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Predictive modelling of growth of *Listeria monocytogenes* The effects on growth of NaCl, pH, storage temperature and $NaNO_2$

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Abstract

The effect of NaCl concentration (5.0–115.0 g/l), pH value (4.0–7.2), temperature (1–35°C) and $NaNO_2$ concentration (0–200 mg/l) on the growth responses of *Listeria monocytogenes* in laboratory medium was investigated. The growth curves generated within this matrix of conditions were fitted using the function of Baranyi and Roberts (1994) and the growth responses modelled using a quadratic polynomial to produce response surfaces. Growth curves could then be regenerated for any set of conditions within the experimental matrix and values predicted for the growth rate, doubling time, lag time and time to 1000-fold increase. The model was validated using data from published literature and was found to give realistic predictions for doubling times in foods, including meat and meat products, milk, dairy products and vegetables. Predictions from this model (Baranyi and Roberts, 1994) compared favourably with those from the models of Buchanan and Phillips (1990), Murphy et al. (1996) and the Food MicroModel. Copyright © 1997 Elsevier Science B.V.

Keywords: Food safety; Growth; *Listeria monocytogenes*; pH; Predictive model; Sodium chloride; Sodium nitrite; Temperature

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1. Introduction

Reports of food-borne transmission of *Listeria monocytogenes* as a cause of human listeriosis have increased in recent years. Between 1949 and 1980 several outbreaks were reported with milk suspected as the vehicle, but none of these has been proven. Since 1981, several outbreaks of foodborne listeriosis have been conclusively documented, implicating coleslaw (Schlech et al., 1983), pasteurized milk (Fleming et al., 1985), Mexican-style cheese (Linnan et al., 1988), jellied pork tongues (Goulet et al., 1993) and pork rillettes (Anonymous, 1993) as the sources of infection. Fortunately, large outbreaks are rare, the majority of cases occurring sporadically, although the outbreak in France involving pork tongues affected 279 people and there were 63 deaths. The primary means of transmission to humans is via foodstuffs contaminated during or after production and processing (WHO Working Group, 1988). Although never proven, the large increase in cases of foodborne listeriosis in the UK during 1988–1989 was attributed to imported paté (Morris and Ribeiro, 1989; McLauchlin et al., 1991). Government health warnings were issued in 1989 to vulnerable groups in the population, drawing attention to 'high risk' foods including soft cheeses, cook-chill foods and paté. This action, coupled with more stringent manufacturing requirements, resulted in a dramatic decrease in the number of cases after this time.

As a consequence of foodborne listeriosis, numerous surveys have determined the extent to which *L. monocytogenes* is present in a wide variety of foods, e.g. raw milk (Liewen and Plautz, 1988), soft and hard cheeses (Pini and Gilbert, 1988), traditional Moroccan dairy products (El Marrakchi et al., 1993), ice cream (Farber et al., 1989), raw liquid whole egg (Leasor and Foegeding, 1989), raw poultry (Bailey et al., 1989), raw red meats (Skovgaard and Morgen, 1988; Lowry and Tiong, 1988), cured meat (Farber et al., 1988, 1989), processed meats (Vorster et al., 1993), frankfurters (Wang and Muriana, 1994), shellfish (Lennon et al., 1984) and pre-packed salads (Sizmur and Walker, 1988).

Because of the widespread distribution of *L. monocytogenes* in food products, it is important to understand the capacity of the microorganism to survive and multiply under conditions associated with their processing, storage and distribution. Assessing the likelihood and extent of growth, in response to a number of key controlling factors acting in combination via mathematical models, is one of several approaches increasingly being used. Such models commonly use response surface regression analysis and have been used to predict the growth, as a function of factors such as pH, NaCl, storage temperature and preservatives, of salmonellae (Gibson et al., 1988; Bratchell et al., 1989), *L. monocytogenes* (Buchanan and Phillips, 1990; Wijtzes et al., 1993; Murphy et al., 1996), *Brochothrix thermosphacta* (McClure et al., 1993) *Bacillus cereus* (Baker and Griffiths, 1993), *Yersinia enterocolitica* (Adams et al., 1991; Hudson, 1993; Sutherland and Bayliss, 1994), *Staphylococcus aureus* (Sutherland et al., 1994), *Escherichia coli* O157:H7 (Sutherland et al., 1995) and *Aspergillus flavus* and related species (Gibson et al., 1994).

Sixteen strains of *L. monocytogenes* were screened for their resistance to low pH and high NaCl and the *L. monocytogenes* strain used in these experiments was the most resistant to both these factors. This paper describes the growth responses of this strain in laboratory medium adjusted to a range of pH, NaCl, and $NaNO_2$ levels, and stored at temperatures between 1 and 35°C. The growth responses are summarised by a quadratic polynomial model which may then be used to predict growth in any combination of conditions within the limits of pH, NaCl, $NaNO_2$ and temperature, where growth was observed experimentally.

2. Materials and methods

2.1. Organism

L. monocytogenes, National Collection of Type Cultures (NCTC) 9863, was used for the experiments and was maintained on nutrient agar (NA; Unipath CM 3) slopes at 4°C.

2.2. Media

NaCl was added to tryptone soya broth (TSB; Unipath CM 129) to give final concentrations of up to 115.0 g/l. Media at each NaCl concentration were adjusted to pH values between 4.5 and 7.5 using 2M HCl or 2M NaOH, dispensed in 330 ml volumes and autoclaved at 121°C for 15 min. Solutions of $NaNO_2$ were filter sterilized (0.22 μm Millipore filter) and added to TSB after autoclaving, but prior to dispensing, to give final concentrations of 50, 100, and 200 μg/ml.

Tryptone soya agar (TSA; Unipath CM 131) was used for recovery of inoculated organisms and maximal recovery diluent (MRD; Unipath CM 733) was used for all serial dilutions for plate counts and for dilution of the inoculum. All media and diluent were autoclaved at 121°C for 15 min.

2.3. Inoculum

L. monocytogenes was grown in TSB (pH 7.3; pH value unadjusted and with no added NaCl) for 24 h at 37°C (ca. 10^8 cells/ml), subcultured on 3 consecutive days, diluted and 1 ml inoculated into each 330 ml volume of TSB, to give a final concentration of approximately 10^3/ml.

2.4. Experimental procedure

TSB in 330 ml volumes was equilibrated overnight at the intended storage temperature. The following day, 20 ml were aseptically withdrawn and the pH

value recorded, but not adjusted. Immediately after inoculation with *L. monocytogenes* and thorough mixing, the TSB was aseptically dispensed in 10 ml volumes, using a peristaltic pump (Accuramatic-5, Jencons, Leighton Buzzard, UK), into sterile 1 oz (28 ml) universal bottles with plastic screw caps (Fisher, Loughborough, UK). Viable numbers in each treatment combination were determined by plating 20 μl volumes on duplicate plates of TSA immediately after dispensing, and the time at which this initial sample was taken was noted. This was the zero time for the purpose of plotting growth curves. The remaining bottles of inoculated broth were returned, within 5 min of completion of dispensing, to the incubator in which the broths had been equilibrated prior to inoculation and were incubated without shaking for the duration of the experiment.

At intervals during storage, decimal dilutions were made from separate bottles of TSB and 20 μl of each plated onto duplicate plates of TSA, recording the time on each occasion of sampling, which was converted to time elapsed since inoculation. After incubation of the plates for 24 h at 37°C, the mean number of colonies on the duplicate plates was determined and the number of colony-forming units per ml calculated and expressed as $\log_{10}$ cfu/ml.

2.5. Determination of pH, NaCl and $NaNO_2$

Measurements of pH were made using a Whatman PHA 230 pH meter. NaCl concentrations of TSB were determined using the official method of the Society for Analytical Chemistry (Hansen, 1973) or by checking the refractive index using a calibrated Atago PR-1 digital refractometer (ChemLab, Hornchurch, UK). Concentrations of $NaNO_2$ were determined by the modified method of Ito et al. (1979).

2.6. Experimental design

The extremes of the experimental plan were chosen after a preliminary screening experiment to determine the growth limits of the strain used.

Two hundred and sixty seven combinations of conditions within the following matrix, representative of most foods, were inoculated:

Storage temperature (°C)	1, 3, 5, 8, 10, 15, 20, 25, 30, 35
pH	4.5, 4.9, 5.3, 6.0, 6.3, 6.7, 7.4
NaCl (g/l)	5, 20, 40, 60, 70, 80, 90, 100, 115
NO_2 (mg/l)	0, 50, 100, 200

The combinations selected (with some duplicates) were evenly spaced between the experimental limits. The distribution of experimental data generated at different concentrations of NO_2 is shown in Fig. 1. A total of 236 growth curves, each comprising between 16 and 30 viable counts, were eventually used to produce the model. For this model, no-growth data were not considered further.

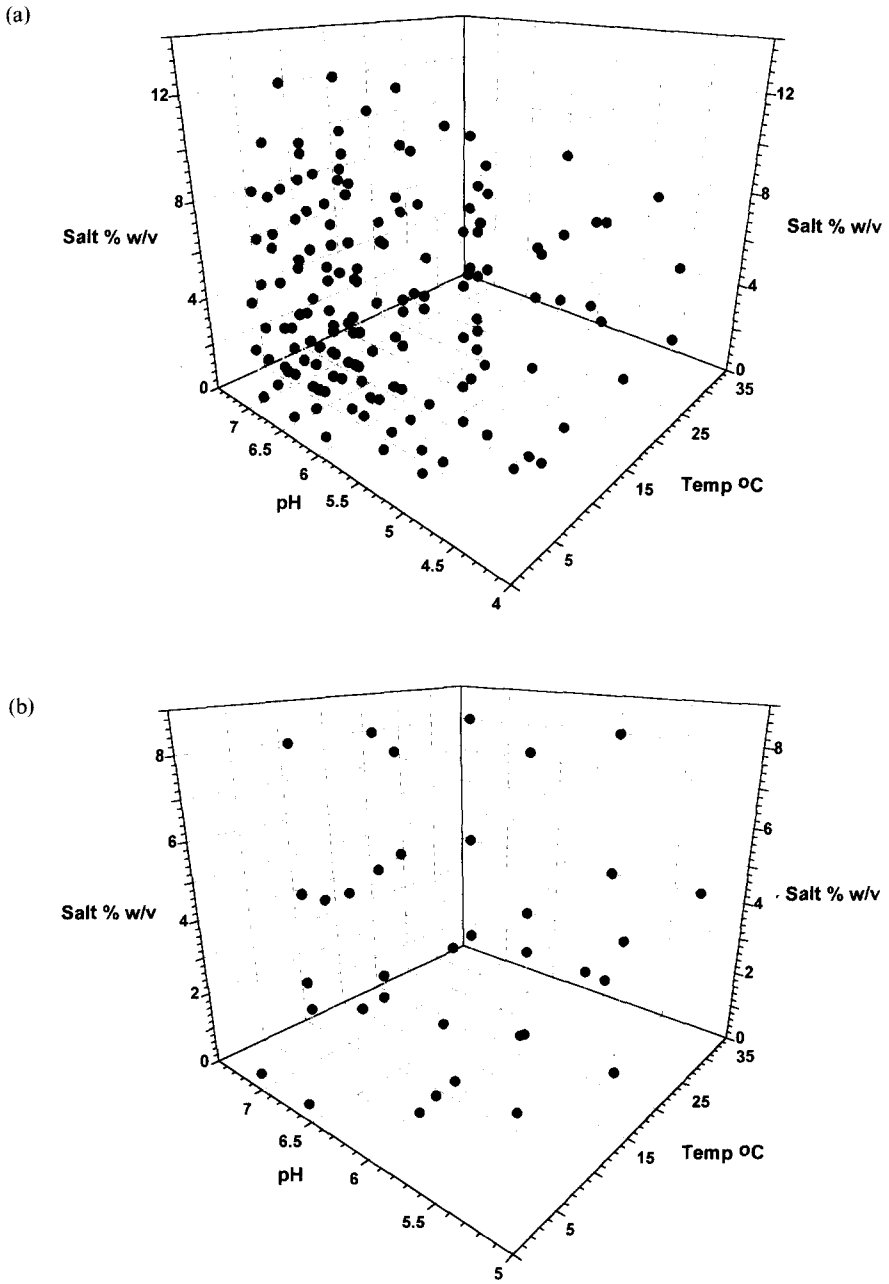

Fig. 1. Combinations of NaCl, pH and temperature used to produce data for modelling growth of *L. monocytogenes* at four concentrations of NO_2. (a) 0 mg/l NO_2; (b) 50 mg/l NO_2; (c) 100 mg/l NO_2 and (d) 200 mg/l NO_2.

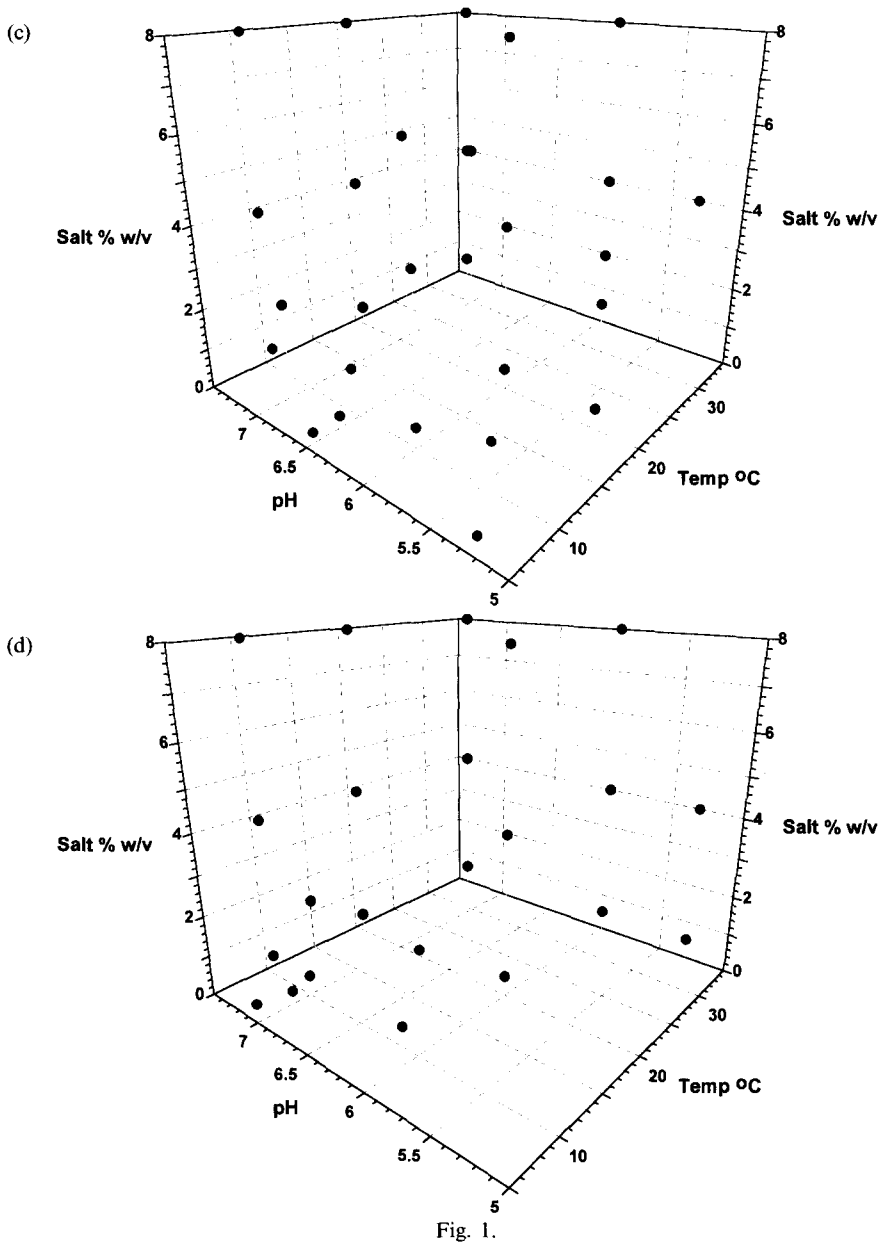

Fig. 1.

3. Statistical modelling

The first of the two stages of modelling involved fitting the bacterial growth curve to the data using Program 2 of McClure et al. (1993). This fits the curve of Baranyi

et al. (1993) using a customised version of Excel 3.0. and the growth rate (μ), lag (λ) and maximum population density (γ) were estimated directly for each growth curve. The second stage of modelling described the variation of the parameters of the growth curve as a function of the growth conditions, using a polynomial of the form:

$$\ln(y) = a + b_1 s + b_2 p + b_3 n + b_4 t + b_5 s^2 + b_6 sp + b_7 sn + b_8 st + b_9 p^2 + b_{10} pn + b_{11} pt + b_{12} n^2 + b_{13} nt + b_{14} t^2 + \varepsilon$$

where $\ln(y)$ is the response variable, i.e. the parameter to be modelled, transformed to the natural logarithm to stabilise the variance; s, NaCl (g/l); p, pH; n, NO_2 (mg/l); t, temperature (°C); ε, random error.

4. Results and discussion

There are two crucial steps in evaluating a predictive model. The first is to ensure that the model accurately describes the data from which it has been generated and represents any biological trends in those data, e.g. optimum growth temperature. The second step in the evaluation of a predictive model is to compare the predictions with other data, generated by other laboratories, in different media/food substrates. In Table 1, observed oubling times of *L. monocytogenes* in selected representative foods reported in the literature are compared with the model based on the function of Baranyi et al. (1993) and the following (Gompertz-based) models: (i) Food MicroModel (which contains data from the Institute of Food Research and other laboratories); (ii) the cubic model of Murphy et al. (1996); and (iii) the model of Buchanan and Phillips (1990), included in the MFS Pathogen Modeling Program (PMP) Version 4.0, 1994. In most cases, for models based on both the Gompertz and Baranyi et al. (1993) functions, predicted doubling times are shorter (i.e. growth rates are faster) than observed values in foods. This is not unexpected, since the liquid (medium or milk-based) experimental systems provide optimal growth conditions, apart from the experimental variations of temperature, pH and NaCl concentration and such models, prepared from data generated in this way, tend to give fail-safe predictions. Many published values are very close to the predicted values (Table 1), supporting the argument that predictive models should be constructed using experimental data in media, which will give the fastest possible growth. In some instances taken from the literature the foods or the packaging systems include factors not encompassed by the model, e.g. CO_2, which are likely to contribute to limitation of growth.

The closeness of the predictions to the observed values of doubling time can be demonstrated using the statistical comparison (McClure et al., 1993):

$$\sum (\ln dt_{publ} - \ln dt_{pred})^2$$

Table 1
Comparison of observed and predicted doubling times using different models for *L. monocytogenes* in food

	Temperature (°C)	pH	NaCl (g/l)	$NaNO^2$ (mg/l)	Published DT (h)	DT predicted-1 (h)	DT predicted-2 (h)	DT predicted-3 (h)	DT predicted-4 (h)	Assumptions[c]	Source[d]	Reference
Vacuum-packed lean beef	5.3	5.60	0.5	0	30	26	19.63	23.76	8.2[a]	NaCl	C	Grau and Vanderlinde (1990)
Minced meat (cooked)	8	5.80	0.5	0	10.84	12.87	9.38	9.95	6.60	NaCl	C	Schillinger et al. (1991)
Cured raw pork	15	6.30	2	40	3.6	4.22	2.88	NP	1.90	None	C	Schillinger et al. (1991)
Minced beef	5	6.27	0.5	0	18.1	17.18	14.83	18.13	10.90	NaCl	C	Shelef and Yang (1991)
Vacuum packed ham	10	6.63	2.77	170	16.4	12.9	11.23	NP	2.6[a]	None	C	Grau and Vanderlinde (1992)
Frankfurters	15	5.80	3.2	156	9	12.56	9.84	NP	5.10	None	C	Berry et al. (1991)
Skimmed milk	8	6.40	0.5	0	12.5	8.93	7.24	8.60	6.00	pH, NaCl	C	Rosenow and Marth (1987)
Cream	13	6.40	0.5	0	6	3.82	2.73	3.30	2.40	pH, NaCl	C	Rosenow and Marth (1987)
UHT milk	5	6.60	0.5	0	18.5[b]	14.51	13.7	17.64	11.20	NaCl	A	Walker et al. (1990)
Non-fat milk	7	6.40	0.5	0	12	10.78	9.02	10.82	7.30	pH, NaCl	C	Petran and Zottola (1988)
Camembert cheese	6	6.10	2.4	0	18	17.2	12.29	13.52	7.60	None	C	Ryser and Marth (1987)
Egg yolk	20	6.20	0.5	0	2.4	1.69	1.06	1.36	0.90	NaCl	A	Sionkowski and Shelef (1990)
Liquid whole egg	4	7.00	0.5	0	24	15.21	16.82	22.71	14.70	NaCl	A	Foegeding and Leasor (1990)
Chicken legs	6	6.52	0.5	0	19.3	12.33	11.04	13.74	9.00	NaCl	B	Zeitoun and Debevere (1991)

Inoculum is 10^2 -10^4/g or per ml, unless otherwise stated.
DT predicted-1, predicted doubling time using function of Baranyi et al. (1993).
DT predicted-2, predicted doubling time using Food MicroModel Version 1.
DT predicted-3, predicted doubling time using model of Murphy et al. (1996).
DT predicted-4, predicted doubling time using MFS model of Buchanan and Phillips (1990).
A, authors' own calculation.
B, calculated from authors' tabulated values.
C, calculated from authors' graphs.
NP, no prediction possible as nitrite not modelled.
[a] Predictions made from anaerobic model.
[b] Mean value of the range 13–24 h.
[c] Assumptions made about conditions of growth in published papers.
[d] Source of information about published generation times.

if the total of the predicted values were exactly equal to the total of the observed values, the sum would be zero. Table 2 shows the comparison of predictions from the four different models to the relatively limited, but representative, range of observations in Table 1.

The model of Baranyi et al. (1993) gave predictions very close to the observed values (Table 2) but three predicted times were greater than the observed times (Table 1). However, even where predictions of doubling time were greater than those observed, they were within an acceptable range. The model of Murphy et al. (1996) provided satisfactory predictions, with no examples of observed doubling time being greater than predicted values. The authors make it clear, however, that the model was devised for dairy products and as such, is not suitable for products containing NO_2. Food MicroModel predicts satisfactorily for products containing nitrite and gave the most conservative (safest) predictions overall. The model of Buchanan and Phillips (1990), formerly available on a free computer disc, also gave very conservative predictions, with no predicted value greater than the equivalent observed value.

There are differences between our approach and that of others. Buchanan and Phillips (1990) used a factorial design supplemented with a central composite design which resulted in data generated in a relatively restricted range of conditions. In our study, the experimental matrix was consciously modified to obtain data under conditions ranging from optimal to those close to the limits of growth. As a consequence, the growth/no growth boundary is better defined using our approach and the models from this study are able to predict growth rates and lag times of *L. monocytogenes* more reliably over a wider range of conditions than that of Buchanan and Phillips (1990).

Another difference between our model and those of Buchanan and Phillips (1990) and Murphy et al. (1996) is that they used a third order polynomial to model their derived parameters, an approach also used by Hudson (1993) for modelling the growth of *Y. enterocolitica*. However, 'more is not necessarily better' in the context of application of orders of magnitude of functions to microbial growth; even if a cubic polynomial response surface provides a better fit to the parameters, the higher the order of the polynomial, the more it tends to follow the random errors of the measured values, rather than eliminating them, which should be the aim (Baranyi

Table 2
Comparison of models

Models	$\sum \ln(dt_{published} - dt_{predicted})^2$
McClure et al., 1993	4.90
Food MicroModel	28.23
Murphy et al. (1996)	7.23
Buchanan and Phillips (1990) MFS model	23.22

and Roberts, 1995). We consider that the quadratic function is, at the present stage of model development, the most satisfactory way of reflecting the observed microbiological response, since the rate of change of growth rate in terms of the environmental factors is monotone, i.e. growth rate increases to a maximum under optimum conditions and declines as conditions increase beyond the optimum (Baranyi and Roberts, 1995).

The four-factor predictive models of Baranyi et al. (1993) and Food Micro-Model, discussed in this paper, will enable food microbiologists to make rapid and realistic predictions of the growth of *L. monocytogenes* in conditions appropriate to a range of foods, limiting the need for extensive challenge testing and enabling any challenge tests deemed necessary to be better targeted.

Some of the predictions in this paper are derived from the 4-factor growth model for *L. monocytogenes*, available on a disc for personal computers (Food Micro-Model Version 1), obtainable through Food MicroModel, Randalls Road, Leatherhead, Surrey KT22 7RY, UK.

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