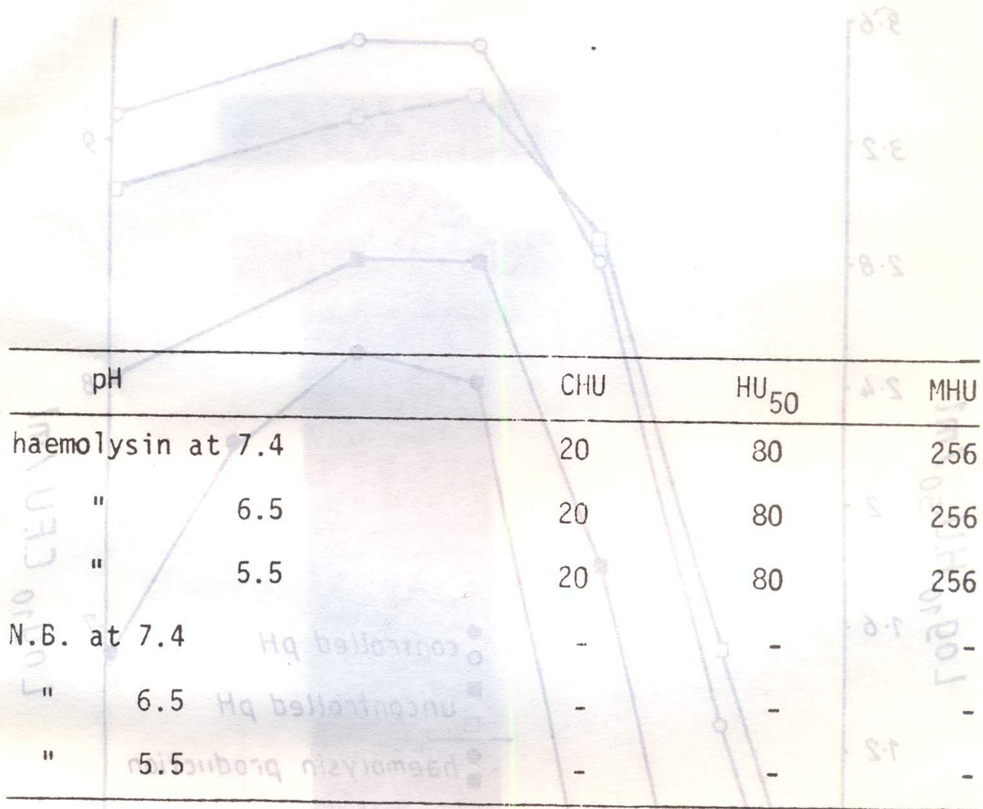


Fig. (5) Effect of glucose on the growth of, and haemolysin production by L. monocytogenes 4379 with controlled and uncontrolled pH of 7.4 and at 37C.



pH		CHU	HU_{50}	MHU
haemolysin at	7.4	20	80	256
"	6.5	20	80	256
"	5.5	20	80	256
N.B. at	7.4	-	-	-
"	6.5	-	-	-
"	5.5	-	-	-

Table (1)

Titration of the same sample of haemolysin adjusted to different pHs (original sample was at pH 7.4) together with nutrient broth adjusted to different pHs.