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The composition of tea infusions examined in relation to the association between mortality and water hardness

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SUMMARY

Recent epidemiological studies have shown that death-rates from certain chronic diseases are higher in areas with soft than in areas with hard drinking-water. In the striking negative correlation found in the county boroughs of England and Wales between cardiovascular mortality and water hardness the important underlying factor is apparently the water calcium. Interest is therefore focused on the dietary significance of calcium present in drinking-water. In relation to that interest, the present report gives a quantitative account of the composition of tea infusions prepared with waters containing different amounts of calcium. It is shown that a substantial part of water calcium is taken up by the tea leaf during the preparation of infusions. The analysis of the infusions covers a wide range of individual components, including trace metals and polyphenolic substances. It appears that the principal change caused in infusion composition by the presence of calcium in the water is a substantial reduction in the relatively high oxalate content. The question is raised whether there may be some connexion between the 'water factor' in cardiovascular disease and the absorption of oxalates from foods.

INTRODUCTION

Attention has been directed in reports from several countries to striking correlations which show that in areas supplied with soft drinking-water the death-rates from cardiovascular disease are generally higher than those in areas supplied with hard water (Kobayashi, 1957; Schroeder, 1960; Morris, Crawford & Heady, 1961, 1962; Biörck, Boström & Widström, 1965). Evidence has also been reported (Turner, 1962) of similar negative correlations between water hardness and mortality from both gastric cancer and primary bone tumours in England and Wales, although with fuller data the association in the case of gastric cancer was later found to have only low statistical significance (Anderson, 1964). More recently Morris and his colleagues (Crawford, Gardner & Morris, 1968) in a thorough epidemiological study covering the period 1958–64 have confirmed that for the county boroughs of England and Wales the association between water hardness and cardiovascular mortality is particularly marked and apparently unrelated to

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social or other environmental factors. These workers conclude that there is an urgent need for further detailed investigation of this problem.

In attempting to relate these associations between mortality and water hardness to chemical entities present in drinking-water, several authors have pointed to trace metals in drinking-water as possibly relevant. It appears, however, that for the associations found in England and Wales the important underlying factor is the water calcium. In particular, it has been shown that cardiovascular mortality is even more highly associated with water calcium (the lower the calcium content the higher the mortality) than with water hardness, and there is no evidence of significant interrelation with any other water component studied (Hollins, 1965; Crawford *et al.* 1968). The important question raised is how might the calcium present in drinking-water perform a protective role.

In hard-water areas of England and Wales the calcium contribution from drinking-water still forms only about a tenth of the average dietary calcium intake (Hollingsworth, 1956). Calcium deficiency is not ordinarily encountered in man (Bronner, 1964) so that it seems unlikely that the small contribution of calcium from drinking-water functions as an essential supplement of dietary calcium for any substantial part of the population in these countries. Attention is therefore turned to the possible ways in which calcium from drinking-water may either modify the composition or influence the absorption of certain dietary components. In this connexion, since a major part of the culinary use of water is associated with the cooking of vegetable materials and particularly with the preparation of beverages such as tea and coffee, information on the interactions of water calcium and the components of these materials is of prime interest. Further interest is directed to the composition of tea infusions by the demonstration, in studies with laboratory animals, that several vegetable tannin extracts possess carcinogenic properties (Kirby, 1960; Korpassy, 1961) and also that tea extracts possess co-carcinogenic properties (Kaiser, 1967).

As a contribution to this field, we have examined quantitatively the interactions which occur between calcium in water and the components of the tea leaf during the preparation of infusions, with attention given both to the movements of calcium between the extracting water and the leaf, and to the influence of water calcium on the composition of the infusions. In the present communication we summarize the results of these studies.

MATERIALS AND METHODS

Preparation of tea infusions

The six samples of black tea used in the present studies were obtained in 1 kg. quantities from a tea broker, and comprised two blends of teas from Ceylon (A, B), two blends of teas from India (C, D), an Assam tea (E), and a China tea (F). An oolong tea from Formosa (G) and a green ('gunpowder') tea from China (H) were also included in some of the work. The samples, which had moisture contents ranging from 3·7 to 5·2%, were stored in air-tight glass jars.

The relative amounts of leaf and water used in ordinary domestic practice by 10 individuals for preparing tea infusions were measured and found to vary between

8 and 21 g. dry leaf/l. water. The mean value of 15 g. leaf per litre of water was selected for use throughout the present studies.* A standard infusion period of 6 min. was used since, with the exception of the pectinic acids which dissolved slowly, the extraction of all the components studied was essentially complete within this period. The infusions were prepared in tared glass beakers by mixing the leaf with boiling water and maintaining the mixture at 95° C for 6 min. Small additions of distilled water were made as required to offset evaporation losses. Control tests showed that neither the amount of calcium taken up by the leaf, nor the extraction of the principal components from the leaf, was measurably affected by gentle stirring, and, for convenience in sampling for the study of uptake and extraction rates, the infusions were generally stirred gently during the extraction period. For the oxalate studies, the procedure used conformed to ordinary domestic practice with brief stirring only at the start and finish of extraction. After extraction, the infusions were separated from the leaves by pouring through either a nylon tea strainer or a pad of glass wool.

Analytical procedures

A.R. grade reagents were used throughout.

Inorganic components

Extensive use was made of emission flame spectrophotometry for element determination, employing a specially constructed flame spectrophotometer, based on a Zeiss total consumption burner, a 'Uvispek' monochromator, and an E.M.I. 13-stage photomultiplier (Hollins, 1965). The methods used were capable of detecting more than forty metals at a level of 0·5 mg. metal/15 g. tea leaf, these metals including the majority of the Group I and Group II metals, the rare earths, and the transition elements of period IV of the periodic table. The calibration curves of the elements analysed were linear over the range of concentrations used and the standard addition technique (Chow & Thompson, 1955) was used to standardize measurements on all solutions. Measurements were made either directly on suitable dilutions of the infusions or on samples of infusion or leaf which had been wet-oxidized with HNO_3 at 'low-temperature' (Middleton & Stuckey, 1954).

The determination of the uptake of calcium by the leaf was based on measurements both of the reduction of Ca concentration in solution and of the increase in Ca content of the leaves. Close agreement was found between Ca loss from solution and Ca gain by leaves, and there was no evidence of measurable loss of Ca by retention on the surfaces of any apparatus used. These results were confirmed by radioactive assay using Ca-47 as a tracer. In preparing infusions with natural waters, the amounts of Ca lost by precipitation as carbonate were negligible when the waters were heated rapidly to boiling point and used immediately.

* The average consumption of tea in the United Kingdom between 1956 and 1965 was 11·3 g. per person per day (National Food Survey Committee, 1967). Since the present studies provide information on the amounts of material extracted from 15 g. leaf, a useful guide to the average daily intake of the different tea components may be obtained by multiplying the amounts reported here by a factor of 0·75.

The pure metal and alloy plates used in testing the corrosive power of infusions were obtained from various metalware manufacturers. Before use, the plates were rubbed down first with 240 grade and then with 400 grade emery paper, and washed thoroughly in a solution of detergent. The area of metal surface immersed during the preparation of 1 litre of infusion was approximately 500 cm^2.

Aluminium determinations were made colorimetrically using the aurintricarboxylate method (Sandell, 1959). Phosphate was determined by the molybdenum-blue colorimetric method, and total nitrogen by the Kjeldahl method, following, in both cases, the procedures described by Pearson (1962).

Organic components

Polyphenol fractions were obtained by ethyl acetate extraction and lead salt precipitation (Roberts, Cartwright & Oldschool, 1957; Vuataz & Brandenburger, 1961), and were analysed by two-dimensional chromatography (Roberts *et al.* 1957). 'Tannin' was determined by the Löwenthal method (Jacobs, 1958), caffeine spectrophotometrically (Polzella, 1961), and phytic acid by the method of McCance & Widdowson (1935). The pectinic acids were converted to pectic acid which was determined gravimetrically as the Ca salt (Ca content approx. 7·5%), following closely the procedure recommended by Kertesz (1951). The method used for oxalate followed the procedure described by Baker (1952) except that calcium salt precipitation from the infusion, which led to persistent contamination of the oxalate, was replaced by ether extraction. Thus, after protein removal, the infusions were acidified to 1 N strength with HCl and continuously extracted with ether for 40 hr. in subdued lighting. The extraction efficiency, which was monitored by radioactive assay of added C-14 labelled oxalic acid, was between 85 and 90%.

RESULTS

The uptake of calcium by tea leaf

Measurements of the calcium content of all eight tea samples gave a mean value of 55 (range 38 – 75) mg. Ca/15 g. dry leaf. Distilled water infusions of these teas contained between 4·2 and 5·8 mg. Ca/l. showing an extraction of only 6·5 – 12·0% of the leaf calcium. With infusions prepared with calcium chloride solutions containing 100 mg. Ca/l. it was found that in each case there was a net uptake of calcium by the leaf amounting to between 16 and 39 (mean 30) mg. Ca/15 g. dry leaf, the uptake value being characteristic of the tea sample. Evidence that these net uptake figures were not substantially smaller than the total calcium uptake values was obtained by activity measurements of Ca-47, added to the calcium solutions prior to preparing the infusions. Thus, it was found that the fractional loss of Ca-47 activity from the solutions was closely similar in magnitude to the fractional loss of stable calcium. More detailed studies of the pattern of calcium uptake were made with four tea types (A, C, E, H) using solutions with calcium contents ranging from 0 to 150 mg. Ca/l. In each it was found that the uptake curve rose steeply over the lower half of the range and flattened out when the solution calcium was at about 100 mg. Ca/l. A typical set of results is shown in Fig. 1.

Tests with solutions of calcium at 100 mg. Ca/l. as sulphate and nitrate showed calcium uptake values which were not significantly different from the corresponding values obtained with calcium chloride solutions. Calcium uptake measurements were also made using samples of three natural drinking-waters in which the bicarbonate content was roughly equal in equivalents to the calcium content (Ion content of waters, mg/l.: (1) Ca, 15; Mg, 3·3; Na, 34; HCO_3, 41: (2) Ca, 66; Mg, 3·4; Na, 20; HCO_3, 222: (3) Ca, 83; Mg, 54; Na, 60; HCO_3, 240). For each water it was found that the uptake was in good agreement with the value expected on the basis of the results for the calcium chloride solutions. The pH values of the infusions

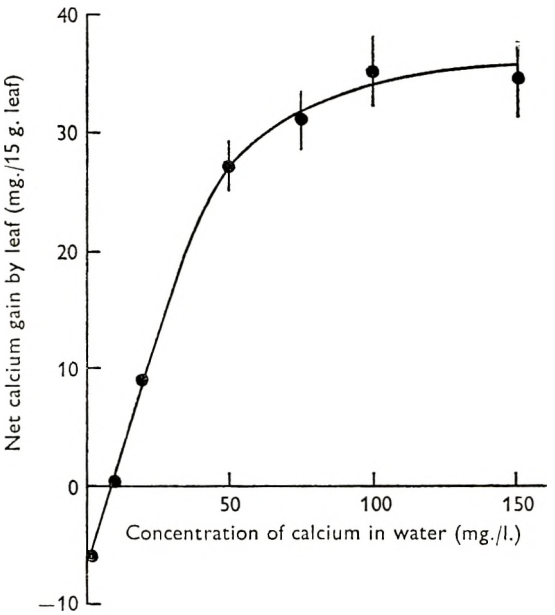

Fig. 1. The relationship between the net amount of calcium taken up by tea leaf during the preparation of infusions and the initial concentration of calcium in the extracting water (tea sample A). The major part of the analytical error in calcium determination was due to the random error of the flame photometric method used; errors shown (2σ) are the total photometric errors introduced into the calculated calcium uptake values.

prepared with the neutral salt solutions were essentially determined by the buffering action of the components extracted from the leaf, and fell within the range pH 4·4–4·9. The pH values of the infusions with natural water samples 2 and 3 were close to 5·3. Further tests were carried out using acetate and collidine buffers (at 0·05 M) to give infusions in which the pH reached 6·5 but no significant effect of pH on the calcium uptake was observed. Thus, in general it appeared that the pattern of calcium uptake (Fig. 1) was not significantly affected by the presence of the other principal ions commonly present in drinking-waters (Taylor, 1958), or by the differences in pH normally encountered in tea infusions.

Studies of infusions prepared with sodium chloride and magnesium chloride solutions showed that there was no analogous uptake of sodium or magnesium by

the leaf. In fact the presence of Mg ions in the starting solution caused an additional release of the magnesium present in the leaf. Thus, solutions of magnesium chloride at 50 mg. Mg/l. extracted about 80 % of the leaf magnesium compared with about 60 % extracted in distilled water infusions.

Further experiments with calcium chloride solutions showed that the calcium uptake process was fairly rapid and essentially complete within 2 min. Tests were also made on leaves recovered from infusions after a 6 min. period. Thus, in experiments in which leaves, separated from infusions with calcium chloride solutions, were subjected to several additional extractions with distilled water, it was found that only a small percentage of the calcium taken up by the leaf was extracted in each successive infusion with distilled water. It was also found that leaves which had been separated from infusions with distilled water showed no appreciable uptake of calcium in subsequent infusions with calcium chloride solutions. Thus, it appeared that the leaf component responsible for the uptake and binding of calcium was either altered or removed during the preparation of a distilled water infusion. Comparative studies were therefore made of the extraction of various components of the tea leaf by waters of different calcium content.

The composition of tea infusions

Preliminary measurements of the total solids present in tea infusions showed that about one-third of the leaf material had been extracted (Table 1), and that there was no large difference between the amounts extracted in distilled water and

Table 1. *General composition of tea infusions*

Component	Average concentration in infusions of 15 g. leaf per litre of distilled water (g./l.) (Figures in brackets represent number of samples studied)	
	Present studies	Previous studies
Total solids	5·10 (8)	—
Inorganic cations	0·23 (8)	0·28 (5)[a]
Polyphenols	2·30 (2)	2·50 (1)[b]
Caffeine	0·53 (3)	0·51 (4)[c]
Protein and amino acids[d]	0·85 (3)	1·00 (5)[a]
Miscellaneous component consisting mainly of sugars (by difference)	1·15 (2)	—

a, McCance & Widdowson, 1956; *b*, Roberts *et al.* 1957; *c*, Smith & Rees, 1963; *d*, Kjeldahl N (corrected for caffeine N) × 6·25.

calcium-loaded infusions. A more detailed set of measurements (Samples A, C) indicated that the presence of calcium in the starting solution (100 mg. Ca/l.) reduced the amount of material extracted by between 2 and 3 %. The results of studies of individual components are now summarized.

Metal ions

Measurements were made of the metal ion content of all eight tea samples, and also of the amounts of these ions extracted in distilled water infusions. The results of these measurements are summarized in Table 2. Attention is drawn to the relatively high levels of aluminium, manganese and zinc found in the samples. Analyses of infusions prepared with solutions of calcium at 100 mg. Ca/l. showed that, except for an increase by about 25 % in the amount of magnesium extracted, the extraction of the metals listed in Table 2 was unaffected by the presence of calcium in the starting solution. As noted above, the extraction of leaf magnesium was also increased to a similar extent by the presence of Mg ions in the starting

Table 2. *Metal ions detected in tea leaf and in distilled water infusions (average of eight tea samples)*

	Amount in dry leaf (mg./15 g.)			Amount in infusion (mg./l.)
Element	Min.	Max.	Mean	Mean
Na	5	10	8·0	7·2
Mg	36	51	44	25
Al[a]	3	14	9·8	2·8
K	135	380	225	182
Ca	38	75	55	5·1
Mn	5	23	18	5·4
Fe	2	5	3·5	< 0·25
Zn	0·5	15	4·0	2·6
Sr	0·18	0·65	0·37	0·06

a, Determinations of Al were made on only 5 tea samples.

solution. A more detailed study, using various ionic concentrations of calcium or magnesium in the starting solutions, showed that the additional release of leaf magnesium caused by Ca ions was closely similar in magnitude to that caused by the same ionic concentrations of Mg ions. It therefore appeared likely that the influence of Ca ions on magnesium extraction was due to an ionic concentration effect on the solubility of the magnesium component present in the leaf, although the possibility that some calcium uptake occurs by ion exchange with leaf magnesium was not fully excluded.

To obtain information on the degree to which the trace metal content of tea infusions may be increased by contamination from metalware currently or formerly used in their preparation, a series of measurements was made of the metal content of distilled water infusions prepared in the presence of various pure metal or alloy plates. No evidence was found of metal contamination at levels above 1 mg. metal/l. infusion from samples of aluminium, stainless steel, modern pewter, silver, or chromium-plated nickel-plated copper. Metal pick-up by infusions was observed from samples of copper (8 mg. Cu/l. infusion), nickel (20 mg. Ni/l.), nickel-silver ([1 mg. Zn + 2 mg. Ni + 7 mg. Cu]/l.), and brass ([3 mg. Zn + 3 mg. Cu]/l.). Infusions were also prepared with calcium-loaded solutions in the presence of nickel and

copper plates but no evidence was found of any marked influence of the calcium on the corrosive power of the infusions. Furthermore, the uptake of calcium by the leaves was unaffected by the presence of the metal plates.

Tests on distilled water infusions prepared in an old, lead-containing, pewter teapot (6 % Pb) showed the persistent occurrence of a comparatively high pick-up of Pb (2–4 mg. Pb/l. infusion) with little tendency for a protective coating to form inside the pot. Infusions prepared with calcium-loaded solutions tended to leave a film on the metal surface and in these infusions the lead content was reduced to about 1 mg. Pb/l.

Polyphenols

A preliminary investigation of the 'tannin' component (reducing polyphenols) of infusions by the Löwenthal method indicated that the presence of calcium in the extracting liquid had no influence on the extraction of this component. The polyphenolic fractions present in distilled water and calcium-loaded infusions of two tea samples (A, C) were separated and analysed by two-dimensional paper chromatography. Detailed examination of the bands and spots on the chromatograms, which displayed the major thearubigin and theaflavin components together with a wide range of flavonoid and other polyphenols, gave no evidence that calcium in the extracting liquid had a marked effect on the extraction of any of these compounds. The total weights of polyphenolic substances isolated from the infusions were 2·10 g. (Sample A) and 2·50 g. (Sample C).

Total nitrogen and caffeine

Measurements of the total N content of the solids recovered from infusions prepared both with distilled water and calcium chloride solutions (100 mg. Ca/l.) indicated that the presence of calcium had no appreciable influence on the extraction of N-containing components (mainly protein, amino acids and caffeine). This indication was confirmed directly in respect of the caffeine content of infusions of three teas (caffeine content of infusions of Samples A, C, E: 0·54, 0·58 and 0·44 g./l.).

Pectinic acids

In preliminary tests on the solubility of the pectic material present in tea, hot extracts of two teas (A, C) were made by stirring samples in distilled water on the boiling water bath for an 8-hr. period. The weights of pectic acid recovered from these extracts amounted to 1·6 and 2·6 % of the dry leaf weight. When similar extracts were made at room temperature there was a tenfold reduction in the amounts of pectic material extracted.

The determination of the pectic material present in normal 6 min. infusions with distilled water gave values for three teas (A, C, E) of 33, 34 and 18 mg. pectic acid/l. When the infusions were prepared with solutions containing calcium at 100 mg. Ca/l., the pectic content was substantially reduced to values of between 5 and 10 mg. pectic acid/l. Control tests showed that the presence of calcium in the infusion did not interfere with these determinations. It appeared therefore that

the precipitation of low-ester calcium pectinates within the leaf would account for some of the calcium uptake from solution, but the quantity of calcium involved in this process would amount to only 1 – 2 mg. Ca/15 g. dry leaf.

Oxalic acid

Determinations of the oxalate content of distilled water infusions of six teas (A, B, C, D, E, F) gave values ranging from 62 to 98 (mean 82·5) mg. oxalate/l. The corresponding values for infusions with calcium chloride solutions (100 mg. Ca/l.) ranged from 39 to 48 (mean 43·5) mg. oxalate/l. Analysis of the results showed

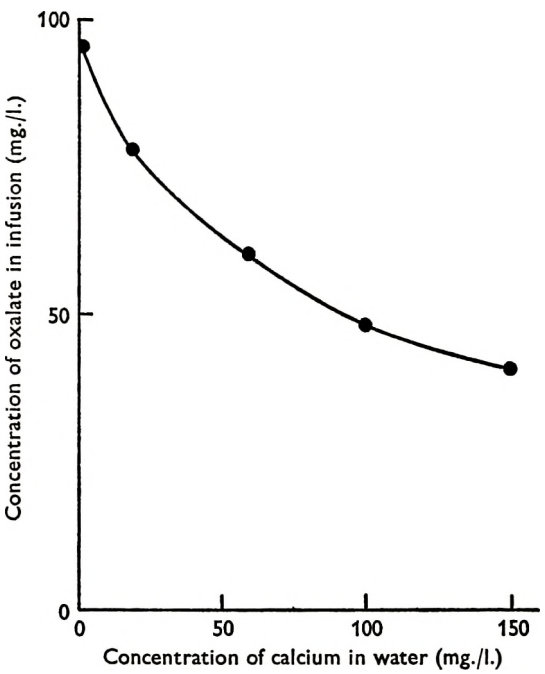

Fig. 2. The relationship between the amount of oxalate ion in tea infusions and the initial concentration of calcium in the extracting water (tea sample A). The overall analytical errors in the oxalate values are estimated to be less than ± 5 % of the plotted values.

that on the average the oxalate content of the calcium-loaded infusions was 53·5 % that of the distilled water infusions. Control tests showed that the presence of calcium in the infusions did not interfere with the oxalate determinations and gave no evidence that calcium oxalate was precipitated from calcium-loaded infusions onto the surfaces of any apparatus used. Further analysis of the results in relation to the values found for calcium uptake by the leaf showed that the deposition of calcium oxalate in the leaf would account for between 42 and 82 (mean 61) % of the calcium uptake values. A series of measurements of the oxalate in infusions prepared with different amounts of calcium in the extracting liquid showed that there was a progressive decrease in the amount of oxalate extracted as the calcium concentration was increased from 0 to 150 mg. Ca/l. (Fig. 2).

Various tests were carried out to discover the fate of the oxalate when milk was admixed with both distilled water infusions and calcium-loaded infusions in the ratio of milk : infusion = 1:5. Following domestic practice, the mixtures were prepared either by pouring the infusions onto the milk, or vice versa, and stirring briefly. The mixtures were then allowed to stand for 10 min. Determination of the oxalate content of the mixtures showed that more than 90 % of the oxalate remained within the body of the mixture. Further checks on the oxalate content both of the undisturbed bottom layers of mixtures held in tea-cups for 10 min., and also of any material deposited on the surfaces of the cups, confirmed that less than 10 % of total oxalate had settled out of the mixtures.

Phytic acid and phosphate

Using a method capable of detecting phytate if present at concentrations above 5 mg./15 g. leaf, no measurable phytate was found in 0·5 N-HCl extracts of four teas (A, C, E, G). This result indicates that, in the process of calcium uptake by the leaf in calcium-loaded infusions, deposition of calcium phytate would not account for more than 1 mg. Ca/15 g. dry leaf. Determinations of the phosphate content of distilled water and calcium-loaded infusions carried out for one tea sample (A) showed that the presence of calcium was without influence on the total phosphate extracted (phosphate content of infusion: 25·0 mg. P/l.).

DISCUSSION

The 'hardness' of drinking waters is due to their content of calcium and magnesium ions. In the majority of waters in the United Kingdom calcium is the principal component of 'hardness', the calcium content ranging from less than 1 mg. Ca per litre in very soft waters to about 150 mg. Ca per litre in the hardest of waters normally supplied (Skeat, 1961). An important fraction of the calcium in most waters is present as calcium bicarbonate ('temporary hardness'), and during the boiling of waters for the preparation of tea infusions some loss of calcium from solution, by deposition as carbonate, will generally occur for waters containing more than about 10 mg. calcium per litre. An indication of the extent of this loss is provided by Hollins (1965) who found that, when solutions containing equivalent amounts of calcium and bicarbonate ions were heated to boiling over a 5 min. period and boiled for a further 3 min., between 10 and 20 % of the calcium was deposited from solutions which had starting concentrations of 50 and 100 mg. calcium per litre. The present studies show that a further substantial loss of calcium occurs from drinking-waters during the preparation of tea infusions due to calcium uptake by the tea leaf. The general pattern of this uptake over the range of calcium concentration normally found in drinking-waters is indicated by the curve in Fig. 1. As outlined in the Introduction, our primary concern here is with the influence of the calcium on the composition of the infusions, and for the present purpose it will suffice to note that calcium uptake was shown in similar degree by all the teas examined and that the uptake process was essentially unaffected by the presence of any of the other principal ions normally found in drinking-waters.

The results found on the composition of the infusions will now be discussed, particularly in relation to the problem of the association between water hardness and mortality.

Several authors (Morris *et al.* 1961; Schroeder, 1966; Davies, 1962) have suggested that the 'water factor' in cardiovascular disease might be related to the trace element content of drinking-waters but no firm evidence has yet been produced to substantiate this view in respect of any single element. Interest in the intake of trace elements from drinking-waters is weakened by the fact that the contribution of these elements from water is generally a small fraction of the total dietary intake. The present results underline this fact specifically for manganese, zinc, and aluminium since the amounts of these metals extracted from the tea leaf into the infusion are higher by an order of magnitude than the corresponding amounts most frequently found in drinking-waters (Schroeder, Balassa & Tipton, 1966; Hollins, 1965; Campbell, Cass, Cholak & Kehoe, 1957). The results of our tests on metal pick-up by infusions in contact with various metallic surfaces indicate that the metalware most commonly used at the present time for the preparation of tea infusions does not give rise to any appreciable contamination of the infusion, although pick-up of copper, nickel, zinc and lead to a level of several milligrams per litre may be expected to occur in infusions prepared in certain types of teapots manufactured at earlier times. Of these various trace metals, lead can be singled out as one carrying a health hazard at a comparatively low intake level (Monier-Williams, 1949), so that any continual use of lead-pewter vessels for preparing tea infusions should be discouraged. In this connexion it should be noted that the spun pewter used in present-day cooking utensils is essentially lead-free. With regard to the other trace metals, it is uncertain (Campbell *et al.* 1957; Underwood, 1962) whether intakes of manganese, zinc, aluminium, copper or nickel have any significant physiological influence at the amounts found for tea infusions although it seems possible that the zinc and manganese contributions may be useful supplements to essential dietary requirements. There is, however, no evidence from the present results of any striking relation between the trace metal composition of the infusions and the hardness of the extracting water. Hard waters tend to extract more magnesium from tea than do soft waters, but the increment involved (about 10 mg. Mg/l. infusion) appears trivial when compared with the normal daily intake of magnesium (about 300 mg.) from other dietary sources (Wacker & Vallee, 1964).

It is also clear from our studies that the calcium in drinking-waters does not have any notable influence on the extraction of the other major tea components listed in Table 1. Special attention was given to the complex polyphenolic flavonoid material present in tea infusions in view of the fact that the chemical units of which this material is mainly composed are closely related in structure to the polyphenolic units present in several carcinogenic vegetable tannin extracts (Kirby, 1960). The analysis of this material, which took account both of the major polyphenolic components in a quantitative way and of the many minor components semi-quantitatively, showed that the presence of calcium in water used to prepare tea infusions has no marked effect on the extraction of any of the polyphenolic

substances studied. There is therefore no evidence from the present study of any interesting link between the supposed water factor in the aetiology of certain forms of malignant and cardiovascular disease, on the one hand, and the polyphenolic substances present in tea infusions, on the other. It may be noted from Table 1 that these polyphenolic flavonoid substances constitute approximately half the total amount of solid material present in the infusions and that the average daily intake of tea polyphenols in the United Kingdom is about 2 g. The recent reports (Korpassy, 1961; Kaiser, 1967) which raise the question whether the continual ingestion of these substances in tea might present either a carcinogenic or co-carcinogenic risk to man should encourage further investigation of their biological activity.

Of the other tea components examined, it will be noted that the presence of calcium in water represses the extraction of both oxalic acid and pectinic acid. It may also be noted that a major part of the calcium uptake process (Fig. 1) is accountable in terms of calcium salt deposition of these two substances in the leaf. It is probable that the remainder of the calcium uptake is due in part to calcium binding by insoluble leaf components, and some uptake by exchange with leaf magnesium may also be involved. The information on the pectinic acid fraction was sought mainly to determine the extent to which this fraction was responsible for calcium uptake, and not by reason of any special pharmacological interest. It is of general interest to note that, in food-canning practice, the process of 'firming' of certain types of fruit and vegetables (e.g. apples and tomatoes) by calcium salt treatment has been ascribed to a calcium uptake related to the deposition of low-ester calcium pectinates within the vegetable tissue (Kertesz, 1951). Evidently a similar type of interaction of water calcium with pectinic acid occurs in tea during infusion preparation but, in the case of tea, deposition of calcium as oxalate forms a more important part of the uptake process. The results found on the oxalate content of the infusions merit further discussion.

Comparatively little information has been published on the oxalate content of tea infusions. Measurements by Bau (1920) indicate the occurrence of about 500 mg. of soluble oxalate per 100 g. tea leaf and Hoover & Karunairatnam (1945) report the presence of about 135 mg. oxalate per litre in a tea infusion. The present values found for six tea samples, showing a range of 62 – 98 mg. oxalate per litre of infusion, are in broad agreement with these earlier results and confirm the general occurrence of appreciable amounts of soluble oxalate in black tea. It is also clear from Fig. 2 that the extraction of oxalate during the preparation of ordinary tea infusions is progressively repressed as the concentration of calcium in the extracting water is increased. It should be noted that in distilled water infusions the oxalate content is in large excess of the calcium content. In infusions prepared with water containing more than about 50 mg. calcium per litre, or in infusions which have been admixed with milk in the ordinary way, the calcium exceeds the oxalate in equivalents. It appears, however, that the oxalate is not readily precipitated from the infusion under ordinary conditions and remains within the body of the liquid after milk addition for periods of at least 10 min. The values found for oxalate in the tea infusions therefore provide a rough guide to oxalate intake from tea. The

results indicate that the average daily intake of oxalate from tea in different parts of the United Kingdom varies inversely with the degree of hardness of the local water supplies, and amounts to some 60 mg. in soft water areas and to half that value in areas supplied with water containing more than about 100 mg. calcium per litre.

Most of the published information on the oxalate content of foods relates to its occurrence in vegetables and fruit. Using the oxalate data of Kohman (1939) together with information on food consumption in the United Kingdom for the period 1956–65 (National Food Survey Committee, 1967) we estimate that the average daily intake of oxalate from vegetables and fruit amounts to some 65 mg., of which about one-third is contributed by potatoes. The figures available for other foods (Jeghers & Murphy, 1945) suggest that cereals, meat, milk, coffee and cocoa, taken as a whole, might contribute a further 50 mg. of oxalate to the average daily intake. These rough estimates indicate that tea infusions are the richest single source of oxalate in the United Kingdom diet. What is not clear is which of the various oxalate sources are the more important ones in terms of the contributions they make to the oxalate level in the blood, for little direct information is available on the complex question of the absorption of these comparatively small amounts of oxalate from different foods.

Various aspects of the metabolism and of the physiological action of oxalates absorbed from food are also problematic. Oxalaemia and oxaluria have frequently been reported as features of a wide variety of diseases, with low-oxalate diets recommended as therapy, although, except in the formation of oxalate calculi and the occurrence of severe renal colic, the relation between the disease states and the tissue oxalate concentrations has generally been indefinite (Jeghers & Murphy, 1945; Nordin & Hodgkinson, 1967). Against this background, it seems appropriate to recommend that, in further research on the significance of dietary oxalates, attention should be given to the oxalate contribution from tea infusions and also to the influence of calcium from drinking-water on both the intake and the absorption of oxalates from foods. An examination of the possibility that there may be some connexion between the 'water factor' in cardiovascular disease and the absorption of oxalates from foods appears to be a valid line for further inquiry.

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Mycoplasmas in cell cultures from rheumatoid synovial membranes

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SUMMARY

Ten strains of myocoplasmas were recovered from cultures of synovium or cultures inoculated with synovial fragments from rheumatoid arthritis and one from osteo-arthritis. The source of the organisms is not known. Patients with rheumatoid arthritis had no complement-fixing antibody and no fluorescent staining antibody against the mycoplasmas isolated and no mycoplasma antigen was detected by immunofluorescence in sections of synovia and in synovial fluids.

The strains isolated were of two main serological types and could be distinguished by direct fluorescent antibody staining from standard types of human commensals and the common tissue-culture contaminants. One may be *Mycoplasma laidlawii*.

INTRODUCTION

Whilst searching in synovium from rheumatoid arthritis for signs of infection by certain enveloped viruses, we noticed that pieces of such synovium always induced acidity in cell cultures into which they were introduced and that mycoplasmas could eventually be recovered from these. Since we had thus inadvertently confirmed observations by Bartholomew (1965) we give our findings here along with some attempts to ascertain the source of the mycoplasmas which were found.

METHODS

Material

Synovium, taken at therapeutic synovectomy from patients with rheumatoid arthritis and from a few operations on osteo-arthritic or injured, non-diseased joints, was placed directly into serum-free sterile cell-culture medium and dealt with in the laboratory within 1 hr. Samples of serum were obtained from the same patients.

Cell cultures

Four systems of cell-culture were inoculated with 10–20 fragments of synovium each about 2 mm. in diameter, because digestion of synovial membrane by trypsin, papain or collagenase did not give a usable cell suspension. Some synovia were put into two or more systems.

System 1. *Inoculation of animal cells.* Fragments of synovium were placed in rabbit, RK 13, and monkey, Vero, cells known to be free of mycoplasmas by staining and by culture and shown subsequently by fluorescent antibody staining to be free also of the types of mycoplasma found in our experiments. Inoculated cells and controls were kept 6 days in growth medium or 14 days in maintenance medium, then scraped off the glass and one-tenth the quantity passed into a fresh monolayer. Six or seven passes were made, the last culture being grown on cover-slips for staining with fluorescent antibodies.

System 2. *Mixed culture with animal cells.* Semi-confluent layers of RK 13 and Vero cells were again used. Since, after 4 days, some outgrowth of cells was visible from synovial fragments, cell suspensions were prepared by trypsinization, divided into three equal volumes and passed in growth medium by conventional methods. At each pass inoculated and control cultures were placed also in maintenance medium and left 12–14 days. Seven, eight or nine such passes were made of various synovial membranes, cover-slips being made of the final culture as in system 1.

System 3. *Human synovial cell cultures.* Synovium did not grow readily in our standard medium, but one of us (P.V.S.) succeeded in initiating cultures in medium containing 30 % foetal calf serum and maintaining them for long periods in Eagle's medium plus 20 % foetal calf serum which was changed at intervals of 4 days for 2 months, then of 10 days for 6 months.

System 4. *Mixed cultures with human cells.* Synovial tissue and cells or supernatant fluids from specimens in System 3 were also added to cultures of human embryo kidney cells and human embryo lung cells prepared in the laboratory by trypsinization of fresh material. Synovial tissue is toxic to these cultures, so the mixed synovial and lung cultures were passed at 4-day intervals in growth medium along with an equal number of fresh lung cells. After 6 or 7 passes, cultures were tested for mycoplasmas.

Culture media

Cells. Eagle's medium (Macpherson & Stoker, 1962) was used throughout. Penicillin only, 100 units/ml., was present in systems 1–3 but mycostatin, 50 units/ml., was added to System 4. Foetal calf serum, tested in liquid medium for absence of mycoplasmas, was used at 2 % concentration for maintenance cultures, 10 % for growth experiments and 20 % or 30 % for synovial cultures. Cells were incubated in 5 % CO_2 at 36° C.

Mycoplasmas. Strains were grown in liquid medium, for inoculation into tissue culture and for use as antigen, and on solid medium for recovery, identification and purification.

Liquid medium was prepared from Difco brain–heart infusion broth, 18·5 g./350 ml. distilled water, autoclaved at 15 lb./in.^{2} and allowed to cool. Sterile, non-inactivated, horse serum No. 3 (Burroughs Wellcome) was added to give a concentration of 20 %, and yeast extract to 10 %. Rabbit serum was used in place of horse serum, when preparing antigen. The yeast extract was prepared from fresh baker's yeast extracted for 30 min. with an equal weight of distilled water at 75° C. at pH 4·5. After centrifugation the supernatant was adjusted to pH 8 and

sterilized by Seitz filtration. Solid medium was prepared from Difco PPLO agar, 17 g./350 ml. distilled water, treated similarly to the liquid medium and supplemented with the same concentration of horse serum and yeast extract.

Ampicillin to a concentration of 1 mg./ml. was added to both media (Hutchinson, 1969).

Attempts were made to isolate mycoplasmas by growing synovial cell colonies in soft agar (Macpherson & Montagnier, 1964) and over cell layers in agarose as described by Zgorniak-Nowosielska, Sedwick, Hummeler & Koprowski (1967).

Strains of mycoplasmas for serological comparison were obtained from the National Collection of Type Cultures as follows: *M. dispar*, NCTC 10125; *M. fermentans*, NCTC 10117; *M. hominis*, NCTC 10111; *M. laidlawii*, NCTC 10116; *M. orale*, Type I, NCTC 10112; *M. pneumoniae*, NCTC 10119; *M. pulmonis*, NCTC V.1145-64. The strain number of *M. agalactiae* is not known. *M. arthritidis* is strain 'Arthr' of Dr Ruth Lemcke and *M. orale II* was provided by Dr B. E. Andrews.

Histology

Sections of synovium were cut in a cryostat and stained conventionally by haematoxylin and eosin, by Giemsa's stain and, after acetone fixation, by fluorescent antibody. Antisera against viruses and mycoplasmas were conjugated by Rinderknecht's method (1962) and applied directly because indirect staining gave interesting, but unwanted complications.

Cytology. Cell cultures on cover-slips were stained by May–Grunwald Giemsa's stain, by prolonged staining with Giemsa's stain at 37° C. or with orcein, and also by the direct method with fluorescent antibody specific for viruses and mycoplasmas. Indirect staining by conjugated anti-human globulin was used on virus and mycoplasma-infected cell cultures, to test for antibody in patient's sera.

Serology of mycoplasmas

Antisera were prepared by subcutaneous inoculation into rabbits of deposits of purified mycoplasma strains grown in medium containing rabbit serum, thrice washed in 0·01 M phosphate buffered saline solution (P.B.S.) and made up in complete Freund's adjuvant. Sera were tested by overnight complement fixation and, after conjugation, by fluorescent staining. Inhibition of growth of mycoplasmas by standard antisera was tested by the disk method on agar (Clyde, 1964).

RESULTS

The isolation of mycoplasma strains

System 1

Of six synovia so treated only one yielded virus or signs of virus antigen. This was a strain of rubella virus, presumed to be a laboratory contaminant.

All cultures inoculated with rheumatic tissue became very acid after 48 hr. incubation. This propensity to form more acid than control cultures was found also in all subcultures after 4 days in growth medium, or 10–12 days in maintenance medium.

Two cultures in maintenance medium showed Giemsa-stained granules in a centrifuged deposit (7000 *g* for 10 min.), and yielded mycoplasmas, but no bacteria, on suitable media. These mycoplasma strains were provisionally designated Belfast one (Bel_1) and Belfast two (Bel_2).

System 2

In nine inoculated cultures no cellular changes were seen that did not also appear in controls. No viruses were recovered. The amount of acid produced by inoculated cultures remained above that of control cultures for three passes and then became similar to controls.

Mycoplasmas were not sought in this series of tests because those found in system 1 were at this time regarded as contaminants, but much later, immunofluorescence tests on stored cover-slips from the seventh pass of two synovia of the series showed mycoplasma antigens of type Bel_1. Corresponding control, seventh pass, uninoculated cells had no stainable mycoplasmas.

System 3

Synovial cultures were prepared by one of us (P.V.S.) and maintained in a separate laboratory from the experiments on animal cells. The supernatant fluids of five synovial cell cultures were tested for virus activity by inoculation into fresh cultures of human embryo kidney cells. No virus was found, but the cultures became very acid. Four out of five cultures yielded mycoplasmas – one cultivated directly from synovial culture, three grown in abundance from the first pass in human embryo kidney cells which, uninoculated, remained free of mycoplasmas. The strain grown directly from synovium was similar to Bel_1 and was isolated from a culture of the same patient's synovium. Two other strains came from the same patients in whom the mixed animal-cell cultures of system 2 ultimately showed type Bel_1 antigen.

System 4

Inoculated and control cultures were tested for the presence of bacteria and mycoplasmas after six or seven passes.

Six more mycoplasma strains were found in inoculated cultures, but no bacteria. Control cultures were free of organisms. The combined recovery rates in the three sorts of experiments are shown in Table 1, the two animal systems being one sort.

Other tests

Five specimens of synovial cells and fragments grown in soft agar and three grown in agarose containing RK 13 cells showed no growth of mycoplasmas. Fifteen other synovia and synovial fluids gave no growth when inoculated directly into mycoplasma medium, yet seven of these gave mycoplasmas in the tissue culture systems.

Table 1. *The proportion* of mycoplasma infections in cultures of joint tissue*

System	Inoculum	No. of cultures	No. yielding mycoplasmas
(1, 2) Animal cells	Rheumatoid synovium	6	2
	None	6	0
(3) Rheumatoid synovial cultures	None	6	5†
(4) Human embryo cells	Rheumatoid synovium	7	5
	Osteo-arthritic synovium	2	1
	Normal synovium	3	0
	None	6	0

* Excludes two fluorescence identifications in system 2.
† Includes the two 'positive' sources of system 1.

Table 2. *Properties of mycoplasma strains Bel_1 and Bel_2*

	Strain	
	Bel_1	Bel_2
Growth rate	Slow. Not on blood agar	Rapid. Adaptable to blood agar
Colony type	Small, granular, indefinite central opacity	Large, clear, marked central opacity
Yeast extract	Required	Not required
Fermentation of glucose in Eagle's medium	Slow	Rapid
Haemolysis of sheep cells	Absent	Present, alpha type
Growth in cell culture	Continuous coating of organism on cell	Lumpy aggregates on cell membrane
Cytopathic effect on human embryo cells and RK13 cells	Very little	Marked destruction
Survival on PPLO agar		
37° C.	14 days	48 days
4° C.	30 days	> 300 days
Antigenicity	Distinct from Bel_2	Distinct from Bel_1

The properties of the mycoplasma strains

Mycoplasma strains were recognized by certain characteristics: the formation of polymorphic structures in liquid media; typical embedded colonies on agar media, suitably enriched; poor staining with Gram's stain; granule-formation with Dienes's stain; and lastly, sensitivity to certain broad-spectrum antibiotics, but not to penicillin (see Marmion, 1967). Their appearance on electron microscopy when stained by negative contrast and also when pelleted and sectioned was also taken into account. The properties of strains Bel_1 and Bel_2 from system 1 were first examined in preparation for comparative studies of the other strains.

It will be seen from Table 2 that the cultural characteristics of Bel_1 and Bel_2 are markedly different. In tissue culture alone, the different forms of adhesion to the cell and the pronounced cytopathic effect of Bel_2 were useful and reproducible

attributes which were always correlated with a clear distinction between Bel_1 and Bel_2 by direct fluorescent staining.

Nevertheless, the organisms did share antigens, as is seen in Table 3. No detailed analysis has been attempted, but guinea-pig antisera (unpublished data) indicate much more antigenic overlap between Bel_1 and Bel_2. The organisms are poor inducers of complement-fixing antibody, for seven rabbits given a course of antigen failed to develop detectable antibody. Four rabbits, however, given antigen in Freund's adjuvant developed antibody after two courses, 5 months apart, of 3 and 4 weekly injections.

Table 3. *Antigenic relationship between mycoplasma strains Bel_1 and Bel_2*

Test and titre	Serum	Bel_1	Bel_2
Complement fixation	Anti-Bel_1	256	32
	Anti-Bel_2	Nil	128
Indirect immunofluorescence	Anti-Bel_1	256	32
	Anti-Bel_2	16	> 256
Direct immunofluorescence	Anti-Bel_1	12	< 3
	Anti-Bel_2	< 3	> 24

Table 4. *Serological relationship of Bel_1 and Bel_2 to other mycoplasmas, tested by direct immunofluorescence, in tissue culture*

Rheumatoid mycoplasma strains	Serum: Anti-Bel_1	Serum: Anti-Bel_2	Standard strains	Serum: Anti-Bel_1	Serum: Anti-Bel_2
LM (2 isolates)	+	0	*M. pneumoniae*	0	0
BW	+	0	*M. hominis*	0	0
JM (2 isolates)	+	0	*M. fermentans*	0	0
TP	+	0	*M. salivarium*	–	–
SS	0	0	*M. orale* (I)	0	0
SO	0	+	*M. orale* (II)	0	0
DG	0	+	*M. arthritidis*	0	0
SM	0	+	*M. pulmonis*	0	0
FL	0	+	*M. agalactiae*	0	0
MC	0	+	*M. dispar*	0	0
Original Bel_1	+	0	*M. laidlawii*	0	+
Original Bel_2	0	+			

+ = Specifically stained; 0 = not stained; – = not done.

Using the direct fluorescent antibody test, we obtained the cross-reactions, shown in Table 4, between antisera to Bel_1 and Bel_2 and the remaining strains, all tested in tissue culture of human lung cells. Four strains resemble Bel_1 and are antigenically unlike five other strains which are similar to Bel_2. One strain was not stained by either serum. The same antisera do not react in the direct test with standard 'human' mycoplasmas nor with some others that have been implicated from time to time as contaminants in tissue-culture experiments. Antiserum to

Bel_2 did react with *M. laidlawii*, which stained more intensely than does Bel_2 itself. The growth of our strains Bel_1 and Bel_2 was not inhibited by standard test antisera to the five human strains tested in Table 4.

Clinical immunology

In order to investigate the clinical significance of strains Bel_1 and Bel_2, sera of patients were examined for specific antibody by indirect immunofluorescence and by complement fixation. In addition, sections of synovial membranes were examined by fluorescent antibody staining for antigen to Bel_1 and Bel_2. Sera, which included all those whose tissues gave mycoplasma-positive cultures, were tested at 1/5 dilution on cell-cultures infected with Bel_1 and Bel_2. Sections of synovia were stained with four times the minimal staining concentration of each conjugate of anti Bel_1 and anti Bel_2 sera.

Table 5. *Clinical immunology of mycoplasma isolates*

Material tested	Tests for antibody: No. tested	Tests for antibody: No. positive to: Bel_1	Tests for antibody: No. positive to: Bel_2	Tests for antigen: No. tested	Tests for antigen: No. positive to: Anti-Bel_1	Tests for antigen: No. positive to: Anti-Bel_2
	Method: immunofluorescence					
Rheumatoid sera	32	1	1	–	–	–
Rheumatoid synovial fluid	4	0	0	6	0	0
Non-rheumatoid sera	21	0	2	–	–	–
Synovial membranes	—	–	–	20	0	0
	Method: complement fixation					
Rheumatoid sera	14	0	0	–	–	–
Rheumatoid synovial fluid	3	0	0	4	0	0
Non-rheumatoid sera	12	0	0	–	–	–

– = Not done.

Table 5 shows that antibody to Bel_1 and Bel_2 is not characteristic of rheumatoid sera, nor do rheumatoid synovia, even allowing for errors of sampling bulky tissue, contain Bel_1 and Bel_2 antigens. No inhibitors of specific fluorescent staining were present in a few sera and a few synovial fluids that were tested.

In addition to the absence of mycoplasma antigens from synovial tissue, we observed that potent specific antisera against the viruses of influenza A, mumps, measles, German measles, respiratory syncytial disease and herpes simplex produced no specific staining of seven rheumatoid synovia, nor any staining of cells into which the same rheumatoid tissues had been inoculated (systems 1 and 2).

DISCUSSION

Mycoplasmas have grown only in cultures of rheumatic synovium or in cultures inoculated with pieces of synovium and not in uninoculated cultures. The circumstances in which they were recovered simulate those which give rise also to

growth of diphtheroids (Duthie, Stewart, Alexander & Dayhoff, 1967) and L-forms of bacteria (Pease, 1969) from similar material; that is to say, prolonged cultivation and repeated passage. Contamination, the first likely source, cannot be ruled out, but this explanation can apply only to rheumatoid tissue and not the cell cultures. The sources of contamination common to all procedures were surgical operation, the initial dissection of the tissue in the laboratory and animal culture media, namely trypsin and calf serum: yet the same trypsin and calf serum were used in control cultures which remained mycoplasma-free.

The absence of mycoplasmas from control experiments, the fact that they are not strains of the common type of laboratory contaminants (Macpherson, 1966), and the duplication of four recoveries in separate rooms by separate workers, are in favour of a synovial origin of the mycoplasma isolates.

Against this conclusion are: failure to find mycoplasmas in primary cell-cultures, or by direct inoculation of mycoplasma culture medium, absence of antibody response in the patients, and lack of mycoplasma antigen in sections of rheumatoid joints.

If our results and those of Bartholomew (1965) are taken first at face-value, we must believe that rheumatoid tissue is apt to carry very small numbers of mycoplasma organisms. If so, the organisms must be practically non-antigenic. They are poor inducers of complement-fixing antibody in rabbits, as we have stated, and in guinea-pigs (unpublished data). More sensitive methods of assay (Taylor-Robinson *et al.* 1965) might reveal specific antibody in the patients.

No occult variety of cellular infection is known which would account for our complete failure to find mycoplasma antigen in synovia by immunofluorescence. There is thus nothing to suggest that the recovered organisms are responsible for the immunological features of the rheumatoid joint. Although some of our specimens contained very numerous foci of large and small mononuclear cells, and also collections of plasma cells, no mycoplasma antigen was found in or near them.

If the mycoplasma strains which we have isolated are contaminants, the fluorescent antibody tests give little indication of their origin. The cross-reaction between Bel_2 and *M. laidlawii* was unexpected and the two organisms have got very similar cultural properties. If Bel_2 is *M. laidlawii*, it should have grown readily in mycoplasma PPLO medium from the original tissue. Although our strains are distinguishable from standard ones by immunofluorescence, they may well be variants of common mycoplasmas, selected by long passage in tissue culture.

It remains possible that poorly antigenic organisms, by persisting somewhere in the immune system, alter immunological function in some way other than by inducing a specific immune response. Our fluorescence tests did not reveal any cross-reaction between specific mycoplasma antibody and joint tissue; tests of cellular hypersensitivity to our strains have not yet been carried out; the effect of the organisms on the patients' responses to other antigens is also unknown. We consider that such possibilities should be investigated before discarding the mycoplasma strains as mere commensals in a tissue whose resistance to infection is somewhat impaired (Editorial, 1967).

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The kinetics of influenza-virus adsorption on iron oxide in the process of viral purification and concentration

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SUMMARY

This paper reports a study carried out to clarify the mechanisms involved in adsorption of influenza A and B viruses on iron oxide. Accordingly, the amounts of virus that are adsorbed from virus suspensions of varying concentrations per unit surface area of magnetic or non-magnetic oxide at fixed temperature and time have been determined. The principles involved are clearly the same as those involved in multiple equilibria during the interaction of particles with a large number of combining sites with different intrinsic affinity. Consequently, the amount of virus that is adsorbed per unit mass of iron oxide depends on the size of the adsorbent area, not on its magnetic property. Owing to a significant difference between the affinities of influenza A and B particles for the binding sites on iron oxide, unit surface area of the adsorbent is invariably capable of adsorbing significantly greater amounts of influenza A than B particles. The practical implications of these findings are that a better understanding of the mechanisms involved in virus adsorption on iron oxide will permit a more efficient separation of virus particles from impurities. The simplicity and the rapidity of the technique and the cheapness of the equipment required suggest that the iron oxide method is of great value for both small- or large-scale viral purification, whether it is used as a single step procedure or as a primary step followed by zonal separation.

INTRODUCTION

This study is concerned with principles involved in adsorption of influenza viruses A and B on iron oxide. Although no generalized theories can be formulated for the process, some practical rules for the use of this compound for viral purification and concentration can be deduced from the experience of the adsorption kinetics gained in this study. The adsorption of myxoviruses on magnetic iron oxide was originally described by Warren, Neal & Rennels (1966), providing a simple method of virus purification and concentration.

MATERIALS AND METHODS

Viruses

The following strains of influenza virus were used in this study: A0/PR8, A1/England/1/51, A2/Singapore/1/57, B/England/101/62 and B/England/13/65. These strains were obtained as allantoic virus from the Virus Reference Laboratory,

Colindale, and the WHO International Reference Centre for Respiratory Diseases, Salisbury. The substrains WS E720, NWS E714 and NWS E691 of influenza A0/WS virus were obtained as allantoic virus from Dr D. Hobson of the University of Liverpool. Virus was grown in the chorioallantoic sac of 10-day-old chick embryos for 48 hr. at 35° C. and the freshly harvested allantoic fluid was clarified by centrifugation at 2000 rev./min. The allantoic virus was used either fresh or after prolonged periods of storage at −20° or −70° C. Purified virus concentrates were prepared using the iron oxide adsorption-elution method of Warren *et al.* (1966). In order to achieve the optimum pH for virus adsorption (pH 7·0–7·2) the purified virus in elution buffer was dialysed against phosphate buffered saline (PBS) or distilled water. The haemagglutinin titres and the number of virus particles per haemagglutinating units were determined by conventional methods (Davenport & Minuse, 1964; Isaacs, 1957).

Iron oxide

Samples of magnetic (γ) and non-magnetic (α) iron oxides were supplied for this study by Minerals, Pigments and Metals Division, Chas. Pfizer and Co., Inc., Easton, Pennsylvania, U.S.A. The samples used in the study were as shown in Table 1.

Table 1. *Samples of iron oxide*

Batch	Formula	Type	Ultimate particle size (μ)	Surface area ($m.^2/g.$)
RX-2165 D	$\alpha Fe_2O_3.H_2O$	Non-magnetic	0·5	16–18
RX-2165 E	$\gamma Fe_2O_3.H_2O$	Magnetic (made from D)		
RX-2165 F	$\alpha Fe_2O_3.H_2O$	Non-magnetic	0·02	80–100
RX-2165 G	$\gamma Fe_2O_3.H_2O$	Magnetic (made from F)		
Mo 9853	$\gamma Fe_2O_3.H_2O$	Magnetic		

Although the ultimate particle size of the oxide is very small, the material tends to aggregate. To break up the aggregates and to provide a smooth dispersion, the above batches of iron oxide were jet-milled at an air pressure of 75 lb./in.^{2} and a feed rate of 1 c.c./15 sec. to achieve average aggregate size of less than 5 μ

Procedure of virus adsorption-elution

Adsorption. An appropriate amount of powdered iron oxide and virus, used either as crude (allantoic fluid) or purified suspensions, were mixed together in a screw-cap bottle or flask and agitated for 30 min. at room temperature using a Griffin Wrist Action Flask Shaker. The mixture was then centrifuged at 2000 rev./min. to separate virus-coated particles of the iron oxide from the supernatant fluid. This procedure was carried out with increasing concentrations of the adsorbent until the uptake per unit mass became constant. This constant uptake was assumed to correspond to the saturation point of the adsorbent.

Elution. The virus-coated iron oxide was suspended in 10% $Na_2HPO_4.7H_2O$ (pH 8·9) and the suspension was agitated for 30 min. at room temperature using

the flask shaker. All the adsorbed virus was recovered in the supernatant after the iron oxide was deposited by light centrifugation.

Protein estimations. The method described by Lowry, Rosebrough, Farr & Randall (1951) was used throughout this study.

RESULTS

Basic observations and expression of results

In this present study the availability of surface area of magnetic and non-magnetic iron oxides, γFe_2O_3 and αFe_2O_3 respectively, was expressed as the ratio of the number of virus particles to unit surface area of the adsorbent. The effect of 'favourable' ratios was that all virus particles were adsorbed, whereas a range of 'unfavourable' ratios gave rise to a pattern in which the amounts of adsorbed virus decreased in proportion to the decrease of available surface area of the adsorbent.

All adsorption-elution experiments employing iron oxide and suspensions of allantoic or purified virus used a batch procedure in which measured amounts of virus and oxide were mixed together under conditions where virus adsorbed quantitatively. The virus-coated iron oxide was then separated by low-speed centrifugation and the virus was eluted under conditions near zero adsorption. Each adsorption-elution experiment was always carried out in triplicate.

Table 2. *Adsorptive power of iron oxide;* tentative observations*

Biological particles (molecules)			
Identity	Size (mμ)	Weight	Adsorption-elution results
E. coli	800×2000	$1{\cdot}5 \times 10^{11}$	Excluded
Influenza virus	80–120	$2{\cdot}8 \times 10^{8}$	Adsorbed-eluted
Rhinovirus 2 (HGP)	20–30	—	
Haemoglobin (fowl)	3×15	$6{\cdot}8$–$7{\cdot}6 \times 10^{4}$	
Ovalbumin	—	4×10^{4}	Excluded

* Relates to the iron oxide amounts as used in influenza virus adsorption.

In general, the adsorption of the virus was very dependent on pH and salt concentration, but did not significantly change when virus particles were suspended in allantoic fluid, distilled water or a 0·1 M phosphate buffer solution over a range pH 6·8–7·3. Tentative observations concerning the adsorptive power of iron oxide indicated that the adsorbable range of biological particles was within 3×15 to 800×2000 mμ (Table 2). It may be emphasized here that the rate at which particulate material is adsorbed is influenced by the rate of contact between the adsorbate and the adsorbent during the adsorption procedure. When allantoic or purified influenza virus was adsorbed on iron oxide at room temperature under the conditions for interaction and binding between the virus and the iron oxide as provided by the Griffin Shaker, the adsorption percentage for two strains of influenza virus was as shown in Table 3.

Egg infectivity titrations showed that the adsorption on iron oxide followed by elution did not affect virus infectivity.

Table 3. *Adsorption times of two influenza virus strains on iron oxide using a Griffin Flask Shaker*

Adsorption time (min.)	Virus particles adsorbed (%)	
	A 2/Singapore/1/57	B/England/13/65
5	50	12·5
10	75	37·5
30	100	100

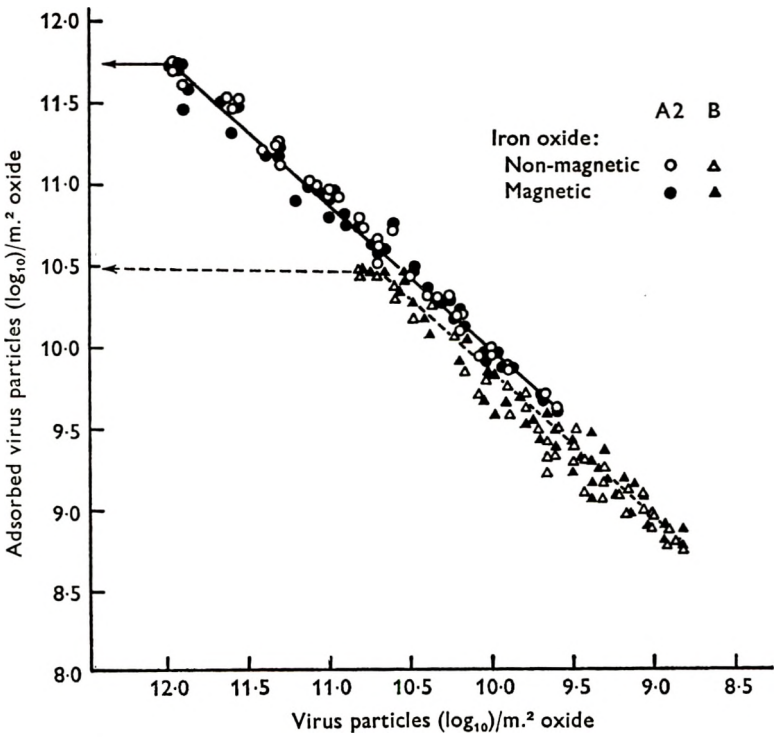

Fig. 1. The patterns of adsorption curves at constant temperature with influenza A 2 and B viruses.

Comparison of the adsorptivity of α and γ iron oxide

Adsorption kinetics of α and γ iron oxides

Originally, the purification of several viruses was accomplished with the use of γFe_2O_3 (magnetic iron oxide) by Warren *et al.* (1966). When it was found in our preliminary experiments that the magnetic property of iron oxide did not influence the rates of virus adsorption and elution and that virus purification could be readily accomplished using non-magnetic iron oxide, the mechanism involved in virus adsorption became of general interest. Work was undertaken therefore to investigate adsorption–elution kinetics of several batches of γ and α oxides and strains of influenza A and B viruses. The effect of six different ratios of virus to oxide surface area on the rate of virus adsorption was determined in triplicate experiments. Figs. 1 and 2 are representative of the results obtained with A 0/PR 8, A 1/England/

1/51, A2/Singapore/1/57, B/England/101/62 and B/England/13/65 strains of influenza virus. The following explanations seem to be sufficient to account for the pattern of adsorption curves plotted in Fig. 1:

(1) It is evident that the virus adsorptivity of magnetic and non-magnetic oxides is virtually identical. Consequently, it appears that the process of virus adsorption is due to forces other than the magnetic property of the adsorbent.

(2) Operations near the saturation point of the adsorbent result in a series of adsorption values indicating that unit surface area of iron oxide is invariably

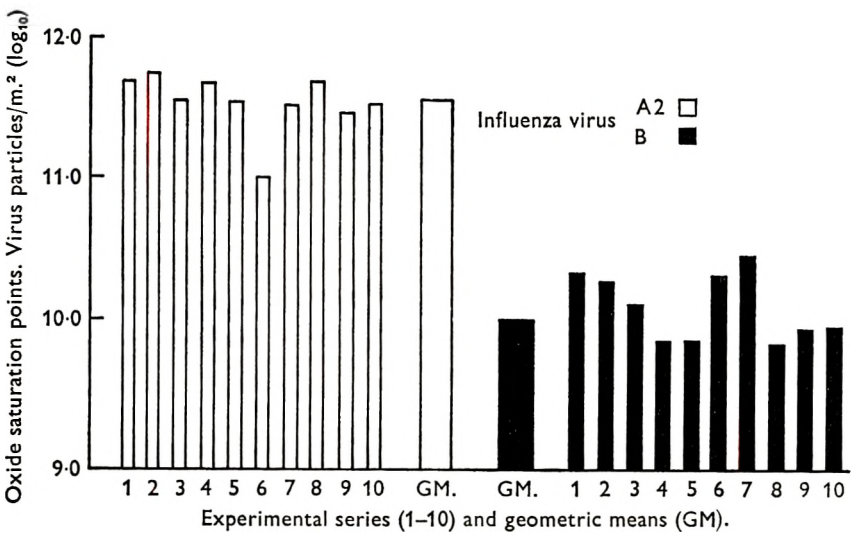

Fig. 2. The uptake of influenza A 2 and B virus particles per unit surface area of iron oxide at the saturation point of the adsorbent.

capable of adsorbing a greater amount of influenza A than B particles. As can be seen in Fig. 2, the amount of influenza B virus adsorbed is only 3·7 % that of A 2 virus. A study of the adsorption kinetics of a limited number of influenza A (including WS and NWS substrains) and B strains showed that strain variations did not affect the adsorption rate of crude or purified virus.

Determination of overall concentration and purification factors with α and γ iron oxide

In this series of experiments, specific activities expressed as HA units per μg protein were observed in the successive purification steps of two influenza virus strains. The results of this experiment revealed that by the use of iron oxide, virus can be efficiently purified and concentrated greater than 19 times as is shown in Table 4.

A comparison was also made between results obtained from the purification and concentration of two strains of influenza B virus, with an eightfold difference in virus particle content. The results, summarized in Table 4, show that purification factors, calculated on haemagglutinin and protein content, are higher with the allantoic fluid containing a greater number of virus particles. This may be due to the high background level of egg protein in virus allantoic fluid compared with virus protein.

Table 4. *Viral material monitored for haemagglutinin and protein content during iron oxide purification*

Strain	Material	HAU/ml.	Protein μg./ml.	HAU/μg. protein	Purification factor
A 2/Singapore/1/57	Crude allantoic virus	5,144	360	14·2	—
	Concentrated eluate from iron oxide	98,304	42	2,340·5	164·8
	Eluate centrifuged at 35,000 rov./min., the virus pellet suspended in PBS, the same volume as the removed supernatant	98,304	36	2,730·6	192·2
B/England/13/65	Crude allantoic virus	128	255	0·5	—
	Concentrated eluate from iron oxide	1,536	80	19·2	38·4
	Eluate centrifuged at 35,000 rov./min., the virus pellet suspended in PBS, the same volume as the removed supernatant	1,200	24	50	100·0
B/England/101/62	Crude allantoic virus	1,024	353	2·9	—
	Concentrated eluate from iron oxide	12,288	32	384	132·4
	Eluate centrifuged at 35,000 rev./min., the virus pellet suspended in PBS, the same volume as the removed supernatant	12,288	24	512	176·6

DISCUSSION

The use of viral adsorption on iron oxide and elution in purifying and concentrating of influenza virus was demonstrated by Warren *et al.* (1966). These authors employed γFe_2O_3, permitting the use of a magnet for the separation of virus-coated particles of iron oxide. The present experiments were conducted (*a*) to study the mechanisms involved in viral adsorption on the iron oxide employing magnetic (γFe_2O_3) and non-magnetic (αFe_2O_3) iron oxides with different surface areas per unit mass for comparison and (*b*) to determine the amounts of influenza A and B viruses adsorbed per unit surface area of γFe_2O_3 and αFe_2O_3 at fixed temperature and time. The data presented show that the adsorption of influenza virus on iron oxide is clearly not associated with its magnetic properties, the mechanisms involved being of the same order as those observed in multiple equilibria of suspended particles with a large number of combining sites. Examination of the adsorptivity of influenza virus in a series of its ratios to iron oxide established that, although the adsorption rates of the same virus were virtually identical with γFe_2O_3 and αFe_2O_3,

there was, in contrast, a significant difference between the adsorption rates of influenza A and B virus particles with each oxide employed. Estimates made on the uptake of virus particles per unit surface area of iron oxide at the saturation point of the adsorbent showed that the uptake of influenza B virus was 3·7 % (P = 0·001) of that of influenza A virus. This finding indicates that these viruses differ significantly in their affinities for iron oxide and suggests a significant difference in surface properties between the viruses in question. Practical implications of all these findings are that they permit a better separation of virus particles from impurities and thus improve the iron oxide method, which offers a practical and useful technique for both small- or large-scale viral purification and concentration.

We are indebted to Dr J. Warren and Mr H. S. Greiner for advice, to Dr R. J. Merrills for protein estimations, to Mr J. E. Jeffries for jet-milling the iron oxide used in these studies, and to Mr J. L. Wood for information concerning the adsorption of *Escherichia coli* on iron oxide.

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Antigenic properties of the envelope of influenza virus rendered soluble by surfactant-solvent systems

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SUMMARY

Dissociating chemical treatments employing surfactant-solvent systems were applied to purified influenza A and B viruses to obtain viral preparations possessing a significantly higher or lower haemagglutinating activity than the intact virus. All preparations, whether with high or low haemagglutinating activity, with the exception of envelope protein solubilized by Triton X-100, were significantly lacking in the ability to excite the formation of haemagglutination-inhibiting and virus-neutralizing antibodies in inoculated ferrets. In contrast to other treatments, Triton X-100 treatment of virus significantly enhanced the antigenicity of viral protein as judged by virus neutralization and haemagglutination inhibition tests. Yet the haemagglutinating activity of the envelope protein solubilized with Triton X-100 was about 1 % that of the intact virus. Results suggest that the correlation assumed to exist between the haemagglutinating activity of influenza virus and its ability to excite the formation of humoral antibodies is coincidental. Another important point is that the specific antigenicity of viral protein may be lost or enhanced owing to effects, other than solubilization, by surface-active agents.

INTRODUCTION

In the present report evidence will be presented suggesting that the correlation assumed to exist between the haemagglutinating activity of influenza virus and its ability to excite the formation of humoral antibodies is coincidental. Evidence will also be presented to show that if viral protein is rendered soluble by surfactants this may result in loss or enhancement of specific viral antigenicity as judged by haemagglutination inhibition and virus neutralization tests. This phenomenon will be discussed with reference to the various biological actions of surfactants (Elworthy, Florence & Macfarlane, 1968). It has been reported that the specific biochemical or biological activity of certain biological membranes rendered soluble by surfactants may be retained, lost or enhanced (Swanson, Bradford & McIlwain, 1964; Bradford, Swanson & Gammack, 1964; Bonsall & Hunt, 1966). Limited investigations of a similar nature have been made with influenza viruses, though there has been continued interest in the antigenicity of the envelope components rendered soluble by surfactants. In general, the application of dissociating chemical treatments to influenza virus has yielded haemagglutinating and non-haemagglutinating antigens which tend to be variable over wide limits in structure, com-

position and antigenicity (Cleeland & Sugg, 1964; Hobson, 1966; Webster & Laver, 1966; Rubin, Pierzchala & Neurath, 1967; Neurath, Rubin & Pierzchala, 1967; Neurath, Rubin & Hartzell, 1969).

METHODS

Virus

The A2/Singapore/1/57 and B/England/101/62 influenza virus strains stored at −60° C. were used in this study. Pools of crude allantoic virus were prepared by inoculating allantoically 10- or 11-day chick embryos with virus diluted 10^{-3} in buffered saline; each embryo was inoculated with 0·2 ml. of diluted virus. After 48–72 hr. incubation at 35° C. the eggs were chilled at 4° C. and the allantoic fluids harvested, pooled and stored at 4° C. until the purification and concentration of virus.

Buffered saline (BS)

The solution designated buffered saline consisted of 0·85 % NaCl buffered with $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.2H_2O$ (0·1 M) at pH 7·2.

Haemagglutination (HA) tests

These tests were done by the 'pattern' method in WHO haemagglutination trays (WHO, 1959). Serial twofold dilutions of virus were made in 0·25 ml. volumes of BS and then equal volumes of a 0·5 % suspension of fowl, human (group O) or guinea-pig erythrocytes were added. Readings were made after 1 hr. and end-points determined by the standard method of interpolation. Titres were expressed in terms of haemagglutinating units (HAU) per 1 ml. of undiluted virus suspension.

Purification and concentration

Pools of crude allantoic virus were clarified by centrifugation at 2000 rev./min. in the MSE-Magnum refrigerated centrifuge for 10 min. Virus was adsorbed from the allantoic fluid at room temperature by addition of Fe_2O_3 (Warren, Neal & Rennels, 1966; Larin & Gallimore, 1971). The suspension was shaken for 30 min. with the use of a Griffin flask shaker and the virus–iron oxide complex was sedimented by centrifugation at 2000 rev./min. for 5 min. Virus was eluted at room temperature and concentrated tenfold by suspending the virus–iron oxide complex in one-tenth the original volume of 10 % Na_2HPO_4 (pH 8·9). This suspension was shaken for 30 min. at room temperature and the iron oxide separated from the eluted virus by low-speed centrifugation. To avoid the effects of excess electrolyte on the solubilization of the viral envelope by surfactants, Na_2HPO_4 was crystallized out by chilling at 4° C. The virus was then sedimented by centrifugation at 60,000 *g* for 2 hr. in an MSE Superspeed 50 centrifuge. The virus pellet was taken up in a volume of BS or de-ionized water equivalent to the original volume of the eluent (10 % Na_2HPO_4). Since the determination of an overall purification factor can be hampered by the extreme lability of highly concentrated virus preparations (Pepper, 1967), the calculations to determine purification factors were made on HA titres immediately before and after purification.

Assuming a particle/HAU ratio of 10^7, the virus concentrates contained $10^{10.4}$ (A 2) and $10^{9.7}$ (B) virus particles/μg. protein. The purified viral concentrates (Table 1) designated as virus particles (VP) were used for the preparation of viral antigens within 24–48 hr.

Table 1. *Purification factors for concentrates of influenza virus used for preparation of viral antigens*

Virus strain	Viral material	HAU/μg protein (Lowry)	Purification factor
A 2/Singapore/1/57	Allantoic fluid	28·2	96·8
	Virus concentrate	2730·6	
B/England/101/62	Allantoic fluid	2·9	176·6
	Virus concentrate	512·0	

Preparation of viral antigens

VP suspensions were divided into the appropriate number of aliquots for use in parallel studies, intact or treated chemically. All these materials, before being used as ferret inocula, were treated with formalin to inactivate residual virus. VP suspensions for treatment with Tween 80-ether were made in BS. For other treatments, VP suspensions were made in de-ionized water or as otherwise indicated in the appropriate sections below.

Tween 80-ether (T 80-E) treatment

The following time-schedules were used for the solubilization of VP envelope with Tween 80-ether at room temperature:

Tween 80	Ether
40 min.	2 hr.
2 hr.	6 hr.
6 hr.	18 hr.

VP suspension were mixed continuously in sealed Erlenmeyer flasks for the required times with Tween 80 at a final concentration of 0·1 % (v/v). Then equal volumes of di-ethyl ether were added to the suspensions and agitation was continued. The material was then centrifuged at 800 *g* in a refrigerated centrifuge. The aqueous phase was collected and cleared of ether by bubbling through nitrogen. The materials obtained by this treatment were designated VP/T 80-E.

Sodium dodecyl sulphate (SDS) treatment

SDS was added to VP suspension to a final concentration of 1 % (w/v). The suspension was shaken for 15 min. at room temperature and then centrifuged at 60,000 *g* for 2 hr. The supernatant obtained by this treatment was designated VP/SDS.

Combined treatment with SDS and sodium deoxycholate (DOC)

SDS and DOC were added to VP suspension to final concentrations of 0·0015 % (w/v) and 0·006 % (w/v), respectively. The mixture was stirred with a magnetic stirrer for 30 min. at 37° C. and then centrifuged at 60,000 *g* for 2 hr. The supernatant obtained was designated VP/SDS-DOC.

Butanol treatment

VP suspension in 0·05 M tris-HCl buffer (pH 7·7) containing 0·001 M EDTA was mixed with 1-butanol in proportions of 1·5:1. The mixture was kept at 4° C. for 30 min. and stirred intermittently with a glass rod. The butanol-aqueous phases were separated by centrifugation in a refrigerated centrifuge at 8000 *g* for 5 min. The butanol treatment of the aqueous phase was repeated twice more as described and the final aqueous phase was designated VP/butanol.

Triton X-100 treatment

Triton X-100 (polyoxyethylated *tert*-octylphenol) was added to VP suspension in tris buffer (pH 7·5) to a final concentration of 5 % (v/v). The mixture was shaken at room temperature for 5 min. and then placed at 4° C. for 10 days, after which it was centrifuged at 60,000 *g* for 2 hr. and the supernatant designated VP/TX-100.

Immunization of ferrets

Ferret inoculations

Young ferrets of both sexes (weight 700–800 g.) were bled before inoculation in pairs with the antigens just described. Each ferret was inoculated intraperitoneally with 1 ml. of the appropriate antigen. Three weeks later this inoculation was repeated and after a further 3 weeks the ferrets were killed. The pre- and post-inoculation sera and the peritoneal effusion, which were collected from each ferret, were stored at −20° C. until tested.

Titrations of antibody in ferret sera and peritoneal effusion

Haemagglutination-inhibition tests (HI). HI tests were carred out in WHO plates. Non-specific inhibitors were removed by trypsin-periodate treatment as described by Fiset (1964). Eight HAU of virus was used in all HI tests.

Virus neutralization (VN) tests in tissue culture. The method described by Hobson, Lane, Beare & Chivers (1964) was used for titrations of VN antibody. VN tests used secondary cultures of Vervet monkey kidney and the haemadsorption technique described by Shelokov, Vogel & Chi (1958). A standard virus dose of 100 haemadsorbing doses per ml. was incubated at 37° C. for 1 hr. with twofold dilutions of ferret serum or peritoneal effusion inactivated at 56° C. for 1 hr. The mixtures were inoculated into roller tubes of monkey kidney tissue culture and incubated at 35° C. for 72 hr. and then they were tested for haemadsorption. Tissue culture and virus challenge dose controls were set up with each experiment. Only complete inhibition of haemadsorption was regarded as evidence that the dilution of serum or peritoneal effusion contained VN antibody.

Protein estimations

All protein estimations in the viral preparations just described were carried out by the method described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Effects of the duration of T 80-E treatment of influenza virus on its haemagglutinin titres and immunogenicity for ferrets

It has been reported that the non-haemagglutinating material of small particle size obtained with the use of trypsin or surfactants and organic solvents, though active serologically, was much less immunogenic in animals (Cleeland & Sugg, 1964; Hobson, 1966; Webster & Laver, 1966; Rubin *et al.* 1967). In view of these findings it was suggested that the integrity of the whole virus or the 70S HA subunits obtained after ether treatment was required for quantitative maintenance of the immunogenic properties of the viral preparations (Neurath *et al.* 1967). But the question arises whether the ability of 'divalent haemagglutinin' (Choppin & Stoeckenius, 1964) to agglutinate erythrocytes necessarily implies ability to induce antibody production when injected into a suitable test animal, e.g. ferret. In this section we describe investigations into the ability of divalent haemagglutinin obtained from influenza virus A 2/Singapore/1/57 by T 80-E treatment for varying times to evoke production of HI and VN antibodies in inoculated ferrets. Based on preliminary experiments, the time-periods chosen for this treatment were those that increased HA titres of the treated virus with fowl, human (group O) and guinea-pig erythrocytes at several consecutive time-points during progressive disruption of the viral envelope. Although the magnitude of HAU increase at given time points varied insignificantly from experiment to experiment using the same type of erythrocytes, the kinetics of the HAU increases were similar to those shown in Table 2 for ferret inocula used in the present experiments.

Using the inocula shown in Table 2, two interesting observations were made. First, with the increased time of T 80-E treatment, the inocula tended to evoke lower antibody titres in inoculated ferrets. This reduction in the capacity to stimulate the production of both HI and VN antibodies (shown in Tables 2 and 3) may be explained by partial inactivation of antigenic protein by the prolonged T 80-E treatment, but owing to the small number of ferrets inoculated with each antigen this can only be a tentative observation rather than an emphatic statement. Secondly, as is seen in Table 3, ferrets inoculated with progressively increasing amounts of divalent haemagglutinin showed progressively decreasing amounts of antibody per 1000 HAU inoculated.

This finding that HI and VN antibody titres are not influenced by the concentration of divalent haemagglutinin in the inocula adds new information concerning the nature of the envelope components responsible for specific viral immunogenicity and haemagglutination. The fact that high levels of divalent haemagglutinin (inoculum VP/T 80-E/3) were less immunogenic in ferrets than lower levels (inoculum VP/T 80-E/1) may contradict the viewpoint that the direct effect of in-

Table 2. *The effects of T 80-E treatment on the capacity of haemagglutinating viral material to excite antibody production in ferrets*

Ferret inocula	Duration of chemical treatment		HAU/ml. of ferret inoculum with different types of erythrocyte			Mean reciprocal antibody titres of sera from ferrets inoculated with undiluted or diluted (1/5) inocula			
						HI antibody		VN antibody	
	Tween 80	Ether	Fowl	Human (group O)	Guinea-pig	Undiluted inocula	Diluted inocula	Undiluted inocula	Diluted inocula
Intact VP	Nil	Nil	8,192	12,288	8,192	320	90	1,408	420
VP/T 80-E/1	40 min.	2 hr.	12,288	49,152	65,536	150	120	720	480
VP/T 80-E/2	2 hr.	6 hr.	16,384	98,304	196,608	120	40	1,200	320
VP/T 80-E/3	6 hr.	18 hr.	24,576	98,304	262,144	120	35	560	180

Table 3. *Reciprocal antibody titres per* 1000 *HAU* (*Rat*/1000) *with fowl, human and guinea-pig erythrocytes*

Ferret inocula	Fowl				Human (group O)				Guinea-pig			
	HI antibody		VN antibody		HI antibody		VN antibody		HI antibody		VN antibody	
	Rat/1000	%	Rat/1000	%	Rat/1000	%	Rat/1000	%	Rat/1000	%	Rat/1000	%
Intact VP	47·5	100	217·1	100	31·0	100	141·2	100	47·5	100	217·1	100
VP/T 80-E/1	30·1	63	125·2	58	7·7	25	31·8	23	5·7	12	23·9	11
VP/T 80-E/2	9·7	20	85·1	39	1·6	5	14·2	10	0·9	2	7·4	3
VP/T 80-E/3	6·0	13	29·8	14	1·5	5	7·6	5	0·6	1	2·3	1

fluenza virus on erythrocytes represents an important entity in the immune mechanism.

Effects of solubilization and lipid extraction on the immunogenicity of viral protein

Fractionation of envelope components of influenza virus to obtain the antigen responsible for specific viral immunogenicity has been attempted by many laboratories over a number of years. It seems that the viral envelope can easily be disrupted to progressively smaller fragments, haemagglutinating or non-haemagglutinating, but such dissociation, brought about by chemical treatment,

Table 4. *The effects of SDS, SDS–DOC, Triton X-100 or butanol on the affinity for erythrocytes and immunogenicity for ferrets of viral protein from A 2/Singapore/1/57*

	HAU/ml. of ferret inocula with different types of erythrocyte			Reciprocal titres of: HI antibody		Reciprocal titres of: VN antibody	
Test material	Fowl	Human (group O)	Guinea-pig	Serum	Peritoneal effusion	Serum	Peritoneal effusion
Intact VP	98,144	98,144	98,144	960	640	24,000	20,480
				960	480	19,200	15,360
VP/TX-100	1,024	512	768	1,920	1,280	30,720	30,720
				5,120	2,560	61,440	61,440
VP/SDS	< 480*	< 480*	< 480*	< 10	NT	38	20
				10	NT	80	30
VP/SDS-DOC	60	16	16	< 10	NT	50	15
				< 10	NT	< 20	17·5
VP/butanol	< 2	< 2	< 2	< 10	NT	40	15
				15	NT	120	25

NT = Not tested.
* Haemolysis in dilutions lower than 1/480.

has so far resulted in a less immunogenic viral material than the intact virus. Further progress seems to depend on dissociating viral lipoprotein without loss of immunogenicity. Yet with the present background there are no conclusive data on the chemical nature of the viral antigen responsible for evoking virus-neutralizing antibodies against influenza, nor universal agreement as to whether or not the envelope lipid is an integral part of the immunogenic antigen. This section describes a critical study of the effects of three different association colloids, the anionic SDS and DOC and non-ionic Triton X-100, on the immunogenicity for ferrets and haemagglutinating properties of the envelope proteins derived from purified VP suspensions of A 2/Singapore/1/57 and B/England/101/62 virus strains. As part of this study the envelope lipid of the above viruses was extracted with butanol and defatted viral material was examined in parallel with the intact virus.

Triton X-100 treatment has been reported from two independent laboratories to enhance the antigenic activity of an iso-antigenic lipoprotein isolated from Sarcoma I (Kandutsch & Stimpfling, 1962) and the enzyme activity of acetylcholine esterase from erythrocyte membranes (Bonsall & Hunt, 1966). We thought that

the mechanisms postulated for these actions by Triton X-100 might also occur in the solubilization by this surfactant of envelope proteins of influenza virus. This prediction proved correct.

Tables 4 and 5 summarize the haemagglutinating activity and immunogenicity for ferrets of the intact VP and VP preparations treated with Triton X-100, SDS, SDS-DOC or butanol. Under treatment conditions a considerable or complete loss of haemagglutinating activity and immunogenicity occurred with the VP preparations treated with the anionic surfactants or defatted with butanol. In contrast, the treatment with Triton X-100 quantitatively removed the haemagglutinating activity without loss of the immunogenicity. Moreover, as is shown in Table 6, the

Table 5. *The effects of SDS, SDS–DOC, Triton X-100 or butanol on the affinity for erythrocytes and immunogenicity for ferrets of viral protein from B/England/100/62*

	HAU/ml. of ferret inocula with different types of erythrocyte			Reciprocal titres of: HI antibody	VN antibody	
Test material	Fowl	Human (group O)	Guinea-pig	Serum	Serum	Peritoneal effusion
Intact VP	12,288	12,288	8,192	480	3,840	> 480
				160	960	50
VP/TX-100	128	256	128	1,280	4,000	> 480
				240	1,920	> 480
VP/SDS	< 480*	< 480*	< 480*	< 10	20	< 20
				< 10	30	< 20
VP/SDS-DOC	40	64	32	15	112	100
				10	200	160
VP/butanol	20	32	16	< 10	48	< 20
				< 10	60	20

* Haemolysis in dilutions lower than 1:480.

Table 6. *Immunogenicity in ferrets of inocula containing equal amounts of viral material; intact or treated with chemicals as indicated*

		Antibody response shown as average reciprocal titres of serum antibody			
		HI		VN	
Influenza virus	Test material	Titre	%	Titre	%
A 2/Sing/1/57	Intact VP	960	100	21,600	100
	VP/TX-100	3,520	366	46,080	213·3
	VP/SDS	5	0·52	59	0·27
	VP/SDS-DOC	< 10	< 1	25	0·12
	VP/butanol	7·5	0·78	80	0·37
B/Eng/101/62	Intact VP	320	100	2,400	100
	VP/TX-100	760	237·5	2,960	123
	VP/SDS	< 10	< 3	25	1·04
	VP/SDS-DOC	12·5	3·91	156	6·5
	VP/butanol	< 10	< 3	54	2·25

Table 7. *Reciprocal antibody titres per* 1000 *HAU* (*Rat*/1000) *ferret inoculum for intact and Triton X*-100 *treated virus*

			HI antibody response				VN antibody response			
			Serum		Peritoneal effusion		Serum		Peritoneal effusion	
Influenza virus	Test material	HAU/ml. in inoculum	Recip. titre	Rat/1000	Recip. titre	Rat/1000	Recip. titre	Rat/1000	Recip. titre	Rat/1000
A2	Intact VP	98,144	960	9·8	560	5·7	21,600	220	17,920	183
	VP/TX-100	1,024	3,520	3,451	1,920	1,882	46,080	45,176	46,080	43,176
B	Intact VP	12,288	320	26	NE	NE	2,400	195	NE	NE
	VP/TX-100	128	760	5,938	NE	NE	2,960	23,195	NE	NE

NE = no end-point obtained (see Table 5).

immunogenicity of viral protein of both virus strains was enhanced, this enhancement being of the order of 237·5–366 % for HI antibodies and 123–213·3 % for VN antibodies. Table 7 summarizes the haemagglutinating activity and immunogenicity of the viral protein before and after Triton X-100 treatment, demonstrating that immunogenicity persists in viral protein which has lost 99 % of its haemagglutinating activity.

DISCUSSION

Our experimental work on antigens of influenza virus has consisted of (1) an attempt to correlate the haemagglutinating power of these antigens with their ability to excite the formation of humoral antibodies, and (2) investigations on the solubilization and defatting of antigenic matter by surfactants and lipid solvents.

The principle underlying the correlation referred to under heading (1) is straightforward. Influenza virus has two distinguishable effects; *in vitro* it agglutinates some species of erythrocytes and *in vivo* it provokes the formation of humoral antibodies, haemagglutination-inhibiting and virus neutralizing. Both humoral antibodies and resistance to infection of the individual occur concomitantly, and the temptation is to believe therefore that immunogenesis is associated with the haemagglutinating power of the antigen, or, as Neurath *et al.* (1967) have suggested, that the integrity of the whole or the 70 S subunit is required for quantitative maintenance of the immunogenic properties of a viral preparation. If either of these interpretations is true, then the haemagglutinating powers of antigenic matter should be closely correlated with its ability to excite the formation of humoral antibodies in animals.

Our experiments described in this present paper failed to uphold this interpretation. Their failure must be attributed to the fact that the term haemagglutinin, 'divalent' or 'monovalent' (Choppin & Stoeckenius, 1964) merely indicates that a particulate complex adsorbs to and agglutinates some species of erythrocyte or adsorbs to erythrocytes without agglutination. Furthermore, the state of 'divalency' or 'monovalency' cannot be expressed in terms of particle size; it may be a reflexion of a preponderance of 'sites' in the particle which are capable of combining with 'receptors' of some species of erythrocytes (Choppin & Stoeckenius, 1964). As is seen in Table 2, none of the high titred haemagglutinins obtained by T 80-E treatment of VP were as immunogenically efficient as the intact VP. A most interesting result of our experiments seen in Table 2 was evidence that antigen VP/T 80-E/3, which contained considerably more 'divalent' haemagglutinin than the intact virus, was yet deficient in the ability to excite the formation of HI and VN antibodies. The most plausible explanation of this fact is that the combining sites necessary for the adsorption to and agglutination of erythrocytes are more resistant to the T 80-E treatment than the lipoprotein assemblage which determines the modality of the antibody response. On the other hand, it can be seen from Tables 4 to 7 that the Triton X-100 treatment has removed 99 % of the haemagglutinating activity of the lipoprotein assemblage without any loss in immunogenicity. Clearly, the immunogenicity and haemagglutinating activity of the lipoprotein assemblage are separable, i.e. immunogenicity persists in viral material

that has lost most (99 %) of its haemagglutinating activity. These results suggest that the correlation assumed to exist between the haemagglutinating activity and immunogenicity of influenza virus is coincidental.

Another point of biological interest that has emerged from this present study is that solubilization of viral protein by surface-active agents is obviously important, not only as a prerequisite to some form of physical or chemical analysis, but also because the immunogenicity of the solubilized viral protein may be expected to vary greatly owing to action of surface-active agents other than solubilization.

In the case of solubilization by Tween 80–ether and anionic surfactants, SDS and DOC, the specific immunogenicity of viral protein was lost, partially or completely (Tables 2, 4, 5, 6). In contrast, the immunogenicity of viral protein solubilized by Triton X-100 was greater than that of the intact virus (Tables 4–7). Although much more work is required to elucidate the 'enhancing' mode of action of surface-active agents like Triton X-100, it would appear from our results that this goal of both theoretical and practical importance is feasible.

Our attempts to extract viral lipid by 1-butanol at 4° C. led to loss of immunogenicity as is shown in Tables 4–6. This finding suggests that the lipid may be an integral part of the envelope lipoprotein assemblage responsible for the formation of VN antibody in the recipient subject. If this is the case, then it may be that our Triton X-100 extracts contained an active water-soluble lipoprotein of the viral envelope.

The methods described in this present paper for the isolation of immunogenically active viral protein were not perfect, nor were the investigations extensive. However, they already demonstrate their usefulness in the following ways:

First, they demonstrated the coincidental nature of the correlation assumed to exist between the haemagglutinating activity and the immunogenicity of influenza virus. Therefore, solubilization and fractionation of viral protein for new improved vaccines should be monitored for specific immunogenicity rather than for HA activity.

Secondly, they showed the variability of the effects other than solubilization by surface-active agents, thus providing some basis for design of methods to produce immunogenic preparations of viral protein.

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A preliminary survey of the epidemiology of bluetongue in Kenya

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SUMMARY

A survey of the species composition and distribution of the *Culicoides* midge populations at a range of sites where bluetongue is enzootic isolated a group of dominant species: *C. cornutus*, *C. grahamii*, *C. magnus*, *C. milnei*, *C. pallidipennis* and *C.* 23.† Monthly light-trap sampling of *Culicoides* showed that the population densities of the dominant species greatly increased after the rain seasons and that these species concentrated around flocks of sheep and cattle. The larval habitats of *C. cornutus* and *C. pallidipennis* were found associated with stock pens. Precipitin tests on blood-fed *Culicoides* showed that most of the dominant species regularly feed on sheep and cattle. Bluetongue virus was isolated from *C. milnei*, *C. pallidipennis* and *C.* 23. Serological surveys of wild and domestic bovids from the enzootic area showed a high proportion with antibody to bluetongue virus. The colonization of *C. cornutus*, a potential vector, is described briefly. A causal relationship between peak rainfall in April–May, peak numbers of *Culicoides* in May–June and peak bluetongue incidence in June–July is postulated. The vector status of the above species and *C. austeni* was evaluated.

INTRODUCTION

In Kenya sporadic outbreaks of bluetongue (B.T.) have been reported in sheep populations since 1909. Control measures are taken by farmers who annually vaccinate their flocks with the attenuated live virus vaccine which contains all those strains so far isolated in Kenya. The efficiency of the vaccine could be limited by the isolation of new virus strains which have to be attenuated before incorporation in the vaccine. Work by Foster, Jones & Luedke (1968) in the U.S.A. has shown that the attenuated virus, on passage through *C. variipennis*, increases in virulence to reproduce clinical B.T. when the midges feed on susceptible sheep. Indiscriminate use of the vaccine in areas where B.T. is not enzootic could spread the disease.

C. variipennis has been incriminated as the vector of B.T. in the U.S.A. (Price & Hardy, 1954; Foster, Jones & McCrory, 1963) and *C. pallidipennis* has been in-

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† A new species, *C. tororoensis*, description not yet published (see Khamala, 1968).

criminated as the vector in S. Africa (Du Toit, 1944, 1962). In both these countries B.T. is characterized by its seasonal incidence, which is associated with seasonal increases in the numbers of the vector populations (Bowne, Luedke, Jochim & Foster, 1964). On the assumption that *Culicoides* are likely to be the vectors in Kenya this study was directed at isolation of the potential vectors and natural virus reservoirs and the study of the ecology and colonization potential of selected *Culicoides* species.

MATERIALS AND METHODS

Sampling and sampling sites

Adult *Culicoides* were sampled with portable light-traps. These were copies of a modified version of the Communicable Diseases Centre mosquito trap that was supplied by A. L. Dyce. *Culicoides* were attracted to the light of a 6 V., 18 W. tungsten filament bulb at the top of the trap and then sucked through the body of the trap and into a collecting jar by a fan driven by a small 6 V. motor. Each trap was powered by a 6 V. car battery and could run for 18 hr. on one battery charge. Samples were caught in alcohol in a plastic jar or, if live samples were required, in a cubical wire-frame cage, 15 cm. dimension and covered with 50 mesh/in. nylon gauze and one clear plastic window. Both jar and cage were connected to the trap by gauze cones. The traps were suspended at 1 m. high from trees, fence wires, etc., or from a tripod of wood poles.

Experiments were carried out to determine the limitations of the traps and the best operating procedure to give standardized results. Simultaneous sampling with light-traps and a Johnson–Taylor suction trap indicated that the light-traps gave a sample of a mixed population of flying *Culicoides* that was not significantly different from the unbiased population samples obtained with the suction trap.

The effective range of the light traps for *Culicoides* was found to be 20–25 m. by performing simple choice-chamber experiments with *C. cornutus* and *C. milnei*. The traps were thus always set up at least 25 m. apart, so that the area over which a trap was independently effective was always greater than the area where it interacted with a trap close to it. Wind speeds greater than 2 m./sec. (4·5 miles/hr.) were found to reduce the flight activity of *Culicoides*, thus sampling-time in which wind speeds were greater than this was not included in the standard period of regular samplings. Strongly moonlit nights were avoided. A study of the nocturnal activity patterns of five of the common species at the Naivasha site showed that they all had a late evening peak. The period from 7 p.m. to 11 p.m. was convenient for the standardized sampling, when meteorological observations had to be made at 20 min. intervals.

To trap samples for the studies of distribution, virus isolation and blood feeding, the traps were set up at sites where large catches were expected – that is, sheltered low-lying wet areas, particularly near stock pens, and were left to run all night. Live catches were recovered soon after dawn, before strong sunlight could affect the catches, and then sorted and processed in the laboratory as soon as possible. To monitor the seasonal population fluctuations and dispersal of *Culicoides* at the fixed sites the traps were set up at least once a month in constant line transects

relative to sheep flocks or larval habitats and operated until a standard total of 20 trapping hours per month was obtained. The location of the regular sampling sites and the sites covered during the distribution survey is shown in Fig. 2.

Potential larval habitats were sampled and larvae and pupae were extracted by washing the samples with water in 100 mesh/in. sieves and then floating them from the clean filtrate with a saturated solution of magnesium sulphate. The immature stages were identified by rearing them through to adults in small emergence traps.

Male and female adult *Culicoides* were identified according to Khamala's treatise (1968).

Precipitin test

Antiovine and antibovine precipitating antisera were prepared in rabbits by the method described by Weitz (1956), and were stored without preservative at $-10°$ C. in 2·5 ml. batches. Light-trap catches of *Culicoides* were searched for specimens that appeared to be engorged; these were aspirated out from the cages, anaesthetized with CO_2, identified and stored in individual tubes at 4° C. Twelve hours before testing individual specimens were placed in separate solid watch-glasses, their abdomens were dissected off and crushed in 0·5 ml. isotonic saline (9 g./l.), where they were left overnight at 4° C. Portions of the blood meal extract were drawn up into 70 × 1 mm. thin-walled capillary tubes and an equal volume of antiserum was drawn up under the blood extract. An air bubble was drawn into the capillary tube, which was then sealed by pushing it into a strip of plasticine mounted on a ruler. The separate capillary tests could be identified by their position on the ruler. After incubation at 35° C. for 2 hr. precipitin rings were studied on the black stage of a binocular microscope with a narrow beam of light shining along the length of the capillary tube. The precipitate, if present, was easily recognized. Each extract was tested against antibovine and antiovine sera. Controls were set up against the homologous and heterologous normal serum at 1/200 dilution and against isotonic saline.

There were slight qualitative differences between the reactions of ovine and bovine normal sera to any one of the antisera; since blood-fed specimens could be selected from sites containing exclusively sheep or cattle the test could effectively be used to determine which species of *Culicoides* feed on sheep or cattle or both.

Virus isolation

Culicoides samples were taken at monthly intervals at Sukari cattle ranch (site 8, see Fig. 2 and Table 1) from the area around a milking shed, and excess colonization material of *C. cornutus* adults from Braemar (site 9) were added to the samples. The samples were transferred to incubation cages; these were of cardboard, 25 × 18 × 15 cm., with a window and with ventilation holes covered by nylon gauze. Droplets of sugar solution and water were suspended from the gauze to sustain the midges and they were kept at about 21° C. 77 % R.H. for 2 days, exclusive of day of capture, to allow for the digestion of any recent blood meals. Batches were then

removed from the cage, anaesthetized with CO_2 and sorted into species by means of a fine aspirator. Each species was placed in a separate bottle and stored at 4° C. Within 2 days the pools of between 20 and 200 midges were ground up in sterile sand with pestle and mortar in a concentrated antibiotic solution containing 500 I.U. penicillin, 500 μg. streptomycin and 25 units mycostatin (Squibb) per ml. The emulsion was then incubated at 37° C. for 2 hr. before centrifugation at 2000 rev./min. for 10 min. Phosphate-buffered saline was added to the supernatant to give a final concentration of 100 I.U. penicillin per ml. This suspension was used as inoculum for eggs by the i.v. route or for tissue culture (BHK cells, 21 c13). These were maintained with Eagle's medium for BHK cells, with 5 % horse serum, 2·5 % tryptose phosphate broth and 25 % sodium bicarbonate (4·4 % soln.). Egg harvest material was inoculated into BHK cells. Any cytopathic agent was identified by the group-specific fluorescent antibody test (F.A.T.) (Pini, Coakley & Ohder, 1966) on flying coverslips using a direct method with conjugates prepared from anti-bluetongue and anti-Nairobi sheep disease sera.

Typing of B.T. isolates was carried out by a plaque inhibition technique (F. G. Davies, in preparation) using L cells (mouse fibroblasts) and antisera specific for the types of B.T.V. (bluetongue virus) so far isolated in Kenya. All sera were examined by an indirect F.A.T. (Pini, Ohder, Whiteland & Lund, 1968) using an anti-bovine conjugate prepared in chickens. Typing was by plaque inhibition test.

RESULTS

Bluetongue incidence

B.T. outbreaks are recorded at the Kenya Veterinary Research Laboratory but it is known that a number of cases are not reported, thus the figures are below actual incidence. The histogram of B.T. outbreaks from 1964 to 1968 (Fig. 1) shows that there is a peak during June and July and that outbreaks have occurred in every month except January. While it can be said that there is a B.T. season in Kenya it is not as clear cut as that in the U.S.A. or in S. Africa.

Postulated B.T. *enzootic area*

Fig. 2 shows the area in Kenya where B.T. is considered likely to be enzootic. It is here defined as the area enclosed by the combined boundaries of three related areas:

(1) The 30–40 in. mean annual rainfall limit (Atlas of Kenya).

(2) The limit of the contiguous ecological zones as defined by Pratt, Greenway & Gwynne (1966); zone II – the forest and grasslands at 5000–7000 ft. and the montane *Acacia* woodland; zone III – the evergreen and semi-evergreen bushland and *Combretum* woodland and savanna; zone IV – the dry transitional *Combretum* savanna and upland *Acacia* woodland.

(3) The limit of the main cattle and sheep farming areas.

The salients to the north are probably just quirks but all recorded enzootic sites have been marked on the map and it can be seen that they all lie within the postulated area.

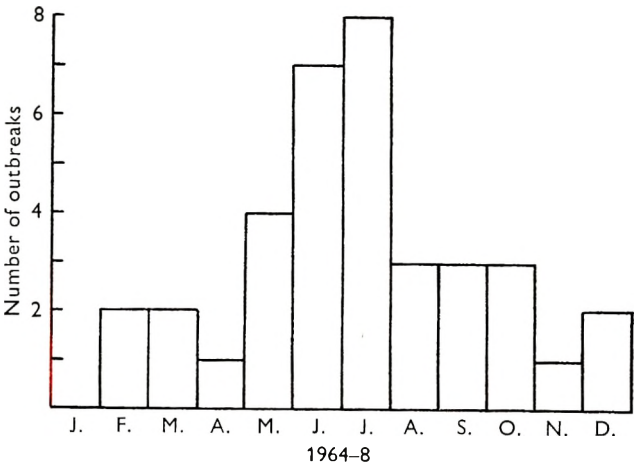

Fig. 1. Bluetongue incidence in Kenya.

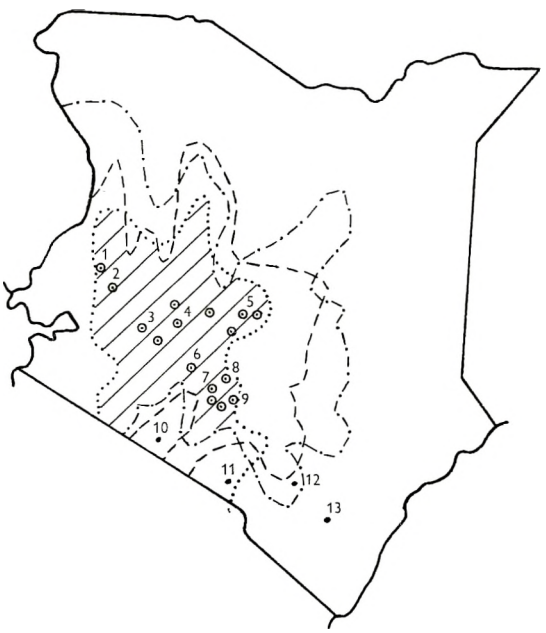

Fig. 2. Postulated enzootic area. —·—, 30–40 in. mean annual rainfall. — —, Ecological zones II, II, IV. ....., sheep- and cattle-rearing area. ○, Recorded enzootic site; sampling sites numbered.

Rainfall

The rainfall data of Figs. 3 and 4 were obtained from the Kenya Meteorological Department for rain-gauges that are maintained within a few miles of the sampling sites. The typical rainfall pattern in Kenya has two distinct and regular peaks: the first, the 'long rains', begin about March and last till May; the second, the less heavy 'short rains', begin in October and are usually over by December. This pattern occurs, with small variations, throughout the postulated enzootic area.

However, the rainfall during 1968–9 was exceptional throughout Kenya. At Naivasha (Fig. 3) both the long and short rains were very heavy and long-lasting, and at Sukari (Fig. 4), although the short rains about November 1968 were normal, the long rains of 1969 virtually failed.

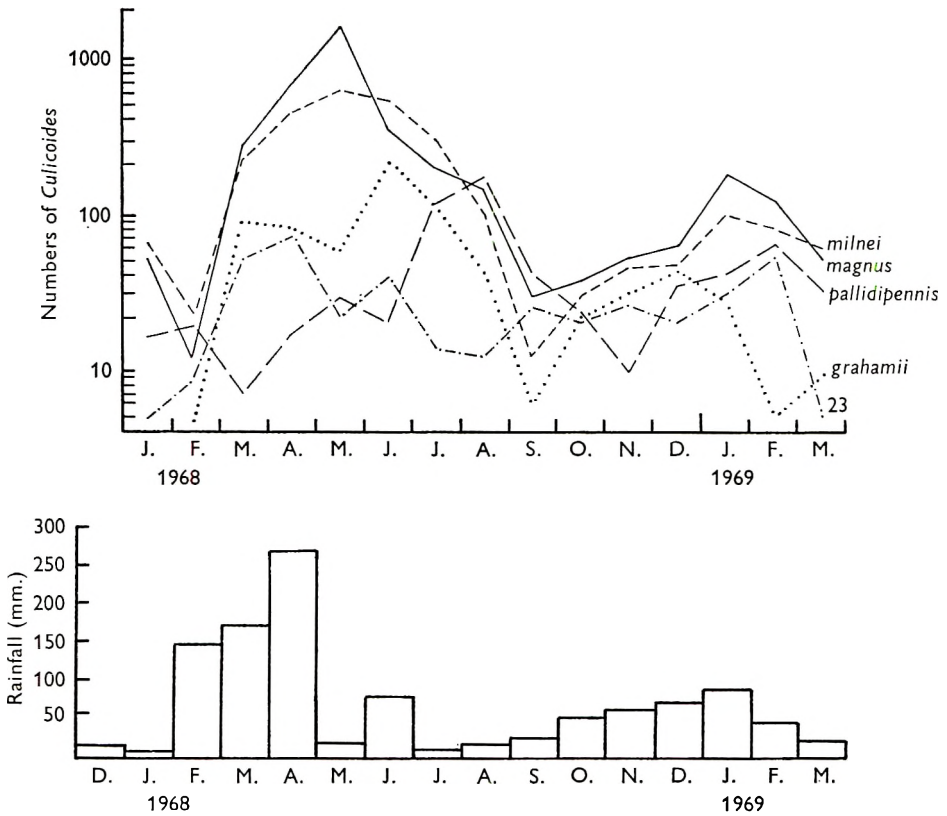

Fig. 3. Population curves of *Culicoides* – Naivasha. Rainfall pattern – Naivasha.

Distribution of Culicoides *in the enzootic area*

Table 1 shows *C. cornutus*, *C. grahamii*, *C. magnus*, *C. milnei*, *C. pallidipennis* and *C.* 23 as the dominant species within the enzootic area. The last two species, although never occurring in large numbers, were widespread and *C. cornutus*, equally widespread, was only found in especially dense localized pockets near stock pens. *C. austeni* is very similar to and closely related to *C. milnei* and it appeared to replace *C. milnei* at the far south-east margin of the enzootic area. The species composition of catches from different sites within the enzootic area were similar, but the *Culicoides* fauna from outside shows little similarity to that within the area.

There was always a preponderance of females caught in the light-traps – from 84 % to 100 %, depending on species and place. However, when immature stages were reared to adults there was always an approximate 1:1 male:female ratio; there was no reason to believe that the natural sex ratios of any of the species dealt with was other than normal.

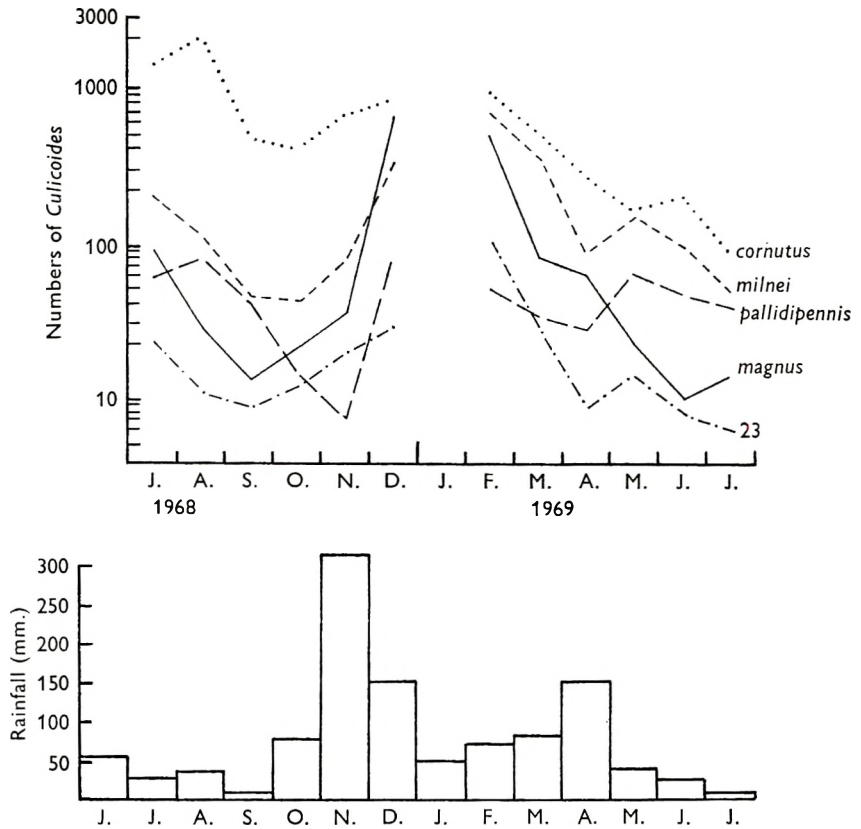

Fig. 4. Population curves of *Culicoides* – Sukari (plus *C. cornutus* from Braemar). Rainfall pattern – Sukari.

Population fluctuations

Fig. 3 shows the results of 15 months sampling at Naivasha, Ol Mogogo Veterinary Department farm (site 6), and Fig. 4 shows the results of 13 months sampling at Nairobi, Sukari Estate (site 8). In Fig. 4 is included the population curve for *C. cornutus* which was sampled simultaneously at Braemar Estate, Nairobi (site 9); no sampling could be done in January 1969. The populations of six species fluctuated considerably and a general pattern is evident in both figures if the curves of Fig. 4 are extrapolated to cover January. Reference to the rainfall histograms shows that a peak in rainfall preceded each total *Culicoides* population peak by 1–2 months and that the greater the rainfall the greater was the population peak. *C. magnus* and *C. milnei* had the most prominent and consistent pattern and together with *C. cornutus* showed the greatest periodic increase in numbers. *C. grahamii*, *C. pallidipennis* and *C.* 23 had more erratic fluctuations but still fit into the overall pattern. None of the rarer species recorded at these sites showed population fluctuations with any consistent or meaningful pattern.

The apparent positive correlation between population expansion and prior heavy rainfall is borne out by the curves for Sukari and Braemar from February 1969 onwards. Although there were rainfall peaks in April and May the amount

Table 1. *Distribution of* Culicoides *species in Kenya*

	Sampling sites*												
	Within enzootic area									Outside enzootic area			
Species	1	2	3	4	5	6	7	8	9	10	11	12	13
austeni	—	—	—	—	—	—	—	—	2	—	4	2	2
bedfordi	—	—	—	—	—	1	—	—	—	—	—	2	1
cornutus	4	2	1	—	—	—	—	—	6	—	—	—	—
fuscicaudae	—	—	—	—	—	< 1	—	—	—	—	—	—	—
grahamii	—	1	—	1	1	1	2	1	1	—	—	1	2
kibatiensis	1	—	—	—	—	1	—	—	—	—	—	—	—
magnus	1	2	3	6	4	5	3	4	1	—	—	—	—
milnei	3	5	6	3	4	3	3	4	—	—	—	—	—
naevii	1	1	—	—	—	—	—	—	—	—	—	—	—
nivosus	—	—	—	—	—	—	—	1	—	—	—	—	—
pallidipennis	2	1	1	2	2	1	3	2	2	—	—	3	2
praetermissus	—	—	—	—	—	< 1	—	—	< 1	—	—	—	—
pycnostictus	—	—	—	—	—	< 1	—	< 1	< 1	—	—	—	—
schultzei	—	—	—	—	—	—	—	—	—	10	6	5	5
23	1	1	—	1	—	1	1	1	1	—	—	—	—

The distribution of each species is shown as the relative density of the species at each site expressed as coded percentages of the total catch: < 1 = < 1 %; 1 = 1–10 %; 2 = 11–20 %; ...up to 10 = 91–100 %.

* Sampling sites: see Fig. 2 for location. 1, Kitale; 2, Eldoret; 3, Molo; 4, Thomson's Falls; 5, Nanyuki; 6, Naivasha; 7, Nairobi, Kabete; 8, Nairobi, Sukari Estate; 9, Nairobi, Braemar Estate; 10, Magadi; 11, Amboseli; 12, Kiboko; 13, Tsavo East.

that fell was appreciably less than that falling in the short rains in November 1968 and there was almost a drought. Apart from minor fluctuations the populations of all species declined in numbers during this period.

The monthly data from Naivasha were obtained by operating the traps in a line transect with traps at 5, 25, 50, 100 and 200 m. from about 450 sheep that were always flocked at the same spot. The totals for each species for the entire sampling period for each transect trap, when plotted as numbers against distance from sheep, give a series of lines representing the distribution of each species relative to the sheep flock. In terms of total numbers caught the species fell into two distinct groups. The group of common species, *C. grahamii*, *C. magnus*, *C. milnei*, *C. pallidipennis* and *C.* 23, all showed a regression of numbers on distance from sheep and the common regression coefficient calculated as the sum of all species (logarithmically transformed to reduce variance) at each trap upon distance gave $b = -0{\cdot}059$, which was highly significant ($P = 0{\cdot}01$–$0{\cdot}001$). Thus from the aspect of populations distributed in a measured area, they concentrated around the sheep. In contrast, the group of rarer species, *C. bedfordi*, *C. fuscicaudae*, *C. kibatiensis*, and *C. pycnostictus*, treated in the same way showed a common regression that was hardly significant ($b = 0{\cdot}017$, $P = 0{\cdot}1$–$0{\cdot}05$); there was no evidence of the concentrating around the sheep. Although the larval habitats of most of the species dealt with were not discovered there was no evidence of any *Culicoides* breeding in high densities within the transect area.

Dispersal from larval habitat

At the Braemer site localized larval habitats of *C. cornutus* and *C. pallidipennis* were found in the mixture of mud and dung surrounding a cattle milking stall. The dispersal of these species was studied by setting up a line transect of six light-traps at 100 m. intervals from the stall on two consecutive calm nights. No other larval habitats were found in the transect area. *C. cornutus* was recovered up to 300 m. from the larval habitat and *C. pallidipennis* at up to 500 m. Although the larval habitats of *C. magnus*, *C. austeni* and *C.* 23 were not definitely established it is probable that they were also near the cattle stall and these species were also recovered at between 300 and 500 m. from the stall.

The dispersal coefficient, i.e. the distance at which the numbers caught fell to one-tenth of their value at the larval habitat and calculated as the reciprocal of the regression coefficient of numbers on distance (Kettle, 1951), for these species was: *C. cornutus* 89·2 m., *C. austeni* 133·3 m., *C. pallidipennis* 264·5 m., *C. magnus* 291·1 m., *C.* 23 420·2 m. These figures are an indication of the distance that each species will actively disperse relative to the others. In view of the wide distribution of B.T. antibodies in wild bovids (see below) and of B.T. enzootic sites it is likely that the vectors of B.T. will have high dispersive capabilities, although it must be remembered that *Culicoides* could be dispersed passively by wind for greater distances. Notably, *C. cornutus*, which was found to be the most localized in distribution, had the lowest dispersal coefficient.

Larval habitats

Although an extensive survey of potential larval habitats was carried out following the rainy seasons of 1968 when high larval densities were expected, the larval habitats of only two of the dominant species, *C. cornutus* and *C. pallidipennis*, were definitely established. The larvae of both species were found regularly and in high concentration in the mixture of fine mud and dung surrounding cattle pens and also in associated effluent ditches. Both *C. milnei* and *C. magnus* larvae were found in a variety of habitats characterized as fine mud with high organic detritus content at the margins of ditches and pools, but these are probably only secondary habitats. No larvae of any species were found in sites that were not at least semi-liquid mud or those that were covered by free water deeper than 1–2 cm.

Blood feeding

Table 2 shows that all of the six species found to predominate numerically and also *C. austeni* will feed on sheep and that all except *C.* 23 feed on cattle. Within the sampling areas these species feed predominantly on sheep and cattle, or possibly in a proportion of cases on closely related wild bovids. No other species were ever found engorged with blood.

Table 2. *Precipitin testing of blood from engorged* Culicoides

Culicoides species	No. tested	No. positive for		No. negative	Approximate % engorged in samples
		Sheep	Cattle		
austeni	7	3	2	2	0·77
cornutus	18	7	10	1	1·8
grahamii	12	7	2	3	2·4
magnus	14	8	5	1	0·93
milnei	43	15	20	8	1·43
pallidipennis	17	6	6	5	1·7
23	7	1	0	6	0·87

It is evident that the proportion of engorged *Culicoides* caught was very low. This is often the case with *Culicoides* (see Kettle, 1969) and could be due to differential responsiveness of engorged and non-engorged *Culicoides* to the light-traps, but tests with captive *C. cornutus* and *C. milnei* indicated that whether engorged or not they were equally susceptible to the traps when actively flying. However, it is unlikely that a midge will disperse far immediately after taking a large blood meal and this will reduce its chances of capture. A consideration of the number and length of ovarian cycles typical of *Culicoides*, each requiring only one blood meal, and the possibility that a proportion of the populations of some of the species are autogenous, leads one to expect that no more than 10% of the female population will be blood-feeding at any one time.

Experiments with captive midges kept in standardized conditions in small holding cages and given blood meals from rabbits showed that individuals of *C. cornutus* (8), *C. milnei* (6), *C. magnus* (5), *C. pallidipennis* (3), and *C.* 23 (4) will

all take at least two blood meals separated by an ovarian cycle lasting the number of days shown in parentheses. No second ovarian cycles or third blood meals were recorded. None of the species seemed to be host-specific; they fed with equal avidity on rabbit, guinea-pig and human hosts.

Virus isolation

The results of virus isolation attempts from the main species at the sampling sites in the Nairobi area are shown in Table 3. The isolation of virus from a *C. pallidipennis* pool in August 1968 can be correlated with a population peak as seen

Table 3. *Virus isolation from* Culicoides

Culicoides species	1968					1969					
	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.
cornutus	—	—	—	—	—	—	—	—	—	—	—
grahamii	—	—	—	—	—	—	—	—	—	—	—
magnus	—	—	—	—	—	—	—	—	—	—	—
milnei	—	—	—	—	—	—	BT 1	—	—	—	—
pallidipennis	BT 4	—	BT 1	—	—	—	BT 1	—	—	—	—
23	—	—	—	NSD	—	—	BT 1	—	—	—	—

Positives are shown as BT, together with the code number of the Howell immunological type, and NSD for Nairobi sheep disease.

Table 4. *Bluetongue antibody tests on wild Bovidae*

Species	No. sera tested	Positive	%
Kongoni, *Alcelaphus buselaphus*	66	57	86
Wildebeeste, *Connochaetes taurinus*	134	81	60
Impala, *Aepyceros melampus*	36	21	58
Eland, *Taurotragus oryx*	20	8	40
Buffalo, *Syncerus caffer*	6	4	67
Topi, *Damaliscus korrigum*	38	11	29
Waterbuck, *Kobus ellipsiprymnus*	3	0	0
Reedbuck, *Redunca fulvorufula*	2	2	100
Bushbuck, *Tragelaphus scriptus*	4	0	0
Thomson's gazelle, *Gazella thomsonii thomsonii*	60	5	8
Domestic cattle	86	46	53

in Fig. 3, and similarly the isolation of virus from *C. pallidipennis*, *C. milnei* and *C.* 23 in February 1969 can be correlated with the population peak of January and February. The isolation of Nairobi sheep disease virus from a *C.* 23 pool is an enigma that prompts further study of *Culicoides* as possible vectors of this disease.

Table 4 shows that a high percentage of wild bovids in the Rift Valley (which runs through the middle of the enzootic area) contain antibodies to B.T.V. and have been infected with the virus, presumably from bites of *Culicoides* vectors. A sample of domestic bovids from the enzootic area showed 53 % with antibody to B.T.V.

Colonization

A colony of *C. cornutus* was established in the laboratory. At least 500 wild caught specimens were placed in conical plastic containers of 20 cm. basal diameter, 8 cm. top diameter and 26 cm. high. The cages were kept at 22° C., 75 % R.H. and the adults were fed on sugar solution and given blood meals on a rabbit held in a special stock above the apical opening in the cage. Large numbers of eggs were laid on moist blotting-paper pads in the cage and these were placed direct into a larval substrate in shallow galvanized iron pans. The substrate was a simulation of the natural habitat but never proved very successful; larval mortality usually exceeded 90 %. It is likely that a microbial broth substrate as described by Jones (1969) would be better. Pupae were produced within 22 days and were extracted from the pans by water flotation. Pupae were placed direct on moist cotton pads and hatched into clean cages. Sufficient 'clean' midges could be obtained for use in transmission experiments; mean adult longevity was 10 days. An improvement of the larval substrate would probably result in a fully self-maintaining colony.

DISCUSSION

A fairly definite pattern of B.T. epidemiology emerges from the results. B.T. is confined in Kenya to one area with specific ecological characteristics. Within this area there are six species of *Culicoides* with a distinct numerical dominance and the species composition of populations from different sites is very similar. Rainfall is the major extrinsic factor affecting the populations of these dominant species. Peak rainfall in April–May facilitates high larval survival and consequent rapid expansion of adult numbers that reaches a peak in May, June and July. During the latter half of this peak there is, significantly, a peak of B.T. outbreaks.

An evaluation of the vector potential of the species studied against the criteria of distribution and time of response to rainfall and also of the nature of larval habitats, distribution about sheep, dispersive powers, blood-feeding habits and B.T.V. content, isolates two distinct groups. In order of decreasing vector potential, *C. milnei*, *C. cornutus*, *C. pallidipennis* and *C. magnus* fall into a group of high potential and *C.* 23, *C. grahamii* and *C. austeni* fall into a group of lower potential.

The number of virus isolations was insufficient to answer many of the questions posed by the study of the epidemiology of B.T. The identification of three of the seven potential vectors as being capable of harbouring B.T.V. is important. The persistence of *Culicoides*, albeit in small numbers, for the whole of the year has led the authors to suspect that in Kenya B.T.V. persists by a repeating midge to cattle or game cycle, with only a silent infection in these hosts. We have no evidence of clinical B.T. in cattle or game in Kenya, but when there is a rise in the total *Culicoides* population after the rains cattle and game probably act as amplifying hosts and after a lag phase increase the proportion of infected *Culicoides* to a stage when the relatively unattractive disease host (Du Toit 1962) is involved in the feeding pattern to a much larger extent. There is some evidence that a constant supply of susceptible amplifying hosts may not be necessary for the propagation of B.T.V.;

recurrent viraemia with the same and different virus strains have been observed in cattle (Owen, Du Toit & Howell, 1965). This might reasonably be expected to occur in the wild bovids. In South Africa and America it is probable that a distinct interepizootic period occurs when *Culicoides* are not present or do not contain the virus, and B.T. is not seen.

The game sera which were taken within the enzootic area gave a high proportion with antibody to B.T. Kongoni, Impala and Wildebeeste appear to be widely exposed to infection, presumably from the bites of *Culicoides*. They are amongst the commonest game in the enzootic area and may be the natural hosts for B.T. in Kenya together with domestic cattle. A better assessment of the role they play must await studies of the duration of viraemia that can be caused by B.T.V. in game animals.

It is felt that further work should be directed towards the colonization of potential vector species, and to transmission experiments to examine further the possibility that vaccine virus reverts to virulence after passage through *Culicoides*. A more detailed study of the ecology of the potential vector species with emphasis on blood-feeding capabilities will help to isolate the definite vectors. The role of wild game and rodents as reservoirs of B.T.V. must be examined and their significance in relation to the role of domestic cattle evaluated.

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The antimicrobial activity of cationic proteins isolated from the cells in bulk milk samples

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SUMMARY

Cationic proteins isolated from the cells in bulk milk samples were shown to inhibit the growth of two pathogenic strains of staphylococci and also *Streptococcus agalactiae* S13. Polyacrylamide gel disk electrophoresis studies on these proteins revealed the presence of at least 9 components some of which had isoelectric pH's between 7·0 and 9·0. Trace amounts of the isolated protein had isoelectric pH's greater than 9·0. Staphylococci incubated with milk-cell cationic proteins absorbed the protein, thereby allowing the organism to be stained with the anionic dye Fast Green FCF. Protein-treated staphylococci in isotonic solutions autoagglutinated. This autoagglutination was more marked in hypo- and hypertonic solutions. Lysozyme was not demonstrated in the isolated protein fractions in assays involving incubation with *Micrococcus lysodeikticus* for 90 min. The antimicrobial activity of the cationic proteins isolated from the bulk milk samples was not destroyed after heating to temperatures up to 70° C for 30 min., whereas at higher temperatures the activity diminished and was almost completely lost at 100° C.

INTRODUCTION

The investigations of many workers suggest that the cells present in the milk of dairy cows may protect the mammary gland from invading micro-organisms (Derbyshire, 1964; Jain & Jasper, 1967; Blobel & Katsube, 1964; Katsube & Blobel, 1964). The part played by the cells in normal milk in this protective role still remains in some doubt. Nevertheless it is conceivable that the neutrophils, which may constitute 40–50 % of the total cell count (Blackburn, Laing & Malcolm, 1955; Dilbat, 1963) could phagocytose bacteria or other debris as demonstrated by Schalm, Lasmanis & Carroll (1964*a*, *b*) in cows suffering from mastitis. The intracellular mechanisms responsible for the killing of the phagocytosed micro-organisms remain incompletely understood. For example, it is difficult to explain why only some of the engulfed organisms are killed. Katsube & Blobel (1964) showed in *in vitro* phagocytosis experiments that milk leucocytes killed coagulase-negative staphylococci, *Streptococcus agalactiae* and *E. coli*, but had little effect on the coagulase-positive staphylococci and *Aerobacter aerogenes*.

In previous studies (Hibbitt & Cole, 1968; Hibbitt, Cole & Reiter, 1969) basic proteins isolated from the teat canal of cows were shown to have a bactericidal effect on two strains of *Staphylococcus aureus* and one strain of *Streptococcus*

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agalactiae. The interest was therefore to study the basic proteins isolated from the cells present in bulk milk samples, paying particular attention to their potential bactericidal activity and the ways in which they affect micro-organisms invading the mammary gland.

MATERIALS AND METHODS

Milk cells

Milk cells were obtained from a commercial dairy. The cells were removed from the bowl of a cream separator at the end of the period of operation and transported immediately on ice to the laboratory for examination and extraction.

Extraction of cationic proteins from milk cells

The cells were washed twice by resuspending in 8 volumes of isotonic saline then centrifuging at 1000 *g* for 15 min. The cationic proteins were extracted from the cells by the same method as that employed for teat-canal keratin (Hibbitt *et al.* 1969) excepting that an extra step was included to reduce the level of casein. The casein was precipitated from the cationic protein extract immediately after dialysis against distilled water by adjusting the pH to 4·6 with 0·1 N-HCl. After standing at 5° C. for 30 min. the casein was sedimented by centrifuging at 1500 *g* for 10 min., the pellet was discarded and the HCl removed from the supernatant by dialysing again for 6 hr. against double glass distilled water before freeze-drying.

Assay of cationic proteins

Antimicrobial activity of the extracted proteins was assayed against *Staph. aureus* 305, *Staph. aureus* Mexi and *Strep. agalactiae* S13 as described previously (Hibbitt, Cole & Reiter, 1969).

Disk electrophoresis

The cationic proteins from milk cells were separated by electrophoresis at pH 3·0, 7·0 and 9·0 in polyacrylamide gels. The separation at pH 3·0 was the same as that described previously for teat-canal proteins (Hibbitt *et al.* 1969). The gels were buffered at pH 7·0 and 9·0 with 0·1 M-KH_2PO_4-NaOH and 0·04 M sodium barbitone-HCl respectively.

Heat-treatment of proteins

Samples of approximately 2·0 mg cationic proteins dissolved in 2 ml. of 0·01 M citric acid NaH_2PO_4 buffer (pH 7·0) were placed in 5 ml. screw-cap glass bottles. The samples were heated for 10 and 30 min. by total immersion in a water bath at temperatures ranging from 50–100° C. in 10° C. stages. The bottles were plunged into ice-cold water for 5 min. at the end of the heating period and the antimicrobial activity of the proteins was determined.

Fast Green FCF staining

Staphylococci incubated for 2 hr. at 37° C. in 0·15 M-NaCl containing dissolved cationic proteins and in 0·15 M-NaCl alone were sedimented by centrifuging at 1500 *g* for 15 min. The pellet was stained for 60 min. by resuspending in a freshly prepared 0·1 % solution of Fast Green FCF in 0·15 M-NaCl adjusted to pH 8·0 with NaOH. Hanging-drop preparations were examined and photographed.

Lysozyme assay

Lysozyme was assayed by the procedure described by Shugar (1952).

RESULTS

The cells obtained in this study were derived from numerous milk samples and consisted of a large proportion of epithelial cells; in addition a variety of other cells were identified which included neutrophils, basophils, lymphocytes and monocytes. An examination of these cells revealed that their proportions approximated those described for normal milk by Zlotnik (1947).

The possibility always exists that cells obtained from milk from a large number of dairy farms may be contaminated with traces of antibiotics which would interfere with the antimicrobial assay. Any contamination of this nature would, however, be removed from the cationic proteins during the process of extraction and purification.

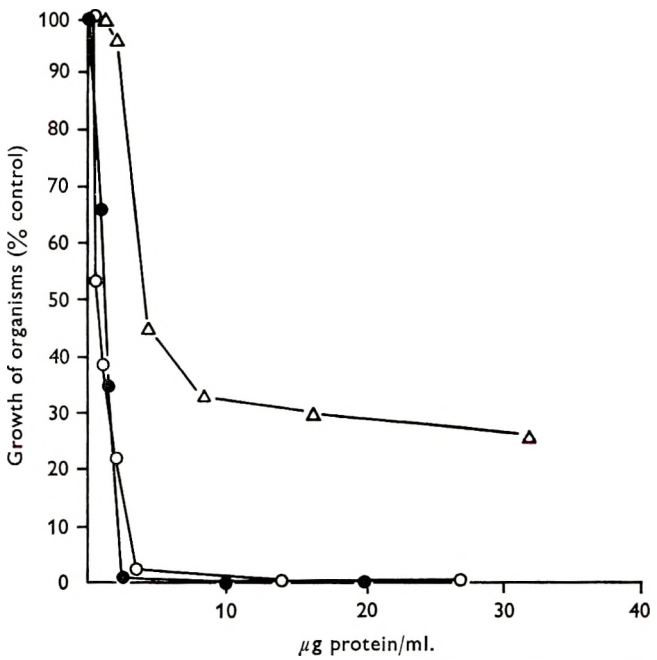

Fig. 1. The effect of cationic proteins isolated from milk cells on streptococci and staphylococci. ○—○, *Staphylococcus aureus* Mexi; ●—●, *Stap. aureus* 305; △—△, *Streptococcus agalactiae* S 13.

Antimicrobial activity of extracted proteins

The cationic proteins from milk cells had a marked effect on the growth of two strains of *Staph. aureus*. Concentrations of less than 1·0 μg./ml. produced 50 % inhibition of growth and complete inhibition was obtained when the protein concentration was increased to 10 μg./ml. *Strep. agalactiae* S13 was also inhibited, 4·0 μg. protein/ml. producing 50 % inhibition of growth. On the other hand, complete inhibition was not obtained even when the protein concentration was increased to 32 μg./ml. The extracted proteins were assayed on four occasions, and Fig. 1 shows a typical result.

Electrophoresis studies

The cationic proteins separated into at least nine bands which migrated towards the cathode on polyacrylamide gels at pH 3·0. As the pH was increased the protein bands moved a shorter distance and appeared to aggregate, so that only three principal bands appeared at pH 7·0. Two gels were used in the experiments at

Fig. 2. The separation of cationic proteins isolated from milk cells on polyacrylamide gels. (*a*) at pH 3·0; (*b*) at pH 7·0; (c 1) and (c 2), at pH 9·0.

pH 9·0, which permitted the current to be run in both directions. At this pH 4 distinct bands were observed which now moved towards the anode with only a very slight trace of cationic material. The results of these electrophoresis experiments which are shown in Fig. 2 suggest that the majority of the isolated protein fractions had isoelectric points less than 9·0, which would indicate the presence of only minimal amounts of lysozyme and other highly basic proteins.

Lysozyme activity

Although lysozyme may play an antimicrobial role in the tissues of most species (Dubos, 1945) it was not detected in the proteins extracted from the cells in cows' milk. With an isoelectric point of 10·5–11·0 (Fevold, 1951) lysozyme, if present, would have appeared as a band migrating towards the cathode at pH 9·0. However, its possible absence on the basis of electrophoresis experiments was confirmed by the failure of the extracted milk cell cationic proteins to lyse *Micrococcus lysodeikticus* in assays involving a 90 min. incubation (Fig. 3).

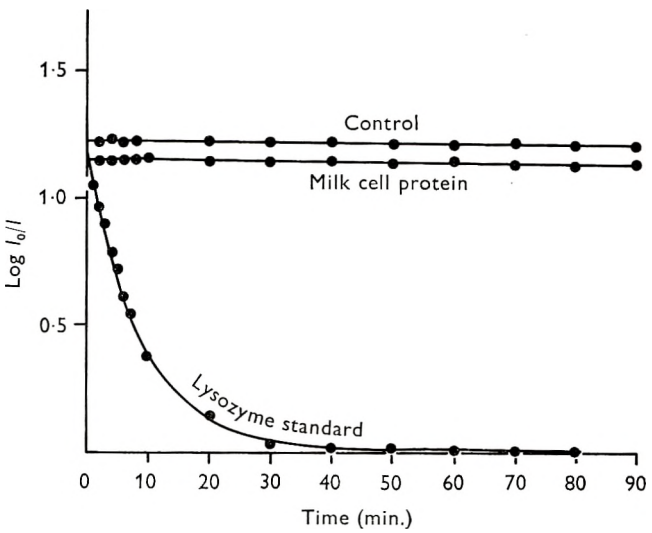

Fig. 3. The lysis of *Micrococcus lysodeikticus* by cationic proteins isolated from milk cells (400 μg/ml.) and a standard lysozyme solution (40 μg/ml.).

The binding of the cationic proteins to Staphylococci

The electrophoresis studies at different pH's indicated that the isolated cationic proteins had isoelectric points between pH 7·0 and 9·0 and would therefore be expected to bind to the surface of the micro-organisms. To investigate this binding, normal staphylococci and protein-treated staphylococci were stained with the anionic dye Fast Green FCF at pH 8·0. Apparently the cationic proteins became firmly bound to the treated staphylococci, which readily absorbed the stain whereas the untreated control group remained unstained (Plate 1).

Staphylococci treated with cationic proteins in these experiments invariably autoagglutinated. Under conditions of hypo- and hypertonicity the autoagglutination was most marked; however, it was not observed in the untreated control experiments. The autoagglutination was not associated with the Fast Green FCF staining or the presence of NaCl since it was also observed with unstained protein-treated-organisms suspended in hypo- and hypertonic sucrose solution.

The effect of heat on the antimicrobial activities of cationic proteins extracted from milk cells

Milk-cell cationic proteins showed little change in antimicrobial activity after being heated to temperatures up to 70° C. for 10 or 30 min. This activity was diminished when the proteins were heated above 70° C. and at 100° C. they retained only 10% of their original activity at 25° C. The results of typical experiments shown in Fig. 4 were obtained with protein solutions of 0·8 μg./ml. The length of time the proteins were exposed to the different temperatures had little effect on their activity apart from producing a slightly accelerated rate of inactivation in the samples treated for 30 min. Experiments with protein solutions of higher and lower concentrations gave similar curves with the exception of a variation in the initial percentage inhibition due to the different protein concentrations.

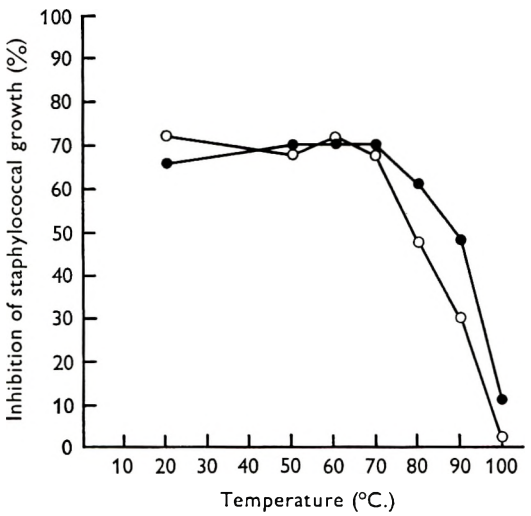

Fig. 4. The effect of heat on the antimicrobial activity of cationic proteins isolated from milk cells. ○—○, Protein solution (0·8 μg./ml.) heated for 30 min. ●—●, Protein solution (0·8 μg./ml.) heated for 10 min.

DISCUSSION

In view of the numerous cell types in milk it was not surprising to find that the extracted cationic proteins from such a heterogeneous population separated into a large number of protein bands during polyacrylamide gel electrophoresis at pH 3·0.

Lysozyme was not one of the major components separated in these electrophoresis experiments at pH 3·0 since in the experiments with gels at pH 9·0 only a trace of diffuse staining material migrated towards the cathode. The apparent absence of lysozyme was confirmed in experiments involving the lysis of *Micrococcus lysodeikticus*. The absence of definite bands of cationic protein at pH 9·0 was a little surprising since the extraction technique employed would be expected to isolate protein fractions of nuclear origin with isoelectric points higher than pH 9·0. The nature of the antimicrobial proteins isolated from bovine milk cells is of

particular interest, since Parry, Chandon & Shahani (1964) isolated lysozyme from bovine skimmed milk. They found that the enzyme was present in the milk at a concentration of 10 μg./100 ml. No indication was given, however, of the location of the enzyme, whether in the milk cells or in a cell-free supernatant. Padgett & Hirsch (1967), on the other hand, in a series of experiments on polymorphonuclear leucocytes, tears, nasal exudates and saliva of cows, were unable to demonstrate any lysozyme activity but they showed nevertheless that phagocytosis and intracellular killing of micro-organisms did not differ from that observed in animals producing lysozyme. They demonstrated the presence of an antimicrobial agent in tears from the cow which, unlike lysozyme, was inactivated by heating to 88° C. for 10 min. at pH 3·0.

In this present study the antimicrobial activity of the isolated cationic proteins from the mixed population of milk cells was considerably reduced after heating to temperatures of over 70° C. But even at 100° C. some antimicrobial activity remained which may be attributed to relatively heat-stable extracted substances such as leukin (Skarnes & Watson, 1956). The loss of the antimicrobial activity as the protein extracts were heated to temperatures between 70° and 100° C. was not unexpected since several antimicrobial fractions were extracted and each would be inactivated at a different temperature.

The cationic proteins isolated from the milk cells had a greater antimicrobial activity than similarly charged proteins isolated from teat canal keratin in an earlier study (Hibbitt *et al.* 1969). The same strains of staphylococci were used in this present study as in the earlier study but with each strain of organism less than 1·0 μg./ml. of the milk cell protein produced a 50% inhibition of growth, whereas the test canal proteins required concentrates of 2–5 μg./ml. to achieve the same effect. *Strep. agalactiae* S13, however, was relatively resistant to these isolated cationic proteins since complete inhibition of growth was not produced even when the protein concentration was increased to 32 μg./ml.

The events causing the death of micro-organisms following treatment with cationic protein from milk cells remain unknown. The experiments with Fast Green FCF staining indicate that the cationic proteins isolated from milk cells bind to the surface of the micro-organisms in the same way as basic polymers from other sources (Bloom, Winters & Watson, 1951; Zeya & Spitznagel, 1966; Hibbitt *et al.* 1969). The autoagglutination of the organisms observed after the cationic protein treatment has been demonstrated by Bloom & Blake (1948), who studied the effects of basic tissue polypeptides on *Staph. aureus*, beta-haemolytic streptococci, *Bacillus megaterium* and *Escherichia coli*. In the present experiments this autoagglutination was most obvious in hypotonic and hypertonic solutions, which may indicate an increased permeability of a damaged microbial plasma membrane with a consequent movement of protoplasmic or cationic protein across the membrane depending on the molarity of the medium.

The importance of these antimicrobial cationic proteins may lie in the fact that they provide an initial form of defence for the mammary gland against invading micro-organisms. Although antimicrobial protein fractions were isolated in the present experiments from the cells in milk it is conceivable that extracellular

destruction of micro-organisms may occur by the release of these cationic fractions into the milk thereby contributing to the poorly defined humoral factors.

We wish to thank Messrs Smith Bros., Grove, Wantage, Berkshire, for the supply of cells from the bulk milk samples, Miss J. Blackford and Miss J. Waite for technical assistance and Mr I. Jebbett for the photomicrographs.

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EXPLANATION OF PLATE

The effect of milk cell cationic proteins on the staining of staphylococci with Fast Green FCF at pH 8·0. (*a*) Protein-treated staphylococci in isotonic saline. (*b*) Untreated staphylococci in isotonic saline.


a
b

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The toxicity of chlorophacinone and warfarin to house mice (*Mus musculus*)

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(*Received* 26 *August* 1970)

SUMMARY

Individually caged house mice (*Mus m. musculus*) were fed 0·025 % warfarin (3(2-acetyl-1-phenylethyl)-4-hydroxycoumarin) or 0·025 % chlorophacinone (2-(1-(*p*-chlorophenyl)-1-phenyl) acetyl-1,3-indandione) for periods varying from 1 to 21 days. In all, 320 mice, 160 of each sex, were tested.

A significant difference was found between the mortalities obtained by the two compounds. In feeding periods varying from 1–5 days chlorophacinone produced a mortality of 60–85 % and warfarin only 5–75 %. Five per cent, however, did survive 21 days feeding on each compound.

A great variation in the susceptibility of individual mice was established for chlorophacinone as well as for warfarin.

INTRODUCTION

In Denmark warfarin was the only anticoagulant in use for the control of house mice during the period 1953–68, but after several cases of mice surviving 10–20 days on 0·025–0·050 % warfarin in the laboratory (Annual Report, 1961–2, 1963) it was decided that it was no longer possible to obtain satisfactory results in practice, where other food sources are always present. And since another poison, crimidin, at the same time turned out to be effective against house mice, it was decided to restrict the use of warfarin to the control of rats only.

In the search for other anticoagulants to replace warfarin in mouse control, clorophacinone was investigated a little further, as it was claimed by the manufacturer to be more effective than warfarin against house mice even at lower concentrations (Technical Report, 1965). This was partially confirmed by Rowe & Redfern (1968).

MATERIALS AND METHODS

The animals used were all house mice (*Mus m. musculus*) bred in the laboratory and fed normal laboratory diet during their entire life. In each test an equal number of males and females was used, and as far as possible pregnant females were excluded. The mice were placed individually in metal cages (15 × 10 × 25 cm) containing a glass or plastic food bowl and a small bottle with cotton as a nesting box. Water was supplied from a bottle in the roof of the cage. The method used in all tests was to offer each mouse for a fixed number of days excess amounts of bait

Table 1. *Toxicity of* 0·025 % *warfarin to* Mus musculus

No. of days feeding	No. of mice dead by end of the test (out of 20)	Mortality (%)	Days to death		Weight of mice		Dosage range that killed (mg./kg.)	Dosage range that failed to kill (mg./kg.)
			Mean	Range	Mean	Range		
1	1	5	8·0	—	18·4	15·0–20·5	30	12–45
2	4	20	4·5	4–7	17·3	15·0–22·0	71–126	48–119
3	7	35	5·4	2–12	15·0	14·0–16·5	76–165	113–191
4	10	50	6·6	3–9	14·9	10·5–22·0	61–180	60–121
5	15	75	6·9	4–10	18·1	13·0–23·0	86–219	97–145
6	16	80	7·9	4–15	12·5	9·5–15·0	163–478	146–213
10	18	90	9·2	6–15	13·7	10·0–20·5	107–690	362–527
21	19	95	9·4	4–18	12·7	9·5–22·5	69–704	832

Table 2. *Toxicity of* 0·025 % *chlorophacinone to* Mus musculus

No. of days feeding	No. of mice dead by end of the test (out of 20)	Mortality (%)	Days to death		Weight of mice		Dosage range that killed (mg./kg.)	Dosage range that failed to kill (mg./kg.)
			Mean	Range	Mean	Range		
1	12	60	6·8	4–12	13·2	8·5–20·5	23–65	9–50
2	15	76	5·9	3–11	19·5	15·0–23·5	47–101	57–76
3	14	70	7·2	3–13	14·8	11·5–19·0	43–195	104–160
4	17	85	7·8	4–14	16·3	12·0–20·5	109–266	140–250
5	17	85	7·1	2–13	15·4	11·0–22·0	62–340	227–332
6	18	90	6·9	4–12	18·4	13·0–23·5	67–325	251–292
10	19	95	8·4	3–14	16·4	12·0–22·0	58–500	500
21	19	95	12·4	6–25	12·5	8·0–18·0	125–1168	906

containing either 0·025 % warfarin (3(2-acetyl-1-phenylethyl)-4-hydroxycoumarin) or 0·025 % chlorophacinone (2-(1-(*p*-chlorophenyl)-1-phenyl)acetyl-1,3-indandione). The warfarin bait was a prepared bait on whole wheat, whereas the chlorophacinone bait was made by mixing 1 part of a 0·25 % oily concentrate with 9 parts of oat groats. In the majority of tests the amount eaten was recorded daily, but in some tests exceeding 6 days the bait eaten was measured only at the end of the feeding period.

After each poison-period the bottom of the cage was removed and the cotton renewed to avoid further contamination of the food. Mice which died during the test period were examined for internal bleeding, and the survivors were fed plain bait for about 14 days.

RESULTS

The results of the toxicity tests are given in Tables 1 and 2. Twenty mice were used in each test in feeding periods varying from 1 to 21 days. In all, 320 mice were tested.

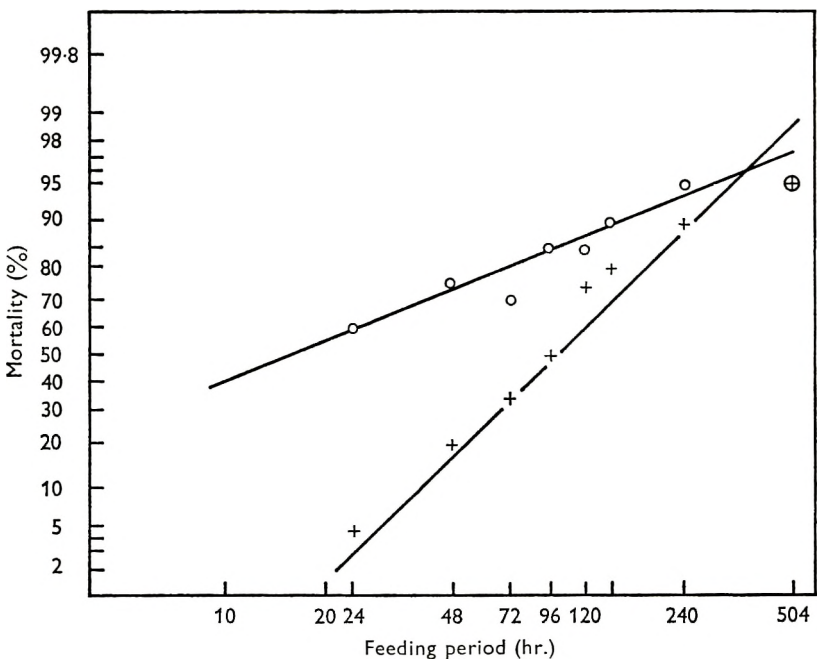

Fig. 1. Effect of two anticoagulants on house mice. ×—×, 0·025 % warfarin; S = 2·04 (1·72 to 2·41). ○—○, 0·025 % chlorophacinone, S = 5·57 (1·92–16·15).

In Fig. 1 the dose/effect lines for the two compounds are given using the method of Litchfield & Wilcoxon (1949). Instead of doses here the feeding periods suggested for anticoagulants by Bentley & Larthe (1959) have been used.

DISCUSSION

Several investigations on the effect of warfarin on house mice have been carried out, especially in England. Bentley & Larthe (1959) in a comparison of five

anticoagulants found a mortality of 90 % (9/10) when feeding house mice for 6 days on 0·025 % warfarin, and a mortality of 100 % when feeding for 7 days. The dosage range that killed varied between 57 and 101 mg./kg., and the highest dose survived was 169 mg./kg. However, rather lower mortalities than these were recorded by Rowe & Redfern (1964). Testing 345 mice from 13 different localities not previously treated with warfarin, they found a considerable individual variation in response, one mouse being killed by a dose of 26·1 mg./kg. and another surviving 1067·2 mg./kg. It was concluded that the lethal feeding period corresponding to a 95 % kill was about 22 days. In a later study (Rowe & Redfern, 1965) it was suggested that probably there are some mice 'resistant' to warfarin in any sizeable population.

In a subsequent comparison of different anticoagulants Rowe & Redfern (1968) repeated these findings with warfarin, whereas chlorophacinone at the same concentration (0·025 %) gave a somewhat better result, e.g. a mortality of 100 % after a 14 days' feeding period. After 3 days' feeding a mortality of 37 % was obtained and it was concluded that chlorophacinone at 0·025 % or 0·0050 % 'is rather more toxic than warfarin at 0·025 %'.

Although the results of the present tests showed a higher mortality to chlorophacinone, no complete kill was obtained with this compound, as with warfarin, even after 21 days' feeding. It seems dubious therefore whether the higher initial kill produced by chlorophacinone can have any practical significance for the control of house mice.

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Investigation into rabbit infusion media for the growth of *Mycoplasma gallisepticum* antigens for inoculation into rabbits

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SUMMARY

Serum cholesterol in rabbits was elevated, by intravenous inoculation of 20% Tween 80, to amounts thought to have been sufficient to support growth of *Mycoplasma gallisepticum* when 20% of such serum was added to rabbit infusion broth.

However, better growth of the organism was obtained with a supplement of 5% rabbit serum and commercial cholesterol. Investigation showed that normal rabbit serum may be inhibitory, which would explain these findings. The commercial cholesterol was not antigenic.

INTRODUCTION

Antigenic purity is an essential feature of mycoplasma antigens intended for animal inoculation for the production of specific antibodies. It has been shown that protein in the mycoplasma culture medium foreign to the animal to be inoculated can give rise to non-specific antibody (Smith, Dunlop & Strout, 1966; Kulasegaram, 1967; Jordan & Kulasegaram, 1968). To overcome this problem, the last-named workers used a medium that contained protein derived entirely from the species to be inoculated (chickens, turkeys and rabbits). In this laboratory *M. gallisepticum* has been grown satisfactorily in chicken and turkey infusion medium supplemented with 20% (v/v) chicken and turkey serum respectively, but difficulty has been experienced with rabbit meat infusion broth supplemented with 20% rabbit serum (Nutor, 1969).

Edward & Fitzgerald (1951*a*, *b*) and Edward (1954) attributed the poor growth of some mycoplasmas in medium supplemented with rabbit serum to a low cholesterol content in the serum. Taylor-Robinson, Somerson, Turner & Chanock (1963) and Eng (1967) successfully grew *M. pneumoniae* by supplementing their medium of rabbit meat infusion and rabbit serum with a suspension of commercially prepared cholesterol.

However, the incorporation of a suspension of cholesterol may not be the ideal way of increasing the supply of sterol because of the possible difficulty in preparing a stable suspension (Edward & Fitzgerald, 1951*b*) and because there may be a tendency for the suspension to sediment with the organism at harvest. In addition, although cholesterol itself is not antigenic, commercial cholesterol, derived from

bovine brain and spinal cord, may contain bovine protein and this might contaminate the prepared mycoplasma antigen (D. H. Roberts, personal communication).

This paper describes an attempt to prepare antigen free from the disadvantages described above in order to produce antisera in rabbits specific to *M. gallisepticum*. The medium for the growth of mycoplasma for this purpose was rabbit meat infusion containing rabbit serum in which the cholesterol concentration had been elevated *in vivo*. Its ability to support growth was compared with an orthodox medium, a rabbit infusion medium containing normal rabbit serum and infusion medium containing commercial cholesterol.

The paper also records the growth inhibitory effect observed in the sera of some normal rabbits.

MATERIALS AND METHODS

Mycoplasma gallisepticum *strains*

The following three strains were used: A514, originally obtained from Dr H. Chu, University of Cambridge, which has now undergone numerous passages in artificial medium in this laboratory; S6M, obtained from Dr D. H. Roberts, Central Veterinary Laboratory, Weybridge; and X95, obtained from Dr R. H. Leach, Wellcome Research Laboratories, Beckenham.

Rabbit sera

After collection these were stored at 0–4° C.

Determination of total cholesterol content in normal rabbit, horse and swine serum

In order to confirm that the serum cholesterol concentrations of our normal rabbits were relatively low, the serum total cholesterol of 20 normal rabbits was determined. For comparative purposes, the concentrations were also estimated in three batches of horse serum and three of swine serum as used in routine mycoplasma culture media.

Determinations were made by the ferrous sulphate* method (Searcy & Bergquist, 1960) and optical densities were measured at 490 nm.

Elevation of serum cholesterol in rabbits

Six rabbits were inoculated intravenously with 20% Tween 80 as described by Kellner, Correll & Ladd (1951). Two were given a single inoculation and blood was collected after 16 hr. The other four received two inoculations with a 16 hr. interval and blood was collected 16 hr. after the first inoculation and 8 hr. after the second.

Media

Broth medium (BM)

The medium used for routine maintenance of cultures was that described by Taylor-Robinson *et al.* (1963) except that it contained 1% nicotinamide adenine

* Hyland Baxter, Thetford, Norfolk.

dinucleotide (NAD), 1 % glucose and 0·002 % phenol red, while amphotericin was omitted.

Rabbit infusion broths (RB 1–3)

The growth-promoting properties of an infusion medium containing rabbit serum with cholesterol elevated *in vivo* was compared with one containing normal rabbit serum and one to which commercial cholesterol and normal rabbit serum had been added.

The basic infusion for all three media was similar to that described by Jordan & Kulasegaram (1968) but using rabbit meat in place of chicken meat, and the infusion was autoclaved at 10 lb./in.^{2}, 114° C., for 30 min. The supplements were as described except that 1 % glucose was used and the pH indicator was phenol red.

To prepare the experimental media the following additions were made to the above infusion:

Rabbit infusion broth 1 (RB 1). 20 % (v/v) rabbit serum with elevated cholesterol (98 mg./100 ml.).

Rabbit infusion broth 2 (RB 2). 20 % (v/v) normal rabbit serum with relatively low total cholesterol (30 mg./100 ml.).

Rabbit infusion broth 3 (RB 3). 5 % (v/v) of the same pool of normal rabbit serum and also 2 % (v/v) of the cholesterol* preparation as described by Taylor-Robinson *et al.* (1963).

For all three media the rabbit serum was heated at 56° C. for 30 min. immediately before incorporation.

Evaluation of the media for the growth of Mycoplasma gallisepticum

This was studied for three strains (A 514, S 6 M and X 95).

Inocula were prepared from a 24 hr. culture of the organisms in BM. The culture was harvested, washed once in 0·5 M sucrose containing 0·01 M phosphate buffer pH 7·0 as described by Rodwell & Abbot (1961), and resuspended to its original volume in phosphate-buffered sucrose.

The inoculum (10 %, v/v) was added to each medium and in addition a sample of the inoculum was immediately titrated for viable organisms by the method described below. The inoculated media were incubated at 37° C., and viable counts were conducted on all samples at 8, 24 and 48 hr.

Viable counts

The 'Most Probable Number' method (Taylor, 1962; Meynell & Meynell, 1965) was employed using colour change in the medium associated with fall in pH by at least half a pH unit as an indication of growth.

A series of tenfold dilutions to 10^{-10} was prepared from the culture in a diluent consisting of distilled water adjusted to pH 7·6–7·8 with 0·01 M-K_2HPO_4 and 1 % (v/v) BM (Butler & Knight, 1960). From every dilution 0·1 ml. was transferred to

* Koch Light, Colnbrook, Bucks.

each of five vials containing 0·9 ml. of BM. All dilutions were then incubated at 37° C. for 10 days.

The value of the Most Probable Number of viable organisms was derived from tables and the validity of the method was confirmed for each strain of *M. gallisepticum* by application of Moran's test to the dilution counts (Moran 1954 *a*, *b*, 1958).

Examination of the cholesterol preparation for bovine protein

The commercial cholesterol preparation was examined for bovine protein contaminants by attempting to produce an anaphylactic response in guinea-pigs, a method that will detect very small amounts of protein (Kabat & Mayer, 1961).

Four guinea-pigs were inoculated intraperitoneally with 5 ml. cholesterol suspension as used in medium RB 3 and four with 2 mg. bovine serum protein. After 14 days, two of the four guinea-pigs given cholesterol and two given bovine serum protein were challenged by intracardiac inoculation of 2·5 ml. cholesterol preparation. The other two of each group were similarly inoculated with 2 mg. bovine serum protein. The two guinea-pigs that were sensitized and also challenged with bovine serum protein were a positive control for the anaphylactic system.

Two normal guinea-pigs were given an intracardiac inoculation of 2·5 ml. of the cholesterol suspension to ensure that large particles or the ethanol content had no adverse effects.

All guinea-pigs were closely observed for signs of anaphylactic shock for 2 hr.

The effect of normal rabbit serum on the growth of Mycoplasma gallisepticum

The possible growth inhibitory effect of normal rabbit serum was examined because better growth was obtained with 5 % rabbit serum (RB 3) than with 20 % (RB 1) although both should have contained adequate cholesterol.

Sera from eight rabbits were tested both unheated and after heating at 56° C. for 30 min. The method employed was a modification of the metabolic-inhibition test in 'microtitre' plates (Taylor-Robinson, Purcell, Wong & Chanock, 1966) using BM, and rabbit serum replacing antiserum. The serum was incorporated in the medium in the following final concentrations: 20 %, 10 %, 5 %, 2·5 % and 1·25 %. Each was tested with two dilutions (10^{-3} and 10^{-4}) of each of the three test organisms. These dilutions were prepared from a 24 hr. culture of the strain in BM and each test-well received 20 % (v/v) of the dilution. Control wells contained the same medium and inoculum without rabbit serum.

Plates were sealed with clear tape, incubated at 34° C. and results were read when the pH of the control wells had fallen by approximately half a pH unit. The end-point for inhibition by a serum was taken as the lowest concentration that inhibited a colour change.

Three batches of horse serum and three of swine were similarly examined and, to ensure that any inhibition of colour change by the different sera was not due to different buffering capacity, this was measured by titration against 0·1 M-HCl.

RESULTS

Determination of total cholesterol content in normal rabbit, horse and swine serum

The distribution of the serum total cholesterol values of 20 normal rabbits is shown in Fig. 1. The mean of the results was 41·0 mg. ± 16·0 mg. (S.D.)/100 ml. and this was considerably lower than the average total cholesterol of the horse and swine serum, 75 and 160 mg./100 ml. respectively.

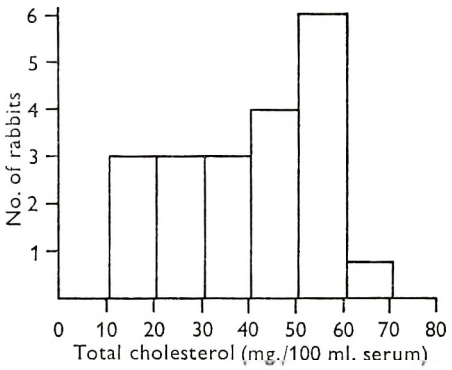

Fig. 1. Serum total cholesterol of 20 normal rabbits.

Table 1. *Serum total cholesterol (mg./100 ml.) of six rabbits inoculated with* 20 % *Tween* 80

Rabbit no.	No. of inoculations	Time after initial inoculation (hr.) 0	16	24
1	1	37	54	.
2	1	61	75	.
3	2	39	65	155
4	2	69	87	104
5	2	34	73	181
6	2	18	ND	98

ND = not determined.

Elevation of serum cholesterol in rabbits

Cholesterol concentrations were considerably raised after a single intravenous inoculation of 20 % Tween 80 and were further elevated after a second inoculation (Table 1).

Evaluation of the media

Table 2 shows the viable counts of A514, S6M and X95 in the four different media, the actual growth curves being shown in Figs. 2–4. Table 3 is a summary of the growth-promoting properties of the media for the three strains.

BM supported growth of all three strains to a higher titre than the other media. RB3 also promoted growth of A514 and S6M, while the titre of X95 was main-

tained for 24 hr. RB1 supported growth of A514 but only appeared to maintain the titre of S6M after 24 hr. and did not support growth of X95. RB2 supported only A514.

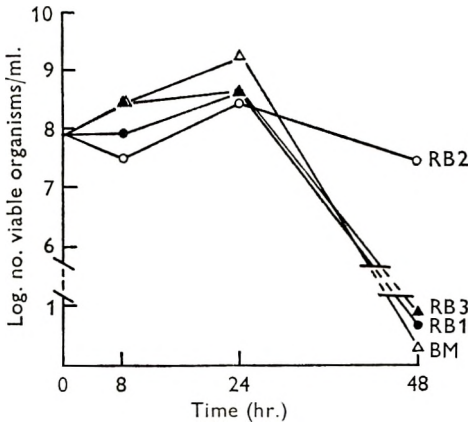

Fig. 2

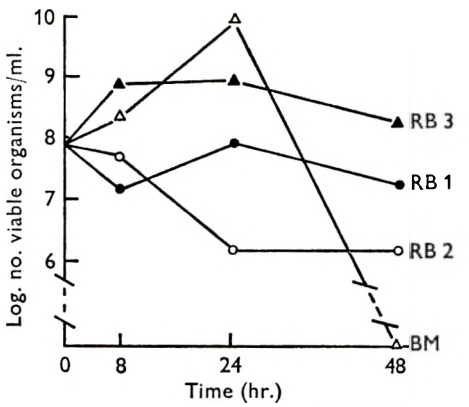

Fig. 3

Fig. 2. Growth of *Mycoplasma gallisepticum* A514. BM = Broth medium; RB1 = rabbit infusion broth 1; RB2 = rabbit infusion broth 2; RB3 = rabbit infusion broth 3.

Fig. 3. Growth of *Mycoplasma gallisepticum* S6M. BM = Broth medium; RB1 = rabbit infusion broth 1; RB2 = rabbit infusion broth 2; RB3 = rabbit infusion broth 3.

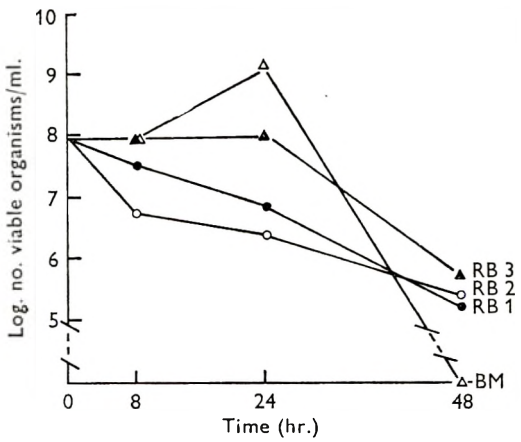

Fig. 4. Growth of *Mycoplasma gallisepticum* X95. BM = Broth medium; RB1 = rabbit infusion broth 1; RB2 = rabbit infusion broth 2; RB3 = rabbit infusion broth 3.

Examination of the cholesterol preparation for bovine protein

Of the ten guinea-pigs, only the two that were sensitized and challenged with bovine serum protein showed signs of anaphylactic shock. These showed classical anaphylactic symptoms and died in less than 5 min.

The guinea-pigs that received intracardiac inoculations of the cholesterol sus-

Table 2. *The number of viable organisms/ml. of* Mycoplasma gallisepticum *A* 514, *S* 6 *M and X* 95 *in media BM, RB_1, RB_2 and RB_3*

M. gallisepticum strain	Incubation time (hr.)	BM	RB_1	RB_2	RB_3
A 514	0	$8{\cdot}3 \times 10^7$	$8{\cdot}3 \times 10^7$	$8{\cdot}3 \times 10^7$	$8{\cdot}3 \times 10^7$
	8	$2{\cdot}9 \times 10^8$	$8{\cdot}3 \times 10^7$	$2{\cdot}9 \times 10^7$	$2{\cdot}9 \times 10^8$
	24	$2{\cdot}3 \times 10^9$	$4{\cdot}9 \times 10^8$	$2{\cdot}9 \times 10^8$	$4{\cdot}9 \times 10^8$
	48	2·0	4·5	$2{\cdot}9 \times 10^7$	7·8
S 6 M	0	$6{\cdot}4 \times 10^7$	$6{\cdot}5 \times 10^7$	$6{\cdot}4 \times 10^7$	$6{\cdot}4 \times 10^7$
	8	$2{\cdot}4 \times 10^8$	$1{\cdot}5 \times 10^7$	$5{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^8$
	24	$9{\cdot}2 \times 10^9$	$8{\cdot}6 \times 10^7$	$1{\cdot}5 \times 10^6$	$9{\cdot}2 \times 10^8$
	48	0	$1{\cdot}7 \times 10^7$	$1{\cdot}5 \times 10^6$	$1{\cdot}6 \times 10^8$
X 95	0	$9{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^7$
	8	$9{\cdot}2 \times 10^7$	$3{\cdot}5 \times 10^7$	$5{\cdot}2 \times 10^6$	$9{\cdot}2 \times 10^7$
	24	$1{\cdot}5 \times 10^9$	$6{\cdot}4 \times 10^6$	$2{\cdot}4 \times 10^6$	$1{\cdot}0 \times 10^8$
	48	0	$1{\cdot}6 \times 10^5$	$2{\cdot}4 \times 10^5$	$5{\cdot}2 \times 10^5$

BM = Broth medium; RB_1 = rabbit infusion broth 1; RB_2 = rabbit infusion broth 2; RB_3 = rabbit infusion broth 3.

Table 3. *Media RM, RB* 1, *RB* 2, *RB* 3 *and the growth of* Mycoplasma gallisepticum, *A* 514, *S* 6 *M and X* 95

	BM	RB 1	RB 2	RB 3
A 514	+	+	+	+
S 6 M	+	±	−	+
X 95	+	−	−	±

+ = growth; − = no growth; ± = viability maintained after 24 hr. incubation.

Table 4. *Growth inhibition of* Mycoplasma gallisepticum *by normal rabbit serum*

Serum	Lowest % of rabbit sera A–H which inhibits growth of strain		
	A 514	S 6 M	X 95
A	20	10	20
A_h	20	20	20
B	20	10	N
B_h	20	20	N
C	20	10	N
C_h	20	10	N
D	N	20	N
D_h	N	20	N
E	10*	10*	N
E_h	20	10*	N
F	20	20	N
F_h	20	20	N
G	20	5*	20
G_h	20	5*	20
H	20	20	N
H_h	20	N	N

h = serum heated at 56° C. for 30 min.; N = serum not inhibitory even at 20 %.
* Bactericidal (no growth after 7 days incubation).

pension, including the two unsensitized ones, showed varying degrees of incoordination for approximately 30 min., which was attributed to the small amount of ethanol in the inoculum.

There was thus no evidence for the presence of bovine protein in the cholesterol preparation.

Inhibitory properties of normal rabbit serum

The results of this investigation are shown in Table 4. Inhibition was found to be independent of the number of organisms in the initial inoculum. Many of the sera were inhibitory to the growth of A514 and S6M at 20% concentration, and some sera were inhibitory at lower concentrations, although none showed any inhibition at below 5%. X95 was inhibited by only two sera and never at less than 20% concentration.

In a few cases there was no mycoplasma growth, even after incubation for 1 week, and these sera were considered to be bactericidal for that organism, whilst others were merely bacteriostatic (Davies, 1969).

The growth-inhibitory effect was occasionally reduced by heating the serum at 56° C., but only once was the effect abolished.

There seemed to be little uniformity in the effect of the sera on the three strains; for example, serum E, which was bactericidal to A514 and S6M, and still inhibitory after heating, did not show any inhibition of X95. None of the horse or swine sera were inhibitory to any of the strains.

The buffering action of the rabbit sera was very similar in all samples, small differences did not correlate with the inhibitory properties and were therefore not considered to have influenced the results significantly.

DISCUSSION

These studies confirm that comparatively low concentrations of total cholesterol are present in normal rabbit sera, and also that they can be raised fairly rapidly by intravenous inoculation of 20% Tween 80. Two inoculations raised serum total cholesterol to values above those found in horse serum but below those in the swine serum. When 20% of such rabbit serum was added to rabbit infusion broth it supported growth of one of three *M. gallisepticum* strains and maintained viability of another. Medium that contained 20% normal rabbit serum supported growth of one of the strains while the other two strains lost viability. However, broth that was supplemented with 5% rabbit serum and a cholesterol suspension supported growth of two strains and maintained viability of the third. Its apparent superiority over that containing rabbit serum in which the cholesterol was elevated *in vivo* may be due to a number of factors. For instance, it is possible that the serum from the inoculated rabbits did not provide a favourable ratio of sterol and phospholipid or fatty acid for the organism (Smith, Lecce & Lynn, 1954; Edward & Freundt, 1956; Smith, 1960; Rodwell, 1963) since intravenous inoculation of Tween 80 elevates serum phospholipid and other lipids as well as total cholesterol.

Another influencing factor may have been the amount of free cholesterol in the

total. The parasitic mycoplasmas incorporate free cholesterol more readily than the esters (Rodwell, 1963; Argaman & Razin, 1965) and in this work the relative amounts of free and esterified cholesterol were not determined. Further, the presence in rabbit serum of natural inhibitors to the growth of *M. gallisepticum* might also explain why the organism was more likely to grow in the medium containing the lower percentage of rabbit serum. Such inhibition has been reported to exist against *M. pneumoniae* (Fernald, Clyde & Denny, 1967) and against *M. gallisepticum* (Friedrich, 1970), and has been confirmed in our studies. In these, growth inhibition of *M. gallisepticum* by rabbit sera varied with the strain of the organism. It is difficult to explain why S6M should be inhibited by all eight sera examined, A514 by seven of the eight while X95 was inhibited by only two of them. It is interesting also that heating the sera had little effect, which is in contrast with the observation of Friedrich (1970) that the inhibitors were heat-labile. The exact nature of the inhibition is not known, but Kenny & Grayston (1965) reported that the sera of some normal rabbits contain complement-fixing antibody to *M. pneumoniae*.

Since sera have been found to be inhibitory to the growth of *M. gallisepticum* when incorporated in the medium at 20 %, 10 % and even in one case at 5 %, it is advisable to screen rabbit serum intended for media supplements.

The rabbit infusion medium that best supported growth of *M. gallisepticum* was that containing commercial cholesterol. It is therefore of value to note that the cholesterol preparation did not evoke an immune response. This means that if commercial cholesterol were used in the growth of *Mycoplasma* and excess was deposited at harvest, it would be unlikely to influence the antigenic properties of the organism.

A report by Sammons, Gardner & Dienst (1968) indicated that a satisfactory high cholesterol serum could be produced by feeding rabbits on a high cholesterol diet. Rabbit infusion broth with 5 % of the resulting serum supported growth of several species of human *Mycoplasma*. No mention was made of inhibitory properties of serum. A comparison of the medium with one containing commercial cholesterol for the growth of *Mycoplasma* would be of value.

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Prevention of airborne contamination and cross-contamination in germ-free mice by laminar flow

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SUMMARY

The efficacy of horizontal and vertical laminar flow units (equipped with high-efficiency air filters) in the prevention of cross-contamination between cages and of contamination from outside has been demonstrated. With germ-free mice and using germ-free standard techniques for sterilization and for the transfer of germ-free mice into the cabinets via a standard entry lock, it was found that during an observation period of 2 weeks the animals remain 'negative'. Other experiments were performed with equally good results in cabinets equipped with a hinged flap, closing 95% of the open front side. When the flap was closed the air flow could be reduced accordingly, thus reducing the noise level and the risk of dehydration.

Experiments made with germ-free mice in a 'down-flow unit' were also invariably good.

In another type of experiment, cages with conventional mice were placed in the cabinets between cages with germ-free animals at varying distances. If all animals were maintained on wire mesh (to minimize the aerosol production of dust) and if the 'conventional' cages were at a distance of 10 cm. from 'germ-free cages' the latter remained bacteria-free during test periods of one week.

The use of 'laminar flow isolators' for the isolation of human patients is mentioned.

INTRODUCTION

Infection frequently complicates experiments with animals in which immune response is decreased by irradiation, chemotherapy or the administration of anti-lymphocyte serum. In most case these infections originate from the digestive tract. By oral administration of antibiotics the digestive tract of conventional animals can be decontaminated, but in a few days these animals become highly susceptible to antibiotic-resistant strains of many bacterial species. Oral contamination with small numbers (< 100 cells) of, for example, *Escherichia coli* invariably results in a rapid and permanent 'take'.

Decontaminated animals to be used for radiation studies must therefore be isolated to prevent contamination and colonization. The standard germ-free isolator is unsuitable for this purpose because antibiotic-resistant organisms, occasionally present in one of the animals, rapidly contaminate the whole group inside the isolator (van der Waaij & Sturm, 1968). This dissemination of a con-

taminant might be prevented by distributing the animals over a number of cages in a cabinet with a 'laminar flow' system (Whitfield, 1962). In such a system, prefiltered air is blown through a 'high efficiency' (HEPA) filter and enters the working area of the cabinet through numerous small holes in a panel in the rear wall. In properly constructed benches the resulting air stream is non-turbulent.

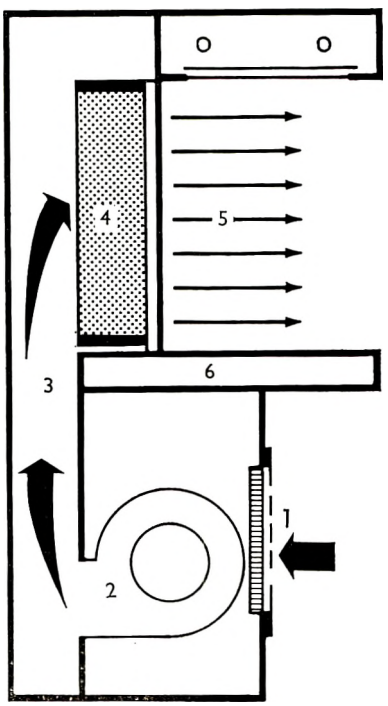


Fig. 1. Diagram of a cross-section of a laminar-flow cabinet with horizontal air flow. 1, Prefilter; 2, blower; 3, pressure box; 4, HEPA filter; 5, sterile area; 6, working table/platform.

The horizontal air flow ('cross-flow') cabinet (Fig. 1) serves to protect material from contamination from outside; vertical air flow in 'down-flow' cabinets, in which a negative pressure is maintained by exhaustion of air under the working area, can be used to prevent contamination of the environment by material in the cabinet (McDade, Sabel, Akers & Walker, 1968; Starzl & Beakly, 1968). Non-turbulent air streams are found to be highly efficient in protecting fine instruments from dust and to improve asepsis in the pharmaceutical and microbiological fields (Favero & Berquist, 1968). McGarrity *et al.* (1969) found that (HEPA-filtered) laminar air flow effectively reduced airborne contamination and factors contributing to the spread of airborne infection. Moreover, clinicians are interested in safe and workable isolation units for nursing patients to be decontaminated with antibiotics (Bodey, Freireich & Frei, 1969; Lidwell & Towers, 1969; Penland & Perry, 1970).

In the present study the protection of germ-free (GF) mice from airborne con-

tamination by laminar air flow was investigated. Both the effect of experimental contamination of the outside air and of the presence of conventional mice inside the clean area of the cabinet were explored.

MATERIALS AND METHODS

Mice

GF mice of the random-bred ND2 stock and conventional mice of the CBA/Rij strain were used. They were housed in transparent polycarbonate cages (18 × 18 × 12 cm). To reduce the amount of dust produced by the animals, sawdust bedding was replaced by a wire-mesh 1 cm above three sheets of filter paper. The animals were housed two per cage.

Cross-flow cabinets

Four standard type 'clean benches'* manufactured by CEAG (Dortmund, W. Germany) and Bassaire Ltd. (Sussex, Great Britain) were operated with an air velocity in the benches of 50 cm./sec. They were modified by building an entry lock as used in germ-free isolators into one side panel. In addition, two of the cabinets were equipped with a hinged transparent polycarbonate flap as suggested by R. Cook (personal communication). When closed, the flap covers about 95 % of the front side. Two entry ports for long neoprene gloves were built into the flap (Plate 1). To keep the surface and the gloves sterile when the flap was lifted, the flap was divided in two equal parts attached to each other by a long horizontal hinge. In the 'open' position the lower half of the flap covered the upper half completely (Plate 1 *B*). The glove entry-ports in the upper half thus became closed by the sterile side of the lower half when the flap was opened and folded. With the flap turned down, the air flow through the cabinet could be reduced to 95 % of its original value; the air velocity in the slit thus became 50 cm./sec. In this way the noise level of the blowers and the risk of dehydration of the animals inside the cabinet could be reduced.

Both the other benches were 'open' and equipped with a wire net to prevent flying insects from entering the clean area.

Down-flow unit

A Bassaire 'portable unit' (with a HEPA filter) was placed on the top of a metal frame to which plastic sheets ending 8 cm. above the floor were fixed to form a tunnel. A standard GF entry-lock and neoprene gloves were built into the side walls to introduce and handle the animals without opening the unit (Plate 2). The cages were placed on a platform 40 cm. above the floor of the room to prevent contamination of the floor surface of the unit. Turbulent air streams in the room in which the unit was placed, caused by opening the doors and walking, resulted in contamination of the floor surface on which the unit was standing.

* Two of these benches have a filter surface of 60 × 120 cm., the others 60 × 180 cm. All cabinets were equipped with HEPA-filters (99·997 % efficiency for particles of > 0·3 μ).

The effect on the air-stream direction of opening one of the side walls of the flexible plastic tunnel was studied by generating smoke inside the unit. It was found that if one of the side walls was opened, the air stream bends inside the unit above the platform and leaves the unit turbulence-free in a horizontal direction. The air flow at the site of the opening was maintained at 50 cm./sec. The down-flow unit then functioned like an open cross-flow bench and was used accordingly. It was tested with both closed and opened side-wall. In the latter situation the unit was only opened during handling of the animals.

Sterilization of the cabinets and the down-flow unit

After the benches and the down-flow unit had been cleaned and the blowers had been functioning for at least 12 hr., the inside was sprayed with a 2% peracetic acid solution.

In the cabinets with a hinged flap, gloves and flap were wiped with cotton pads soaked in 2% peracetic acid just before the flap was closed. About ½ hr. thereafter GF mice, food, cages and drinking water were introduced from a GF isolator via the entry lock. In experiments with 'open type' benches, GF mice were introduced via the entry lock, and cages with conventional animals through the open front side; they were placed between those containing GF mice. During manipulations inside the sterile chambers of open benches and the down-flow unit, long neoprene gloves (wiped with 2% peracetic acid just before entering the clean area), a face mask and a plastic apron were worn. For obvious reasons the gloves were re-sterilized with peracetic acid between handling the mice of different cages.

Bacteriological investigation of faeces

To investigate whether the GF animals became contaminated, fresh faeces were collected daily from each cage. The faeces were transferred to culture tubes of brain heart infusion broth (Oxoid) and of Brewer's semisolid thioglycolate medium (Difco). These cultures were incubated for 2 weeks at 37° and 21° C. If no growth was observed, the cultures were stirred and subcultured on blood agar plates. These were incubated under aerobic and anaerobic conditions. Faeces were also examined microscopically.

EXPERIMENTAL DESIGN AND RESULTS

In the evaluation of the laminar-flow cabinets, both the protection against contamination from outside and the occurrence of cross-contamination between cages inside the cabinets were investigated.

Contamination from outside

Cages with GF mice were distributed in two rows over the platform of the down-flow unit and the working area of the 'open' and 'closed' cross-flow benches. In the cross-flow benches, cages were also placed on a shelf at the rear end of the working-area half the height of the filter surface (Plate 1A). In the 'closed'

benches the flap was opened only during sampling of the faeces. In this type of cabinet cleaning of cages, etc., was performed with the gloves built into the flap.

The down-flow unit was tested for two periods of 6 weeks. In the first period, sampling and handling were done with the gloves built into the unit. The unit was never opened during this period. In the second 6-week experiment the unit was used in the manner described for the 'closed type' bench. Sampling was performed with long sterile gloves after one of the side walls was opened and the air velocity had been adjusted accordingly.

In the first week of all experiments, the air in front of the air inlet filter was contaminated twice by nebulizing suspensions of *Escherichia coli*. These suspensions were also nebulized in front of the open type benches. In each case 10 ml. of suspensions containing 5×10^9 organisms/ml. were nebulized in about 30 sec.

Two weeks after introducing the GF mice the blowers of the 'open type' cabinets were switched off. The bacteriological investigation of the animals was then continued for a few days to see whether contamination from outside occurred.

Results

The experiments in the down-flow unit were very satisfactory. During their 3 months stay in the unit none of the mice became contaminated. In the cabinets with a hinged flap no cage was found to be contaminated during an observation period of 2 weeks.

Results with the 'open' cabinets were different. Animals in cages directly in front of the filter remained sterile. However, cages in the front row, located near the open side, were found to be contaminated after various intervals. After the blowers had been switched off, all the cages became positive within 2 days. This shows that the laminar flow of sterile air had been essential in protecting the area in front of the filter panel from contamination from outside.

Cross-contamination

In this type of experiment a stream of non-turbulent air of sufficient velocity between the cages is critical. Therefore only the 'open type' benches were used. The air velocity was maintained at 50 cm./sec. Four cages with GF mice were placed directly in front of the filter wall at a distance of about 32 cm. from each other. Cages with conventional mice were then placed between them. The cage distance thus became about 10 cm. After 1 week the distance was reduced to 7 cm., after 2 weeks to 5 cm. and in the third week to 3 cm.

Results

In four identical experiments no cross-contamination occurred in the first week when the cages distance was 10 cm. When the cage-distance was reduced to 7 cm. in the second week, one cage became contaminated in one of the four experiments. The contaminated cage was then replaced. In the third week most cages became positive and were also replaced. When the cage distance finally was 3 cm., all cages were found to be contaminated within a few days. From these results it was evident

that cage distance was a predominant factor in protecting from cross-contamination. When the importance of cage distance was realized, three 'open type' cross-flow benches could be used during the past year in antibiotic decontamination experiments in mice without any spread of contamination.

DISCUSSION

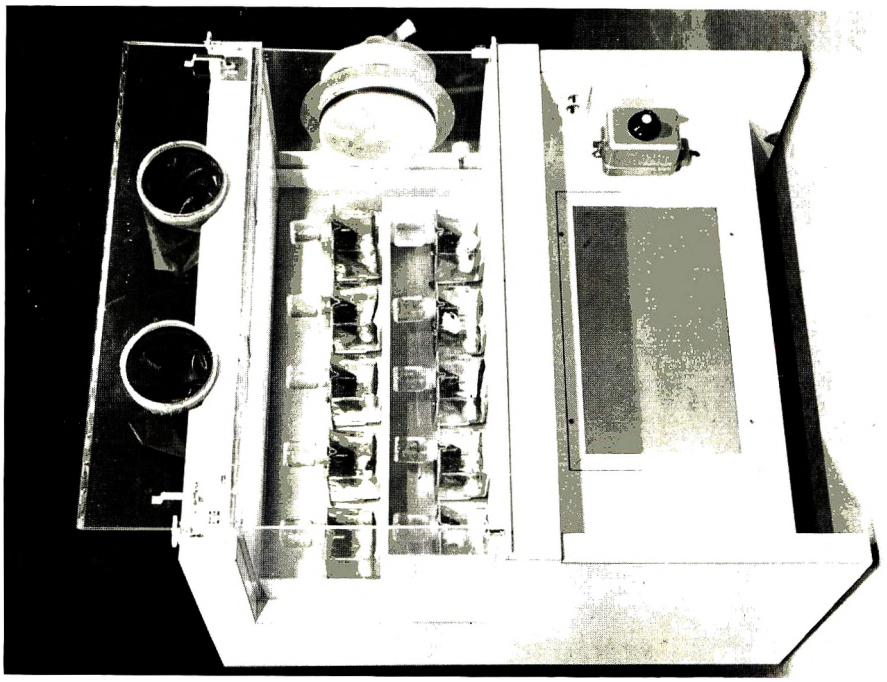
It can be concluded from the results reported that laminar-flow cabinets equipped with HEPA air filters and modified in the way described can afford adequate protection against airborne contamination for a short period of time. In the 'open type' benches the protected area is limited to the rear of the working area directly in front of the filter panel. The cabinets modified with a hinged flap, however, gave protection against airborne contamination from outside over the entire working area. Cages located in the front row also remained free from bacterial contamination. For this reason the 'closed type' benches are preferable to 'open' benches when only prevention of contamination from outside is sought. Accordingly, they have been used in our Institute for short-term surgical experiments in germ-free mice.

The successful isolation provided by the 'open type' benches could have been positively influenced by the fact that the bacterial concentration of the air in the room was low. This was because all four cabinets were located in the same room and the air of that room was continuously recirculated through the cabinet filters. With a slit-sampler, between 5 and 50 colony-forming units were found per 500 l. of air. In a comparable room without cabinets the bacterial concentration was ten time higher.

The down-flow unit described and tested in this study also provided adequate protection from airborne contamination during 3 months. It is stressed, however, that neither the down-flow unit nor the 'closed type' cabinets can (in their present form) replace the standard type of GF isolator. The benches, for example, do not prevent ants and other small insects from entering the sterile chamber. The 'laminar-flow isolator' (closed type, benches and the down-flow unit) may, nevertheless, find useful applications in some short-term experiments.

In the 'open type' benches the area directly in front of the filter panel was not only protected from contaminations from outside, but also from cross-contamination between cages. The latter depends, however, on the cage distance. No cross-contamination from cage to cage occurred, if the air stream could pass between the cages over a distance of at least 10 cm. After the benches were tested with GF and conventional mice they were used in decontamination experiments. No cross-contamination has been seen since. So far, mice were decontaminated in standard laboratory racks closed at the sides with sheets of plastic (van der Waaij & Sturm, 1968). Although airborne gross contamination was limited in this way, contaminations with antibiotic-resistant bacteria were seen from time to time under these isolation conditions.

On the basis of experiments performed with animals in the 'closed type' benches, a cross-flow cabinet with a hinged flap was used with good success for



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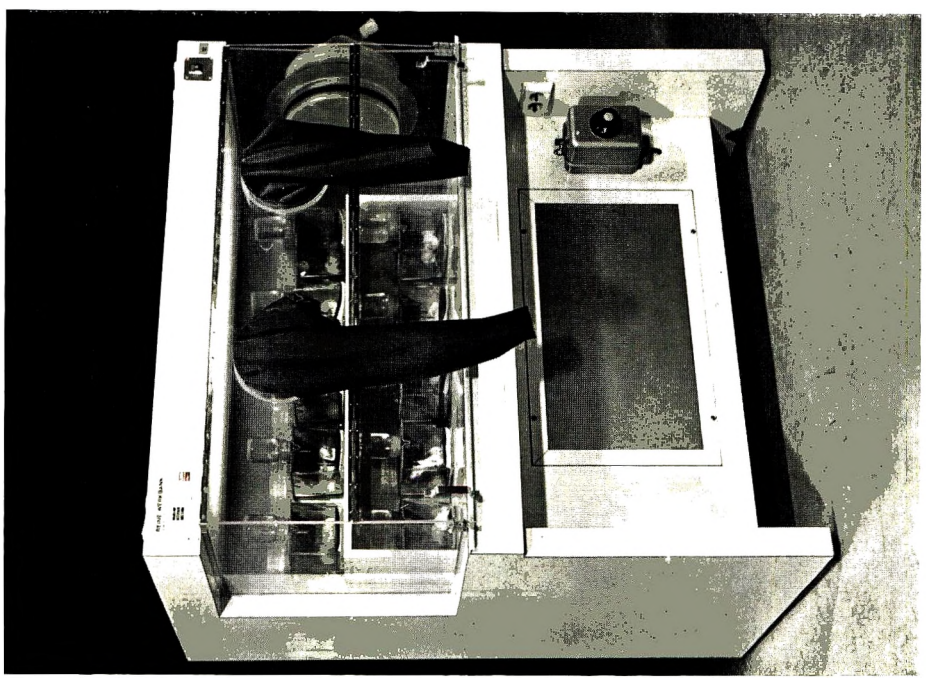

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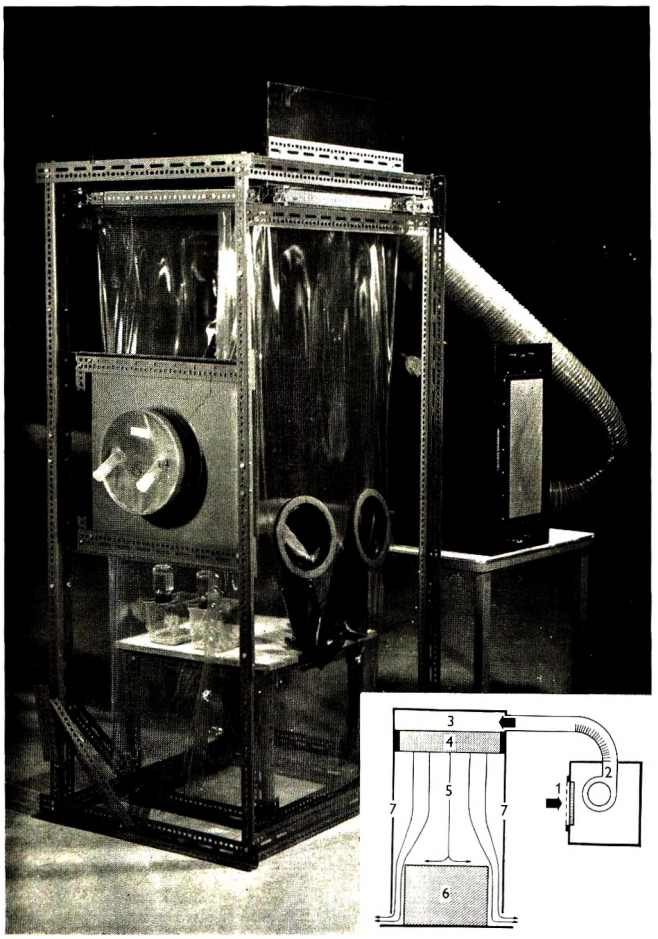
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clinical purposes. A human baby suffering from congenital Swiss-type agammaglobulinaemia has been isolated for 6 months in such an isolation system (de Koning *et al.* 1969). Furthermore, from the results obtained with the down-flow unit, it was concluded that a down-flow unit with a raised floor area (to be used only by the patient) can be recommended for strict isolation of human patients. A unit of this type has been in use now in the paediatric department of the Medical School of Rotterdam for a period of over 8 months for the isolation of a 12-year-old boy. This boy was isolated and (antibiotic) decontaminated because of heavily infected *Pemphigus vulgaris* lesions of the skin and oral mucosa.

We thank Miss J. M. de Vries and Mrs J. E. C. Lekkerkerk for their skilful assistance in microbiological testing.

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EXPLANATION OF PLATES

Plate 1

A, Laminar-flow cabinet modified with a hinged flap with gloves and GF-type entry-lock in the side wall. B, Laminar-flow cabinet with an 'opened' hinged flap (note that the lower half of the flap closes the glove entry ports; in this way the inner side of the flap and the gloves are maintained sterile).

Plate 2

Down-flow unit with a raised floor, a GF entry-lock and long neoprene gloves, built into the side wall for handling. 1, Prefilter; 2, blower; 3, pressure box; 4, HEPA filter; 5, sterile area; 6, working table/platform; 7, flexible plastic side walls.

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Prevalence of cytomegalovirus in France*

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(*Received* 15 *September* 1970)

SUMMARY

Between October 1968 and February 1970, 30 strains of cytomegalovirus were isolated from the urine of children admitted to hospitals in Lyon. Three groups of children up to the age of 14 years have been investigated.

The first group consisted of 304 newborns and infants up to the age of 1 year; cytomegalovirus was grown from five of these (1·6 %). Among these five children, two had cerebral disorders. None of them had ever shown any sign of typical CMV infection.

The second group comprised 102 children between the ages of 1 and 14 years, from a special service for neurological and mental diseases. Cytomegalovirus was grown from 19 (18·6 %).

The third group was 27 children also between 1 and 14 years of age, admitted to hospital for miscellaneous diseases excluding cerebral disorders; cytomegalovirus was grown from six (22·2 %).

It appears that cytomegalovirus has a very low incidence in neonatal disease. The virus spreads at a higher rate in children 1–14 years old. No difference has so far been shown in the excretion rates of two groups of children, one with cerebral disorders and one with other diseases, but the number of children in the last group is too small to allow definite conclusions to be drawn.

INTRODUCTION

Several studies carried out in different countries have shown that cytomegalovirus (CMV) may be involved in pathological processes in new-born as well as in older children. Among the older children the virus has been incriminated as the origin of mental defects.

In a previous study (Jeddi, Gaudin, Terraillon & Sohier, 1969) we have investigated the immunity to CMV of a sample of the French population by means of the complement fixation test. We found that the percentage of positive sera increased with age at roughly the same rate in France as in other countries. At the age of 20, 50 % of the population have acquired CMV antibodies.

The present paper reports a study set up in Lyon on different groups of patients to establish the frequency of infection during childhood.

* Presented at the 1st International Conference on Cytomegalovirus Infections, St Gall, Switzerland, 1–3 April 1970.

MATERIALS AND METHODS

Collection of urine

Urines were collected from 433 children for first isolation. In addition, eight samples were collected for re-isolation of the virus from children who had been shown to be excreting. Samples were collected in sterile containers, refrigerated and rapidly transported to the laboratory, where cultures were put up within 3–4 hr. after collection.

Tissue culture

The cell line W.I. 38 was grown in plastic bottles.* The growth medium was Eagle's medium† to which 10% calf serum was added. For maintenance, medium 199‡ was supplemented with 2% foetal bovine serum.§

Virus isolation

Each sample of urine was treated with antibiotics, the following amounts being added to 3 ml. of urine: penicillin, 10,000 units; streptomycin, 10 mg.; kanamycin, 5 mg.; Negram (naladixic acid), 2 mg.; nystatin, 5000 units. The samples were then kept at 4° C. for 30 min., after which 0·3 ml. of each urine was allowed to adsorb on a cell sheet of 25 cm.^{2}. After incubation at 37° C. for 3 hr. the medium was changed. The cultures were observed for cytopathic effect (CPE) for a period of 2 months, during which the medium was changed twice weekly. At the end of the observation period about 100 TCID 50 of echovirus type 11 was inoculated on the cells which showed no CPE, to test the susceptibility of the cells at the end of the experiment and also to detect any possible interfering agent. The strains of virus were characterized by the appearance of the degeneration and cytology after staining with Erythrosin Orange G Toluidin Blue.

RESULTS

Isolation of cytomegalovirus

Group 1*;* 304 *newborns and infants.* All these patients, under 1 year of age, nad been admitted to a paediatric clinic for miscellaneous syndromes excluding typical CMV inclusion disease at birth. Five strains were isolated (Table 1). The clinical histories of the five positive children may be summarized as follows: (1) 1 year old: lower respiratory infection; (2) 1 year old: neonatal cerebral defect; (3) 1 year old: neonatal cerebral defect; (4) 10 months old: mild gastroenteritis; (5) 7 months old: vomiting at the time of isolation of CMV; hepatitis occurred 3 months later.

Groups 2 *and* 3*; children from* 1 *to* 14 *years of age.* Group 2 consists of 102 children admitted for long-term stay in a service for neurological and mental diseases, and group 3 consists of 27 children admitted for miscellaneous diseases, excluding cerebral disorder. The results are shown in Table 1. It is seen that the percentage of

* Falcon Plastics (Becton Dickinson).
† Dried MEM medium from Wellcome Laboratories, London.
‡ Ten times concentrated, from B.D. Morieux, Lyon.
§ From SORGA laboratories, Paris.

positives is in each case much higher than in group 1; it is roughly the same in these two groups, but the small number of children examined in group 3 prevents any definite conclusions.

Table 1. *Results of isolation of cytomegalovirus in 3 groups of children*

	Total no. of children investigated	No. of children excreting cytomegalovirus	Percentage
Newborns and infants (1 year)	304	5	1·6
Children 1–14 years with cerebral disorders	102	19	18·6
Children 1–14 years without cerebral disorders	27	6	22·2
Total	433	30	

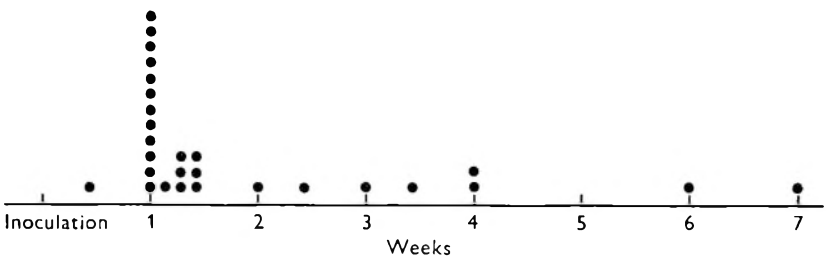

Fig. 1. Rate of appearance of cytopathic changes in W.I. 38 cell cultures at first isolation of cytomegalovirus from urine.

Speed of appearance of cytopathic effect in tissue culture

Fig. 1 shows the time of first appearance of CPE on W.I. 38 cells. It is seen that most of the viruses can be detected in 7–10 days after inoculation, though a few may show a delay of several weeks.

Duration of excretion of cytomegalovirus

In eight cases it was possible to get a second specimen of urine from which the virus was isolated again. In three of these eight cases a third specimen was obtained; these three were positive at 101, 116 and 182 days after the first evidence of viral infection.

DISCUSSION

The incidence of cytomegalovirus infection in neonatal disease does not appear very important in spite of the percentage of infants excreting the virus. This confirms the findings of other authors in Europe and North America (Cherry, Soriano, Jahn & Wis, 1968; Feldman, 1969; Krech, Jung, Bärlocher & Sege, 1968; Sanders & Cramblett, 1968; Starr & Gold, 1968; Stern, 1968), although a higher incidence of CMV has been found in Japan by Chiba, Osaki, Hanazono & Nakao (1968).

No clear evidence of disease related to CMV has been found in the course of our

study, with the possible exception of the two cases of cerebral defect among the five positive infants in the first group, and also the case of hepatitis which developed 3 months after first isolation of the virus. It should be noted that in this last case no typical inclusions have been shown by histological examination of a liver biopsy. Some previous reports have mentioned the possibility of cerebral lesions (Crome & France, 1959; De Fouquet, 1956; Marie *et al.* 1957; Nezelof, Gaquière & Brousse, 1961), and more recently Hanshaw (1966) and Stern, Elek, Booth & Fleck (1969) have drawn attention to a possible connexion of CMV with mental defects. We are not yet able to confirm this connexion, and in the two groups of children between 1 and 14 years of age which we investigated the excretion rates for CMV appear to be roughly the same.

Although our control group is as yet too small to provide quite reliable figures, we wonder whether the conditions of life of mentally retarded children in specialized communities may not be responsible for the high prevalence of CMV among them, since the wide spread of viruses in hospital wards is a well-known phenomenon. A similar percentage of excreters has been found in a population of poor economic status by Li & Hanshaw (1967).

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A note on the safety testing of vaccines

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SUMMARY

The use of the hypergeometric distribution in calculations of sample volumes for safety testing is criticized. Alternative interpretations of the concept of 'infective ml. doses' lead to slightly different solutions, the simplest of which, involving the exponential function, is recommended for general use.

In a recent paper Anderson, Capstick, Mowat & Leech (1970) consider the testing of batches of vaccine to detect small amounts of residual live virus. Given a batch of volume N ml., with 'an assumed number of infective ml. doses', m, the probability P of failing to detect this amount of infection in a test on n ml. is given by these authors (p. 167) as

$$P = \frac{(N-m)!(N-n)!}{N!(N-m-n)!}. \tag{1}$$

This equation enables n to be calculated in terms of N, m and P, and thus provides guidance about the amount of testing necessary if a certain level of contamination is to be detected with reasonably high probability.

The authors do not clearly define an 'infective ml. dose', and the purpose of this note is to point out that alternative interpretations lead to formulae different from (1), although the practical consequences of these differences are usually small.

The basis of (1) appears to be the following model. Suppose the whole volume of N ml. is subdivided into N boxes, each of 1 ml., precisely m of which are 'infective' and $N-m$ of which are 'non-infective'. A random selection is made of n of these N boxes. The test fails to detect the infection if the boxes can be classified as follows.

	In sample	Not in sample	Total
Infective	0	m	m
Non-infective	n	$N-m-n$	$N-m$
Total	n	$N-n$	N

The probability of such an outcome is the term in the hypergeometric distribution, (1).

This model seems inappropriate, because the volume of N ml. is not a finite population of 1 ml. boxes. Thus, the sample of n ml. can be drawn in an infinite number of ways; further, the infective particles are presumably distributed irregularly in space—perhaps in an approximately uniform manner—and it seems

artificial to choose one particular partition into N boxes labelled as 'infective' or 'non-infective'.

In any realistic model involving a random distribution of infective particles, the number of 'infective ml. doses' is most naturally thought of as a random variable. Anderson *et al.*, however, clearly regard m as a parameter of the model, not subject to random variation. We therefore consider some alternative models in which m is given various interpretations as a parameter. We assume throughout that infective particles follow a uniform random distribution in space.

Model 1. There are exactly m particles in the volume of N ml. A random selection of n ml. is made. The probability of a negative test result is

$$P = (1-n/N)^m. \tag{2}$$

(This model is used by Peto & Maidment (1969); see the footnote on p. 170 of Anderson *et al.* (1970).)

Model 2. The volume of N ml. under consideration has been effectively selected from a much larger volume in which the density of infective particles is m per N ml. The actual number of infective particles in the batch under consideration is then Poissonly distributed about m. The required probability is

$$P = e^{-mn/N}. \tag{3}$$

Model 1A. There are exactly m' particles in the volume of N ml. An 'infective dose' is defined as a dose containing at least one infective particle, and m/N is defined as the probability that a 1 ml. dose is infective. Then

$$\frac{m}{N} = 1-\left(1-\frac{1}{N}\right)^{m'},$$

whence

$$m' = \frac{\log\{1-(m/N)\}}{\log\{1-(1/N)\}} \tag{4}$$

and, by analogy with (2),

$$P = (1-n/N)^{m'}. \tag{5}$$

Model 2A. An 'infective dose' is defined as in 1A, and m/N is again the probability that a 1 ml. dose is infective, but, as in Model 2, the number of infective particles in the batch is Poissonly distributed with mean m'. Then,

$$\frac{m}{N} = 1-e^{-m'/N},$$

and, by analogy with (3),

$$P = e^{-m'n/N} = \left(1-\frac{m}{N}\right)^n. \tag{6}$$

Equations (1), (2), (3), (5) and (6) give different relationships between P, m, n and N. When m and n are very small in comparison with N (as seems to happen in practical applications), all the formulae tend to the exponential version (3), which has the additional merit of simplicity. Unless there are clear arguments in favour of one of the other models, it would seem sensible to base calculations on (3).

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An antigenic variant of the Hong Kong/68 influenza A 2 virus

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SUMMARY

Both the haemagglutinin and the neuraminidase of influenza A2 virus have undergone progressive antigenic drift since this subtype first appeared in 1957.

In 1968 a strain showing a much greater antigenic difference was isolated in Hong Kong. This variant has been responsible for epidemics throughout the world in the past 2 years.

In the second year of its prevalence a small proportion of strains isolated showed a small but definite antigenic change, related principally to the haemagglutinin component.

This new variant formed about 4 % of all strains investigated and was detected in Britain, Portugal, France and New Zealand.

The influenza A virus undergoes major antigenic changes which occur at intervals of 10 years or more. Such changes result in the appearance of a new virus subtype which shows considerable immunological differences from previously circulating strains in its surface antigens, namely the haemagglutinin and neuraminidase. Such changes are usually easily detected since they are associated with widespread epidemics which occur because the human population is entirely susceptible to the new virus subtype. Besides these major changes, minor changes known as 'antigenic drift' occur during the prevalence of each subtype. These are less easy to detect since the antigenic differences are small. The appearance of variants showing such minor antigenic changes is unlikely to be associated with extensive epidemics because the antigenic differences between the variant and its predecessor are small enough for the two viruses to show cross-immunity.

The gradual but steady drift among influenza A2 viruses since the first strains were isolated in 1957 was observed in several laboratories (Isaacs, Hart & Law, 1962; Morris *et al.* 1963; Weinberger, Buescher, McCown & Gauld, 1963; Pereira, Pereira & Law, 1964). The usual pattern has been the detection in an epidemic of a proportion of the isolated strains showing a significant difference from the majority. Thus in a single epidemic two easily distinguishable strains may be circulating together. The chances of detecting this antigenic drift depend partly on the total

number of strains available for antigenic analysis. For this reason the existence of many virus laboratories in a country, as in Britain, increases the opportunities for observing it.

Isaacs *et al.* (1962) considered that influenza A1 virus showed more antigenic variability in the first 4 years of its prevalence than influenza A2 virus, which remained antigenically uniform from 1957 until 1961, when the first small but distinct deviation was demonstrable in some strains. The replacement of the original 1957 strains by the variant was not a rapid process and both were circulating concurrently during the epidemics of both 1960–1 and 1962–3, the new variant providing in Britain about one-quarter of the total of 157 strains isolated in the first epidemic and one-quarter of 128 strains isolated in the second. However, the viruses isolated in the winter of 1963–4 showed a further antigenic change. Of the 51 strains tested in England all showed this new development. None were like the original 1957 strains, which were, in fact, never again isolated in Britain. The epidemics of the following years up to 1968 were associated with A2 strains showing only minor differences from the 1964 variant until the winter of 1967–8, when this variant was joined by another one first isolated in Tokyo in 1967 (A2/Tokyo/3/67) and showing a much increased difference. These two variants circulated in the proportion of 3:1 and together were responsible for an epidemic of considerable size. These studies on the antigenic changes in the A2 viruses have been largely based on haemagglutination-inhibition tests and the antigenic changes observed thus reflect changes in the virus haemagglutinin. More recently, comparative studies on the immunological reactions of the neuraminidases of the influenza A2 viruses have been carried out. The neuraminidase is an envelope antigen which is immunologically distinct from the haemagglutinin (Webster & Laver, 1967). Such studies provided evidence that, like the haemagglutinins, the neuraminidases of the A2 viruses have undergone progressive antigenic drift since 1957, when the first A2 strains were isolated. Thus only minor cross-reactions in neuraminidase inhibition tests were detected between the 1957 and the 1967 A2 virus strains (Coleman *et al.* 1968; Schild & Newman, 1969*a*).

The appearance of yet another variant in Hong Kong in 1968 introduced a strain showing a much greater antigenic difference in its haemagglutinin than had been observed previously among A2 variants; however, its neuraminidase was closely related to that of A2/Tokyo/67 (Coleman *et al.* 1968; Schild & Newman, 1969*b*; Schulmann & Kilbourne, 1969). A2/Hong Kong/68 behaved as a new subtype in that it spread rapidly round the world causing epidemics of greater or lesser extent, replacing completely the previous variants in the northern hemisphere, though the A2/Tokyo/67 variant was still isolated for a period in countries in the southern hemisphere.

In Britain in the winter of 1968–9, 900 strains of influenza virus were examined antigenically during the long drawn-out epidemic of influenza A2. All these strains were antigenically identical with the prototype virus A2/Hong Kong/68. In the following winter of 1969–70 over 800 strains were isolated during an epidemic of a very different kind with an explosive onset, widespread morbidity and a sharp rise in the death-rate. The majority of these strains were indistinguishable from the

strains isolated the previous year and were typical Hong Kong/68 variants. However, early in the epidemic a strain was noted (numbered A2/England/878/69) which did not conform exactly. Cross haemagglutination-inhibition tests indicated a definite antigenic change (Table 1). The degree of difference was small and

Table 1. *Cross-reactions of human A 2 viruses in haemagglutination-inhibition tests*

(Reciprocal of serum dilution producing 50 % inhibition of eight agglutinating doses of virus.)

	Ferret sera				
Virus	A2/ Singapore/ 1/57	A2/ England/ 12/64	A2/ Tokyo/ 3/67	A2/ Hong Kong/ 1/68	A2/ England/ 878/69
A2/Singapore/1/57	**5120**	1280	10	320	480
A2/England/12/64	640	**5120**	320	160	120
A2/Tokyo/3/67	80	320	**640**	40	10
A2/Hong Kong/1/68	120	40	10	**5120**	640
A2/England/878/69	120	40	10	1280	**2560**

Table 2. *Cross-reactions of human A 2 viruses in neuraminidase-inhibition tests**

	Rabbit sera		
Source of neuraminidase	Anti-purified A2/Singapore/1/57 neuraminidase	Anti-purified A2/Tokyo/67 virus	Anti-purified A2/Hong Kong/1/68 virus
A2/Singapore/1/57	**3000**†	125	100
A2/Tokyo/3/67	125	**1200**	2500
A2/Hong Kong/1/68	85	1000	3500
A2/England/878/69	75	850	3500
A0/BEL/42	< 10	< 10	< 10

* Tests performed as described by Schild & Newman (1969*b*).

† Reciprocal of serum dilution producing 50 % inhibition of 1–2 units of neuraminidase.

could well have been masked if broader-reacting antisera from hyperimmunized animals had been used instead of the highly specific post-infection ferret antisera. Neuraminidase-inhibition tests indicated that the variant contained neuraminidase which was closely related to that of A2/Hong Kong and A2/Tokyo/3/67 but which, like A2/Hong Kong, showed only minor cross-reactions with that of A2/Singapore/1/57 (Table 2). The antigenic changes shown by A2/England/878/69 thus appeared to be restricted to its haemagglutinin.

By the end of the epidemic of 1969–70, 35 strains, amounting to 4 % of the total, were found to be similar to this new variant, showing the same antigenic difference from the Hong Kong/68 strain. The first of these variants was isolated from a nurse at a London hospital, ill in December 1969, and the rest appeared irregularly throughout the following weeks. The source of the strains is shown in Table 3.

It can be seen that they form a varying proportion of the strains isolated in any laboratory and their distribution is wide. Several laboratories did not provide any of the variants.

There did not seem to be any association of these strains with the age-incidence of cases or with the severity of illness and it is unlikely that this new variant played any part in causing the greatly increased severity of the second epidemic as compared with the first.

Table 3. *Sources of strains of the new influenza virus variant*

Laboratory	No. strains sent	No. similar to the A2/England/878/69	
		Adult	Child
London Hospital	3	1	—
Stafford	21	1	1
Leicester	89	1*	1*
Worcester	28	1	2
Bath	75	1	3
Reading	2	—	1
Coventry	11	1	—
Bristol	88	3 (1*)	—
Bedford	10	—	1
Manchester	18	—	2
Cirencester	125	3	—
Nottingham	60	4 (3*)	—
Derby	15	1	—
Brompton Hospital, London	67	3	—
Guildford	6	—	1
Leeds	41	1	1
Norwich	9	1	—
Total	668	35	

* Fatal case.

A total of 1169 strains of the Hong Kong/68 virus isolated between November 1969 and June 1970 were received for identification from 34 countries throughout the world. Of these, 41 resembled the variant strain A2/England/878/69. They were made up as follows: 35 of a total of 851 from England and Wales; 1 of 53 strains from Scotland; 2 of 25 from France; 2 of 33 from Portugal; and 1 of 4 from New Zealand. It will be of considerable interest to determine which of the viruses A2/Hong Kong or its variant A2/England/878/69 becomes the predominant type of A2 virus in future epidemics of influenza. The degree of antigenic difference shown by the variant is probably not sufficient to suggest it should be included in current vaccines.

We are grateful for the co-operation of the laboratories which submitted strains of influenza virus for identification.

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A reappraisal of the role of mosquitoes in the transmission of myxomatosis in Britain

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SUMMARY

Field experiments were made in southern England to re-examine the possibility that mosquitoes in Britain might feed on wild rabbits and hence be vectors of myxomatosis. Mosquitoes of several species were attracted to rabbits enclosed in cylindrical traps and in a trap in which the animal was placed in a wire mesh cage. Substantial numbers of mosquitoes were also caught biting, or attempting to bite, tethered rabbits which were not in cages or traps. Evidence that mosquitoes fed on wild rabbits under natural conditions was obtained from results of precipitin tests made on blood-smears collected from mosquitoes caught resting amongst vegetation. On a few evenings mosquitoes were seen to be attracted to healthy wild rabbits and apparently attempting to feed on them. Batches of two mosquito species collected from the field were infected with myxoma virus.

It was concluded that contrary to previous beliefs mosquitoes in Britain feed to a certain extent on wild rabbits, and therefore are potential vectors of myxomatosis. No attempts were made to assess their relative importance in the transmission of the disease, which in Britain is transmitted mainly by the rabbit flea.

INTRODUCTION

Mosquitoes are the primary vectors of myxomatosis in wild rabbits in Australia and they are recognized as vectors in France, and are considered to be important in causing summer epizootics (Fenner & Ratcliffe, 1965; Joubert, Oudar, Mouchet & Hannoun, 1967; Roubaud cited by Jacotot, Toumanoff, Vallée & Virat, 1954), but they are not considered to be vectors amongst wild rabbits in Britain. Their unimportance in the transmission of the disease in Britain, except possibly to domestic rabbits (Muirhead-Thomson, 1956*a*), is based almost entirely on investigations undertaken soon after the disease first appeared in Britain. None of the six sentinel rabbits exposed during March to October 1954 in a wood near Edenbridge in Kent contracted myxomatosis despite the presence of large numbers of rabbits suffering from the disease (Armour & Thompson, 1955). In the same area Muirhead-Thomson (1956*b*) concluded from field experiments that mosquitoes did not feed on wild rabbits.

The principal vector in Britain is the rabbit flea, *Spilopsyllus cuniculi* (Lockley, 1954; Andrewes, Thompson & Mansi, 1959), which until now has been considered

the only important vector. However, during recent ecological studies on mosquitoes in the Poole area of Dorset (Service, 1969*a*, *b*) it became evident that mosquitoes did in fact feed on rabbits. During the next 4 years further information was obtained concerning the ability of mosquitoes to feed on wild rabbits; results are presented here.

RABBIT-BAITED TRAPS

Cylindrical traps

Two metal cylinders about 35 cm. long, 25 cm. in diameter and with inwardly projecting wire mesh funnels at both ends were each baited with a rabbit and placed amongst vegetation on Brownsea Island in Poole Harbour, Dorset. Detailed results of these, and other trapping methods, have been given already (Service, 1969*b*); summarized results are that from 38 catches during June–September, 5 *Anopheles plumbeus* Stephens, 8 *Aëdes punctor* (Kirby), 166 *Aë. detritus* (Haliday), 6 *Aë. cinereus* Meigen, 36 *Culiseta annulata* (Schrank), 205 *C. morsitans* (Theobald), 102 *Culex pipiens* L., 4 *C. torrentium* Martini and 448 *Mansonia richiardii* (Ficalbi) were caught. None of the adults of *Culex torrentium* and very few of those of *Culiseta morsitans* and *Culex pipiens* had fed on the rabbits in the traps. All three species are known to be avian feeders (Service, 1969*a*), and it is thought that they may have been attracted to the trap by a localized artificially high concentration of carbon dioxide or temperature. Several adults of the other species, which normally feed mainly on mammals, were shown by precipitin tests to have fed on the rabbits.

Baited suction traps

Because some types of animal-baited traps, including the design just described, may not give representative samples of the mosquito species normally feeding on the bait animals, a new type of trap was designed to present a rabbit under more natural conditions (Plate 1). This consisted of a cage measuring $38 \times 38 \times 50$ cm. with the four sides and top covered with 1 in. wire netting and was baited with a rabbit and placed about 35 cm. from the ground amongst scrub vegetation in Monks Wood Nature Reserve, Huntingdon. One end of a 1·75 m. length of 25 cm. diameter flexible tubing rested on top of the cage, while the other end was connected to the top of a 9 in. (23 cm.) diameter Johnson–Taylor suction trap (Johnson, 1950). The fan of the suction trap operated on a repetitive cycle of 7 min. off and 3 min. on. This allowed host-seeking mosquitoes to be attracted to the rabbit, after which they were sucked into the collecting magazine situated at the base of the suction trap. An electrical timing device allowed metal disks to drop and segregate the catch into 50 min. samples. Because mosquitoes in the area were most active at night the trap was operated from 1600 to 0900 hr. B.S.T.

Since unbaited suction traps have been used successfully to sample mosquito populations (Service, 1969*c*), the trap was operated on 8 nights without rabbits to discover whether those caught represented just randomly flying mosquitoes or those definitely attracted to the rabbit. Only two unfed *Aë. cantans* were caught in these unbaited catches, whereas in 20 catches during June–August, 1970, with a rabbit, 383 *Aë. cantans*, 88 *Aë. geniculatus* (Olivier), 1 *Aë. rusticus* (Rossi), 77 *An.*

plumbeus, 1 *An. claviger* Meigen and 5 *Culiseta annulata* and 18 *M. richiardii* were collected. All were females and most were unfed, but a few blood-fed adults of *Aë. cantans*, *Aë. geniculatus* and *M. richiardii* were caught. Precipitin tests on 9 *Aë. cantans*, 3 *Aë. geniculatus* and 1 *M. richiardii* showed they had fed on rabbits.

Segregation of the catch into 50 min. intervals showed that 77 % of *Aë. cantans*, 62 % of *Aë. geniculatus* and 71 % of *An. plumbeus* were attracted to the rabbit between 1740 and 2010 hr. Only 10 % of the mosquitoes caught were collected after 2100 hr. Mosquitoes appeared to feed on rabbits mainly in the early evening, at a time when they are active above ground.

TETHERED RABBITS

In Australia Myers (1956) collected mosquitoes attracted to rabbits by lowering cone-shaped traps at intervals over rabbits firmly pinioned to a board. Although the rabbits were not enclosed in traps they nevertheless had very little freedom of movement. To try and catch mosquitoes attracted to rabbits presented in a more natural manner, a rabbit was tied by 1 m. length of lead to a tree and visited at 10 min. intervals so that any mosquitoes attracted to it could be caught in aspirators. Such bait rabbits had considerable freedom of movement. In the first series of 9 catches on Brownsea Island, 7 *An. plumbeus*, 7 *Ae. punctor*, 164 *Aë. detritus*, 15 *Culiseta annulata*, 12 *M. richiardii* and 1 *C. morsitans* were caught (Service, 1969*b*). In four new catches in a small copse at Arne Peninsular in Dorset, 108 *Aë cantans*, 57 *Aë. punctor*, 28 *Aë. cinereus*, 6 *Aë. detritus*, 4 *Culiseta annulata*, 11 *An. claviger*, and 18 *Aë. geniculatus* were caught. This gave further evidence that British mosquitoes are attracted to rabbits.

PRECIPITIN TESTS

An alternative method to using traps for determining whether mosquitoes feed on rabbits is to identify by precipitin tests blood-meals of engorged mosquitoes collected from natural resting places. Lee, Clinton & O'Gower (1954) used the method in Australia to discover the species feeding on rabbits. Blood-fed mosquitoes were collected, therefore, by sweeping various types of vegetation in areas of southern England where rabbits were known to occur, and Dr P. F. L. Boreham kindly agreed to perform precipitin tests on the blood-fed mosquitoes caught. The following lists show the numbers of blood-meals positive for rabbit blood out of the totals tested from different areas. **Woodwalton Fen and Monks Wood, Huntingdon:** 25/26 *Aë. cantans*, 0/1 *Aë. annulipes*, 0/2 *An. maculipennis* complex, 4/4 *Aë. cinereus*. **Surlingham Fen, Norfolk:** 14/14 *An. claviger*. **Arne and Studland Heath, Dorset:** 7/77 *Aë. cantans*, 0/7 *Aë. detritus*, 0/11 *An. plumbeus*, 0/3 *Aë. geniculatus*, 1/16 *Culiseta morsitans*, 0/29 *M. richiardii*, 1/2 *Culex pipiens* or *C. molestus* Forskål, 3/149 *Aë. cinereus*, 0/195 *Aë. punctor*, 0/20 *C. annulata*, 5/40 *Aë. dorsalis*, 0/5 *An. claviger* and 0/6 *Aë. annulipes*.

Six hundred and fifty-five blood-smears from 14 species were tested and 58 of these from six species were positive for rabbit blood. It is just conceivable, though I consider it most unlikely, that these feeds on rabbits were from lethargic animals suffering from myxomatosis, and that mosquitoes cannot feed on healthy active

wild rabbits. A search for blood-fed mosquitoes was made therefore on Lundy Island which is 20 km. from the mainland of Devon, and which has a very large myxomatosis-free rabbit population. Unfortunately only 12 blood-fed *Aë. punctor* were found, but precipitin tests showed that six of these had in fact fed on rabbits; two of these had also fed on other mammals. It is inconceivable that all these had fed on the mainland on diseased rabbits, and subsequently dispersed to the island. Further evidence was therefore obtained of mosquitoes feeding on healthy rabbits.

NATURAL INFECTIONS OF VIRUS IN MOSQUITOES

In France myxoma virus has been found in wild caught adults of *Aë. caspius* (Pallas), *Aë. detritus*, *An. maculipennis* complex and *C. modestus* (Ficalbi); the first species being considered the most important vector of summer epizootics of myxomatosis in the Camargue area (Joubert *et al.* 1967). In England Muirhead-Thomson found natural infections in *Aë. cantans* and *Aë. annulipes* (1956*b*) and in *An. labranchiae atroparvus* van Thiel (1956*a*), and I have caught adults of *Aë. cantans* from Monks Wood and *An. claviger* from Surlingham which were found to be infected with myxoma virus by Dr J. Ross of the Infestation Control Laboratories of the Ministry of Agriculture, Fisheries and Food at Worplesdon. As transmission by arthropods is apparently mechanical (Fenner & Ratcliffe, 1965) then any mosquito feeding on rabbits is a potential vector. The presence of the virus in mosquitoes only indicates that the species concerned are attracted to rabbits and can feed at least on those infected with myxomatosis.

DIRECT OBSERVATIONS

On Brownsea Island adults of *Aë. detritus* and *M. richiardii* were often observed feeding on tame rabbits kept out of doors in rabbit hutches. Because of the rabbit's movements and twitchings they appeared to have some difficulty in getting a complete blood-meal, and many were seen to probe at several sites before becoming replete. As observed in France by Jacotot *et al.* (1954) nearly all biting was restricted to around the eyes and nose.

It is difficult to observe mosquitoes feeding on wild rabbits, but on several evenings at Arne in Dorset and on one evening on Lundy Island mosquitoes were observed hovering over and settling on the heads of grazing rabbits. It seems reasonable to assume that they were attempting to feed on them.

DISCUSSION

The rabbit flea, an important vector of myxomatosis in Europe, is absent from Australia where the most important vectors are *Culex annulirostris* Skuse, *C. pipiens australicus* Dobrotworsky and Drummond, and *An. annulipes* Walker. The last mosquito is a particularly well adapted vector as adults rest during the day in rabbit burrows, and feed on rabbits both above and below ground (Myers, 1956). In England mosquitoes, including blood-fed specimens, have been found in a fox hole by Shute (Service, 1969*a*), but Muirhead-Thomson (1956*b*) was unable to

find any in rabbit burrows. I have examined rabbit burrows on many occasions but have found mosquitoes in them only once. This was during a very dry spell in the summer when *Aëdes* species were resting in several burrows in Monks Wood. It rained heavily in the evening and none could be found on the following days. Normally adults of British *Aëdes* species rest during the day amongst the shelter provided by grassy and scrub vegetation, but it is possible that they retreat to rodent holes during adverse dry weather. It is not known whether they feed on rabbits in their burrows.

Results from experiments with baited and tethered rabbits indicate that British mosquitoes are attracted to rabbits, and direct observations on feeding and the identification of natural hosts by precipitin tests proves without doubt that mosquitoes do feed on rabbits. This conclusion disagrees with those reached by Muirhead-Thomson (1956*b*). Working in a wood harbouring large numbers of man-biting *Aëdes* species, he found no evidence that they were attracted to rabbits either in, or outside, traps. He considered that the identification of blood-meals from wild caught mosquitoes would give the most convincing demonstration of any natural feeding on wild rabbits. Unfortunately he was unable to find any blood-engorged specimens. The reasons why the rabbit-baited traps of Muirhead-Thomson failed to attract mosquitoes and why he was unable to find blood-fed specimens is not understood. It is possible, however, that trap design and siting are important. I have found that within woods there are favoured, and often very selective, sites and types of vegetation in which mosquitoes rest during the day, and Muirhead-Thomson may have failed to locate these sites.

It is established from the present study that mosquitoes in Britain can feed on healthy wild rabbits, but their importance as vectors of myxomatosis depends on several factors. For example, examination of the results of precipitin tests indicates that rabbits were preferred hosts when there were few, or no, alternative larger hosts, such as domestic livestock (e.g. in Monks Wood and at Surlingham) but when these were present (e.g. at Arne) considerably fewer appeared to have fed on rabbits. The incidence of feeding on rabbits may therefore depend on the availability of alternative hosts. It is also possible that in areas where myxomatosis is present mosquitoes may find it easier to feed on comatose diseased rabbits than healthy ones, thus leading to a disproportionate number of feeds on diseased rabbits. A high percentage of mosquitoes will consequently become infected, but the frequency of transmission to healthy individuals may be lowered. On the other hand, because mosquitoes seem to need several probes on the same, or different rabbits, before getting a complete blood-meal the chances of transmission should be increased.

The time during June to September in England required for blood digestion and ovarian development is about 5–7 days for those species investigated (Service, 1968). It is not known whether there is any marked delay between the completion of ovarian development and oviposition, or between oviposition and refeeding, but it is generally assumed that these intervals are minimal in mosquitoes. If so, then adults will take blood-meals at about weekly intervals. Age grading techniques on British species have shown that large numbers of mosquitoes feed at least twice in

their lifetime, and a number feed four or more times. Because of this longevity and the fact that myxoma virus can remain infective on mosquitoes for nearly 6 weeks in the summer and possibly much longer in the cooler winter months (Andrewes, Muirhead-Thomson & Stevenson, 1954), there is ample opportunity for mosquitoes to become infected. Mosquitoes feeding through normal rabbit skin do not become infective but those feeding on primary lesions or on well-developed secondary lesions nearly always do (Fenner & Woodroofe, 1953; Fenner, Day & Woodroofe, 1952, 1956). Since mosquitoes mainly feed around the eyes and nose of rabbits, regions in which tumours and lesions usually occur, they therefore select the most infective areas.

There have been no detailed studies on the distances covered by mosquitoes in Britain, although there are a few records of individuals travelling at least 4–8 km. (Covell & Shute, 1962; Service, 1969*a*) and up to 5 km. soon after taking a blood-meal (Service, 1969*a*). It is likely that mosquitoes in Britain disperse greater distances. It is possible that the sudden re-appearance of myxomatosis in areas from which it has been absent for some time is due to re-infection by mosquitoes originating from areas harbouring the disease.

The above experiments have shown, contrary to previous belief, that in Britain mosquitoes feed on healthy rabbits. Therefore the possibility that they transmit myxomatosis to wild rabbits should be reconsidered.

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EXPLANATION OF PLATE

Plate 1. Suction trap fitted with flexible tubing for catching mosquitoes attracted to rabbits.

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Nasal acquisition of *Staphylococcus aureus* in partly divided wards

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SUMMARY

The spread of coagulase-positive staphylococci has been studied in a modern hospital in which most of the patients were nursed in 4-bed rooms separated from a common corridor only by low dividing walls. Acquisition of new nasal strains from patients in other bedrooms was nearly as easy as from patients in the neighbouring beds. There was no indication that subdivision of this type hindered the spread of nasal strains as compared with open wards of the 'Nightingale' pattern.

INTRODUCTION

In recent years most newly built hospital wards have been subdivided in one way or another. This has been done for aesthetic, management or hygienic reasons and it is not known to what extent, if any, the different forms of subdivision may affect the risk to a patient of acquiring an infection. As part of an extended study of this question a survey of nasal carriage and acquisition of *Staphylococcus aureus* in two medical wards of the Queen Elizabeth II Hospital, Welwyn Garden City, was carried out during the two years 1965–6.

METHODS

Organization

Both the wards studied contained 29 beds distributed in six 4-bed rooms and five 1-bed rooms (Fig. 1). The latter were proper rooms completely separate from the rest of the ward, but the 4-bed rooms had only three complete walls, the fourth side being open to the corridor except for low dividing walls, about 3 ft. high, on each side of the entrance from the ward corridor.

Bacteriological samples were taken and the patient records kept by a nurse

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investigator appointed for the purpose. The samples were examined and primary isolations made by an experienced laboratory technician also appointed for the purpose. Both worked at the hospital under the general direction of the hospital clinical pathologist. Strains of *Staphylococcus aureus* were sent to the Central Public Health Laboratory for phage typing.

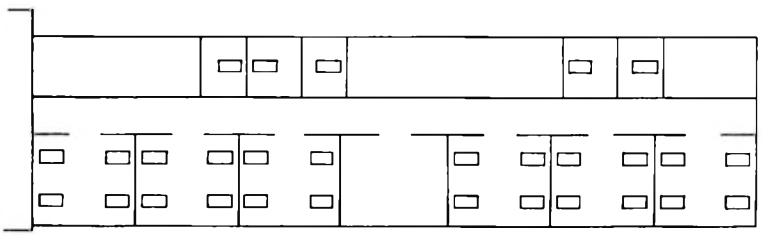

Fig. 1. Plan of one of the wards (the other was a mirror image on the same floor of the building on the left-hand side of the central area).

Observations in the wards

Nasal swabbing

Both nostrils were sampled with one dry swab, two circular excursions being made around each nasal vestibule.

Patients. A nasal swab was taken as soon as possible after the patient was admitted to the ward. If this was not done within 3 days of admission the patient was considered not to have had an admission swab. Subsequently a swab was taken from each patient on a set day every week. A patient was defined as anyone who spent a night in a hospital bed.

Staff. A nasal swab was taken weekly on a set day whenever possible. Staff regularly absent on the swabbing day were swabbed on another convenient day with the object of spacing the swabbing evenly. Anyone working in the ward more than 6 hr. a week was considered to be staff.

Air sampling

On the day on which the weekly nasal swabs were taken from the patients, $5\frac{1}{2}$ in. diameter (14 cm) Petri dishes filled with phenolphthalein-phosphate serum agar were exposed in each ward. Four Petri dishes were exposed for 8 hr. during the day and four more for 16 hr. during the following night. The four rooms examined each week were selected from the 11 rooms in each ward according to a randomized scheme, two being 1-bed and two 4-bed rooms.

General information

Records were made daily of the bed position of individual patients in the wards, of their mobility (i.e. whether confined to bed, up for toilet purposes only or fully ambulant) and of antibiotic treatment.

Analysis

Assessment of acquisition of new strains of *Staphylococcus aureus* in the noses of the patients was made in the same way and using similar conventions to those employed in previous studies (Lidwell *et al.* 1966). An attempt was also made to assess the effect of such factors as the age, sex, disease and nasal carriage state of the patient, as well as antibiotic treatment and environmental circumstances, on the nasal acquisition rate. Since many of these factors are intercorrelated the data were examined using a stepwise multiregression analysis programme, BMD 02R (Biomedical Series, 1965). The unit of data for this analysis was the patient-week of exposure in the ward. In earlier studies the interval between admission and the first weekly nasal swab had been regarded as equivalent to a full week for purposes of analysis, although it is in fact variable and averaged only $3\frac{1}{2}$ days. Separate analyses were performed in the present instance recording the above interval as either equivalent to a whole week or as equivalent to only one-half a week's exposure in the ward. The computations were carried out at the Medical Research Council's Computer Unit.

RESULTS

Nasal carriage rates

The nasal carriage state of the patients after varying length of stay in the ward is shown in Fig. 2. Both the overall carriage rate and in particular carriage of strains resistant to tetracycline increased with time in the ward.

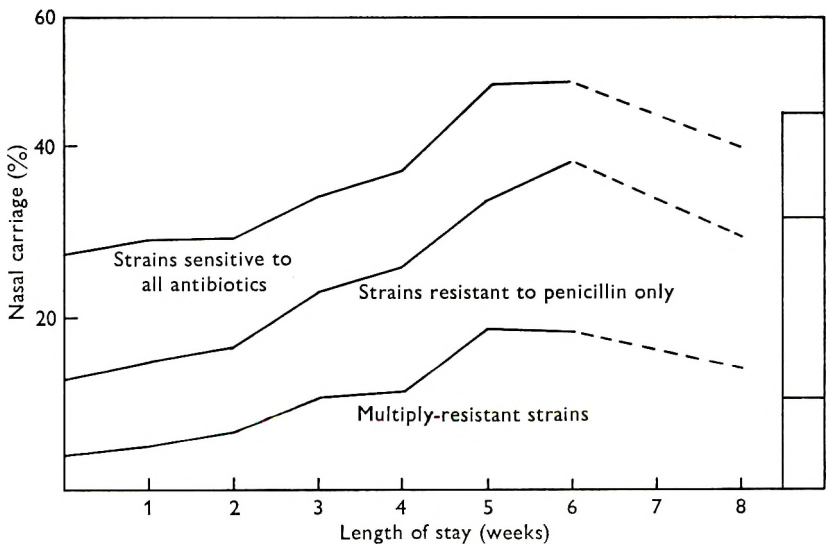

Fig. 2. Change in nasal carriage of *Staph. aureus* during hospital stay. The top line shows the percentage of patients carrying *Staph. aureus* in their noses after varying lengths of stay in the ward. The bottom line shows the percentage carrying strains resistant to tetracycline. The middle line shows the percentage carrying strains resistant to penicillin together with the small proportion carrying strains sensitive to penicillin but resistant to tetracycline. The histogram shows the average rate of nasal carriage by members of the staff.

Distribution of airborne Staphylococcus aureus

The results of the air sampling are shown in Table 1. As the air samples were taken on the same day as the patients noses were swabbed the carriage state of each patient at this time was known. Each colony isolated from an air-sampling plate, or each group of colonies indistinguishable by phage typing, was then considered in relation to the strains carried by the patients and the staff. About 16 % of the colonies isolated could not be related to any known source in the ward. In the case of rather more than 50 % of colonies there was more than one possible

Table 1. *Number and sources of airborne strains of* Staphylococcus aureus

Probable source	No. settling per 1000 sq.ft. min.		No. of possible sources			No. settling per possible source per 1000 sq.ft. min.	
				Carriers			
	All strains	T strains	Persons	All strains	T strains	All strains	T strains
Patient(s) in same room or bay							
Single rooms	48·2	3·9	1·0	0·4	0·1	120	39
4-Bed bays	15·6	1·5	3·56	1·2	0·3	12·9	5·0
Patient(s) in other rooms or bays	16·6	9·0	22·3	7·0	1·7	2·4	5·3
Staff carriers	9·0	4·0	—	10·3	2·5	0·9	1·6
No known source	8·7	3·7	—	—	—	—	—
Total	55·1	18·6	—	19·0	4·4	3·1	4·2

T strains = strains resistant to tetracycline.

The numbers of possible sources are average over the period of observations.

In forming the totals the values for the 4-bed bays and the single rooms have been weighted in proportion to the number of patient-weeks of experience in the different locations.

1000 sq.ft./min. = 93 m^2/min.

source. In compiling the table these have been allocated to the different sources in the same proportion as the distribution of those from unambiguously located possible sources. The much larger number of colonies found in the single rooms, and probably originating from a patient carrier in that room (48·2/1000 sq.ft. min.), compared with that found in the 4-bed bays, probably originating from patient carriers in beds in the same bay (15·6), is a reflexion of the much smaller air volume into which the organisms were dispersed in the single rooms. The central three columns in Table 1 show the spatial distribution of the carriers who could have acted as sources for the strains found in the air samples. The fractional figures arise from averaging the numbers over the period of observation; that is, the average bed occupancy in the 4-bed bays was 89 %, hence the average number of persons in these bays was $4 \times 0{\cdot}89 = 3{\cdot}56$ (single rooms were included only when they were occupied). Since the average carrier state among the patients was a little over 30 % for all strains and about 8 % for strains resistant to tetracycline, calculation then gives the average number of carriers shown in the different situations, e.g. 30 % of 22·3 $\simeq$ 7·0 (small differences arise from the different rates of

carriage found among patients nursed in the different rooms of the ward). The actual number of staff was variable and records were only kept of the numbers of nasal carriers. Dividing the numbers of colonies found in the air samples by the number of possible sources from which it might be presumed they had derived produced the figures given in the last two columns of the table; e.g. the number of colonies of strains resistant to tetracycline probably arising from patient carriers being nursed in beds in other parts of the ward than that where the sample was taken was 9·0/1000 sq.ft. min. There were, on average, 1·7 nasal carriers of such strains among the patients in beds in other parts of the ward. The average number of colonies of these strains reaching the sampling position from a single patient carrier in other parts of the ward was then 9·0/1·7 = 5·3/1000 sq.ft. min. Overall, a patient in a 4-bed bay was exposed to airborne staphylococci from a carrier in the same room in numbers about 5 times as great as from a carrier in another part of the ward. The dispersal of tetracycline strains, however, appeared to be more uniform throughout the ward area.

The exposure of a patient in a single room to airborne staphylococci from patient carriers elsewhere in the ward (not given separately in the table) was actually greater than that of patients in the 4-bed bays. The day-time numbers of *Staph. aureus* in the air were about twice the night-time values.

Nasal acquisition of Staphylococcus aureus

The rates of acquisition of new strains of *Staph. aureus* are given in Table 2. Of the 257 apparent acquisitions on which the table is based, 91 (35 %) could not be related to known sources in the ward, although 18 of these were with strains identical with strains present at the same time in the other of the two wards studied. Thus 73 (28 %) apparent acquisitions were without any known possible source.

Table 2. *Rates of nasal acquisition of* Staphylococcus aureus

	Acquisition rate per 1000 patient-weeks		No. of possible sources (carriers)		Acquisition rate per possible source per 1000 patient-weeks	
Probable source	All strains	T strains	All strains	T strains	All strains	T strains
Other patients in same bay	3·4	1·3	0·8	0·2	4·3	6·5
Patients in other rooms or bays	22·0	11·0	7·0	1·7	3·2	6·5
Staff carriers	24·6	10·7	10·3	2·5	2·4	4·3
All known possible sources	50·0	22·9	18·0	4·4	2·8	5·2
No known source	27·3	6·3	—	—	—	—
Probably real	6·9	3·3	—	—	—	—
Probably spurious	20·4	3·0	—	—	—	—
Total	77·4	29·0	—	—	—	—

The total experience comprised 3327 patient-weeks, of which 2750 was in 4-bed bays (the interval between the admission swabs and the 1st regular weekly swab has been counted as a full week). There were 257 apparent acquisitions.

When the distribution of antibiotic sensitivity among the apparent acquisitions without sources is considered (see Lidwell *et al.* 1966) it seems probable that no more than 23 represent genuine acquisitions of new strains and that the remaining 50 are probably spurious, due to failure to isolate the strain on the previous swabbing. Overall, for 81 acquisitions there was more than one possible source. These have been allocated in compiling the table in the same way as was done for air strains in Table 1. The central two columns in the table show, in the same way as the corresponding columns in Table 1, the spatial distribution of carriers from whom the acquisitions might have derived. Since a patient cannot be the source of his own acquisition the number of possible sources is less than those given for the airborne samples, e.g. there can be no source in the same room for a patient in a single room and only three at most in the same bay for a patient in a 4-bed bay. The actual figures for bed occupance bring this down to $3{\cdot}56-1 = 2{\cdot}56$ and the average number of carriers who could have been sources becomes $1{\cdot}2(2{\cdot}56/3{\cdot}65) = 0{\cdot}86$ (rounding-off errors account for the difference from the more accurate figure of 0·8 given in Table 2). The corresponding value for those carrying tetracycline-resistant strains is similarly given by $0{\cdot}3\ (2{\cdot}56/3{\cdot}56) = 0{\cdot}2$. The figures in the last two columns are then derived, as in Table 1, by dividing the acquisition rates by the number of carriers from whom acquisitions of the kind could have arisen. For example, the rate of acquisition, all strains, probably arising from nasal carriers among the staff, was 24·6/1000 patient-weeks, and the average number of staff carriers was 10·3. Hence the acquisition rate from a single staff carrier was $24{\cdot}6/10{\cdot}3 = 2{\cdot}4$/1000 patient-weeks. It is clear that acquisition from a patient carrier in another part of the ward, 3·2/1000 patient-weeks, was almost as likely as when the carrier was in the same 4-bed bay, 4·3/1000 patient-weeks.

Patients in the single rooms acquired strains from other patients at almost the same rate as patients in the other parts of the ward (23·9/1000 weeks compared with 24·1/1000 weeks). These figures are not shown in the table.

Exposure to airborne staphylococci and risk of nasal acquisition

When the rates of nasal acquisition from single sources (shown in the two right-hand columns of Table 2) are plotted against exposure to airborne *Staph. aureus* derived from individual source carriers a consistent relationship is obtained (Fig. 3). As in the previous studies in which this method of analysis was employed (Lidwell *et al.* 1966; Lidwell *et al.* 1970) the overall risk of nasal acquisition increases much less than proportionally to the increase in exposure and is considerably greater for a given airborne exposure in the case of the tetracycline-resistant strains. The slope of the line drawn in the Fig. 3 is 0·22, compared with 0·20 and 0·6 in the two studies referred to above, and the ratio of the rates of acquisition of tetracycline-resistant strains to that for all strains at similar levels of airborne exposure is 1·7, compared with 2·7 and rather over 2.

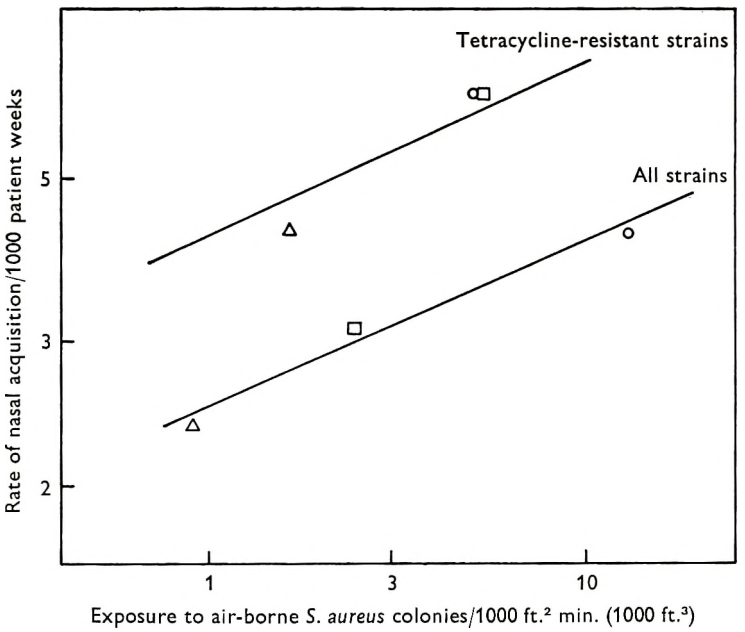

Fig. 3. Relation between risk of nasal acquisition and exposure to airborne *Staph. aureus*. Logarithmic scales for both coordinates. Risk of nasal acquisition: per potential source (carrier) per 1000 patient-weeks. Exposure to airborne *Staph. aureus*: colony count per carrier per 1000 sq. ft (93 m.²) minutes of exposed settling plates – approximately equivalent to 1000 cu. ft. (29 m.³) of air. ○, Acquisitions from other patients in the same bay; □, acquisitions from other patients in other parts of the ward; △, acquisitions from staff carriers. The lower line refers to all strains of *Staph. aureus*, the upper line to those strains resistant to tetracycline.

Effect of patient and environmental factors on nasal carriage and acquisition

The following factors were considered in relation to nasal carriage and nasal acquisition of *Staph. aureus*, and where no assessment of the effect of any one of them is given in the tables it implies that this was not significant, i.e. it did not reach its estimated standard error. The factors were: age, sex, diagnosis, nasal-carriage state (for effect on acquisition of new strains), antibiotic treatment, week of stay in ward, mobility, nursed in a 1-bed room, and week preceding death (for patients who died in hospital). The diagnostic groups considered were (*a*) diseases of the heart and circulatory system, (*b*) diseases of the respiratory system, (*c*) diseases involving the stomach and duodenum, (*d*) diseases of the cerebral vascular system, (*e*) disorders of the urogenital system, (*f*) skin conditions, (*g*) neoplasm, (*h*) diabetes, (*i*) rheumatoid arthritis, (*j*) other conditions not comprised in any of the above groups. Table 3 shows the differences in nasal carriage rates in relation to some of the above factors. Carriage rates for tetracycline-resistant strains but not for all strains together were clearly higher in the elderly, in those receiving antibiotics and during the week preceding death. There were also substantial differences, especially in relation to tetracycline-resistant strains, for some diagnostic groups, although the number of individuals involved was sometimes rather small. The effects of length of stay in the ward have already been presented in Fig. 2.

A similar examination of the rates of nasal acquisition is presented in Table 4. The rates are greater for the elderly and very much greater with regard to acquisition of tetracycline-resistant strains among patients receiving antibiotics. It is clear that the elderly, for example, often stay longer in the ward and may be liable to receive antibiotics more often than younger patients. The multiregression analysis was undertaken in order to determine which of the effects apparent in the simple analysis represented independent influences. This is particularly relevant

Table 3. *Association of various factors with the mean nasal-carriage rate*

Factor	Reference group	Relative carriage rate	
		All strains	T strains
Age			
Under 40	40–60	1·1	1·2
Over 60	40–60	1·1	2·5
Sex, female	Males	1·0	0·9
Diagnosis			
A	All patients	1·1–1·8	1·2–3·0
B	All patients	0·9–1·2	0·8–1·3
C	All patients	0·2	0·8
Received penicillin	Received no antibiotics	0·9	2·1
Received antibiotic other than penicillin	Received no antibiotics	0·9	2·5
In single room	In 4-bed bay	1·2	1·4
Week before death	All patients	1·2	2·3

The figures are the ratio of the carriage rates in the 'factor' groups to those in the reference groups.

Diagnostic set A includes skin conditions (41), diabetes (48), disorders of the urogenital system (108) and diseases of the cerebrovascular system (535). Set B includes respiratory diseases (510), diseases of the stomach and duodenum (124), neoplasm (286) and diseases of the heart and circulatory system (885), together with conditions not specifically classified (1181). Set C was rheumatoid arthritis (132). The numbers in parentheses give the patient-weeks experience for each group. The diagnoses are here listed in order of decreasing carriage rate of T (tetracycline-resistant) strains. The range of values given for the relative carriage rates is the spread for the several diagnoses included in the particular set.

Table 4. *Association of various factors with the nasal-acquisition rate*

Factor	Reference group	Relative acquisition rate	
		All strains	T strains
Age, over 60	Under 60	1·4	1·6
Received penicillin	Received no antibiotics	1·0 (0·6)	2·6
Received other antibiotic	Received no antibiotics	1·3 (0 5)	3·9
In single room	In 4-bed bay	1·1	1·3
Confined to bed	All patients	0·9	—
Fully ambulant	All patients	1·0	—
Week before death	All patients	0·8	1·0

The figures are the ratio of the nasal acquisition rates in the 'factor' groups to those in the reference groups.

The figures in parentheses are the relative acquisition rates for strains sensitive to all antibiotics or resistant to penicillin only.

to the comparison of cross-infection in hospitals of differing layout and construction as it will be essential to take into account any differences in the patients or their treatment that can be shown to influence their acquisition of new nasal strains of *Staph. aureus*.

Table 5. *Factors influencing the rate of nasal acquisition of* Staphylococcus aureus: *coefficients of the multiregression analysis*

		Probable sources					
		All known sources		Other patient carriers		No known source	
Factor	Proportion (%)	S+P strains	T strains	S+P strains	T strains	S+P strains	T strains
Age							
Under 60	57	**25**	**42**	6	3	(−1)	6
Over 60	43	11	**32**	(4)	(2)	(−2)	(4)
Diagnostic group							
Heart and circulation	25	—	—	(−6)	—	—	(−5)
Respiratory	15	—	—	—	**12**	—	—
Cerebro-vascular	16	(12)	—	—	—	17	—
Neoplasm	8	**34**	—	—	—	—	—
Others (except skin conditions and unclassified)	12	—	(−14)	(−7)	—	—	—
In single room	13	—	—	12	—	(11)	—
Non-carrier	68	—	—	—	—	—	—
Received no antibiotics	43	(10)	(−12)	10	—	**19**	—
Received antibiotics (other than penicillin)	15	—	**20**	—	5	—	**12**
Carrier	32	—	—	—	—	—	—
Received no antibiotics	21	—	−27	—	—	—	—
Received antibiotics (other than penicillin)	7	(−18)	—	—	—	—	13
1st week in ward	35	—	−14	—	—	**29**	—
Mean acquisition rate	—	27	23	9	5	21	7
Multiple correlation coefficient	—	0·18	0·19	0·12	0·10	0·19	0·11
No. of acquisitions	[257]	88	77	28	17	70	22

The figures in the table, apart from the last three rows and the first two columns, are the coefficients of the linear regression equation giving the nasal acquisition rate per 1000 patient-weeks. The values in bold figures exceed three times their standard errors while the values in parentheses lie between 1 and 2 times their standard error. A dash indicates that the regression analysis terminated without involving the factor concerned, none of those omitted attaining a regression coefficient equal to or greater than its standard error if included in the analysis.

S+P strains are those sensitive to all antibiotics or resistant to penicillin only. T strains are those resistant to tetracycline. The strains were only tested against penicillin and tetracycline.

The results of the computer analysis are given in Table 5. The second column shows the proportion of the population (each patient-week record is a separate unit) to whom the factor in the first column relates, e.g. 57 % of the patient-week records related to patients under 60; the 32 % with positive nasal swabs (carriers) included 7 % who had received an antibiotic, other than penicillin, at some time during the preceding week. The rate of acquisition among elderly patients was greater than among patients under 60, independently of the effect of other factors. The difference is equivalent to a rate of acquisition between 1½ and 2 times greater. The substantial increase in the rate of acquisition during the first week of stay in the ward of strains sensitive to all antibiotics or resistant to penicillin only, which could not be related to any known source, confirms the impression that a high proportion of these were spurious. No improvement in the overall correlation was obtained by treating the interval between admission and the first weekly swab as a half rather than a whole week of patient stay. The figures given in Table 5 and elsewhere are therefore based on the earlier practice of treating the interval between admission swab and first regular weekly swab as equivalent to the intervals between weekly swabs and recording these as 'weeks'. Nasal carriers not receiving antibiotics were much less likely and non-carriers receiving antibiotics were much more likely to acquire resistant strains. The only other notable factor apparently affecting the rate of nasal acquisition was the increase in this rate among cancer patients with regard to strains fully sensitive to antibiotics or resistant to penicillin only.

DISCUSSION

The increase in nasal carriage rates with length of stay in the ward, the fairly high rates of acquisition of new strains, over three times that found in the divided thoracic surgery ward (Lidwell *et al.* 1966), and the relatively small differences between the risk of acquisition from a carrier in a nearby bed, i.e. in the same 4-bed bay, or from one in a more remote part of the ward are all characteristics common to the pattern of nasal acquisition with *Staph. aureus* in open wards of the 'Nightingale' pattern.

This confirms the belief that the partial subdivision of a ward, as in the Queen Elizabeth II Hospital, is without effect on the spread of infection.

The nursing of patients in single rooms opening off the general ward area and not mechanically ventilated also appears to have had no detectable effect on the spread of staphylococci to these patients.

The multiregression analysis of factors that might affect the rates of nasal acquisition did not produce any substantial additional information but confirmed that age and antibiotic treatment are the most important factors so far discovered.

Our thanks are due to the patients and staff of the hospital, to Mrs Vick, Mrs Grinrod and Mrs Peirce who collected the bacteriological specimens and recorded patient and staff data, and to the North West Metropolitan Regional Hospital Board for their co-operation and financial support from their research funds.

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Infections in a hospital for patients with diseases of the skin

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SUMMARY

Studies on infections in a hospital for diseases of the skin are described. Patients were shown to acquire staphylococci in the groin and on the chest at about the same rate as in the nose. In contrast to surgical wards, many staphylococci were resistant to tetracycline but sensitive to penicillin. Nevertheless, much of the epidemic spread of staphylococci was with typical surgical-ward strains rather than with phage group II strains which might be thought typical of skin diseases.

INTRODUCTION

Much has been written on the incidence and mechanism of cross-infection in surgical patients and a little has been carried out on patients with diseases of the skin (Biro, Gibbs & Leider, 1960; Biro, Buchbinder *et al.* 1960; Selwyn, 1963, 1965; Hellerstrom, Linneroth & Nilzen, 1966). Such patients present many problems in the field of cross-infection for they may carry large numbers of potentially pathogenic bacteria on apparently normal skin; they may be admitted to hospital already carrying pathogenic bacteria on their lesions; and they may be more susceptible to colonization and infection whilst in hospital than are surgical patients.

This paper reports some of the findings which have emerged from studies on the transmission of organisms within a hospital for patients with diseases of the skin.

MATERIALS AND METHODS

During the period of this study the hospital had four wards each housing 16 or 17 patients. These wards were of the conventional open type. In addition, each of the two female wards had a single-bed side ward attached to it. The two wards for males, A and B, were on the ground floor and shared a treatment room situated between the two wards. Although there was no direct contact between the patients in this treatment room there was ample opportunity for airborne spread of bacteria to occur. One ward for females (C) was situated on the floor above wards A and B. The fourth ward (D) was in a different wing of the hospital. Wards C and D did not share any facilities.

Initially, patients were swabbed once a week, but this was later increased to

twice a week (Fig. 1) and throughout this paper the results are based on a twice-weekly swabbing regimen.

Because it seemed likely that patients would carry pathogenic organisms at sites other than the nose, the patients were swabbed in the nose, chest and groin using cotton-tipped swabs moistened with broth. An attempt was made to obtain true perineal swabs but this proved socially unacceptable and the groin was sampled instead. The chest was used as a convenient site of normal glabrous skin; skin lesions were avoided, an attempt being made always to sample normal skin. Swabs were inoculated on blood agar plates and incubated aerobically at 37° C. for 18–24 hr. Potentially pathogenic organisms were tested for sensitivity to a

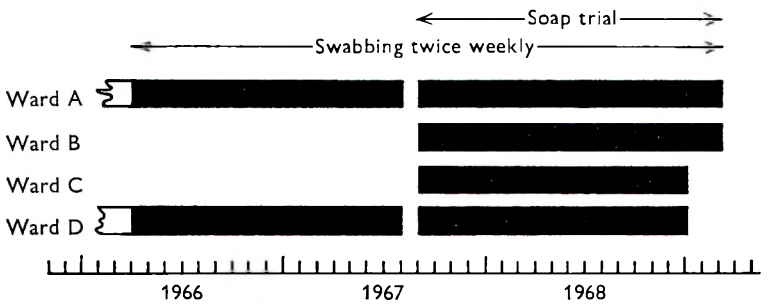

Fig. 1. Calender of investigation.

standard panel of antibiotics (penicillin, tetracycline, cloxacillin and neomycin in the case of Gram-positive cocci, and ampicillin, sulphonamides, thiosporin, colistin, gentamicin, furadantoin, kanamycin and soframycin in the case of Gram-negative rods) and subcultured on agar slopes. All tetracycline-resistant staphylococci were phage-typed. Pathogens isolated from routine clinical bacteriology specimens were treated in the same manner although tested with a greater range of antibiotics.

Upon discharge of a patient, details of treatment, diagnosis, age, etc., were abstracted from the patient's notes and all the data transferred to edge-punch cards.

During the second period of these investigations a trial of antibacterial soaps was carried out in the wards. Whilst this practice had a marked effect on the acquisition rates for *Staphylococcus aureus*, the effect was balanced between the wards and does not influence the results quoted here. The results of the soap trial are published separately (Wilson, 1970).

RESULTS

The individual wards differed slightly in the type of patient admitted (Table 1) and this was reflected in duration of the patient's stay in hospital. Two diagnostic categories, eczema and psoriasis, contributed more than half of the patients. Light-sensitivity patients, who had the shortest stay, were admitted mainly to wards B and D.

Carrier status

Table 2 shows the carrier status on admission for patients in the different diagnostic categories. Patients with eczema more often carried staphylococci on admission than did other patients, particularly in the chest and groin. The carriage of tetracycline-resistant staphylococci was greatest in patients with leg ulcers – this may be related to the prolonged therapy and multiple admissions sustained by these patients.

Table 1. *Distribution of diagnoses between wards*

	Distribution (%)					
Ward	Eczema	Psoriasis	Light sensitivity	Ulcer	Other	Total patients
A (male)	40	20	2	3	35	569
B (male)	30	27	9	1	33	276
C (female)	30	35	2	4	29	236
D (female)	21	28	8	6	36	467

Table 2. *Carriage of* Staphylococcus aureus *on admission*

	Carriage on admission (%)					
Staphylococcus	Eczema	Psoriasis	Light sensitivity	Leg ulcer	Other	Total
Nose S/PT	38	32	14	17	23	28
R/P	13	13	15	7	10	11
R/T	9	3	4	12	5	5
R/PT	6	3	3	7	5	4
Chest S/PT	32	15	11	2	12	16
R/P	11	7	6	0	7	6
R/T	5	2	3	14	3	3
R/PT	5	1	3	9	4	3
Groin S/PT	21	12	8	14	12	12
R/P	9	5	4	0	4	5
R/T	5	2	0	5	4	3
R/PT	4	2	3	10	4	3
Total patients	457	389	80	57	509	1492*

* Because of occasional weekend admissions, some patients were not swabbed on admission or within 48 hr. of admission and have been excluded from this analysis.

S/P, Sensitive to penicillin; S/T, sensitive to tetracycline; R/P, resistant to penicillin; R/T, resistant to tetracycline.

Acquisition of an organism was defined by the recovery on culture medium of six or more colonies of an organism not previously cultured from that site. The organisms were distinguished on the basis of their antibiotic-sensitivity pattern where phage typing was not carried out. This procedure probably seriously underestimates the acquisition rates for tetracycline-sensitive strains.

Acquisition at all sites proceeded at an even rate, the chance of acquiring an organism being linearly related to the duration of stay in hospital for about the first 4 weeks of stay, thereafter a drop in the rate occurred. In the two largest

homogeneous groups, eczema and psoriasis, acquisitions in the nose and in the groin proceeded at the same rates but eczema patients more often acquired staphylococci on the chest than did patients with psoriasis (Fig. 2). This may reflect fundamental differences in the 'normal' skin of these patients or it may be a consequence of the greater local environmental contamination of eczema patients. That chest acquisitions were not simply a reflexion of acquisition at other sites is demonstrated by the fact that 27 % of the 483 chest acquisitions were of strains not carried on admission at any site or acquired in the nose or groin during the hospital stay.

Topical steroids are used extensively in the management of skin disease and it has not proved possible to analyse these results in terms of steroid prescription; 80 % of patients received topical steroids, those who did not were mainly light-

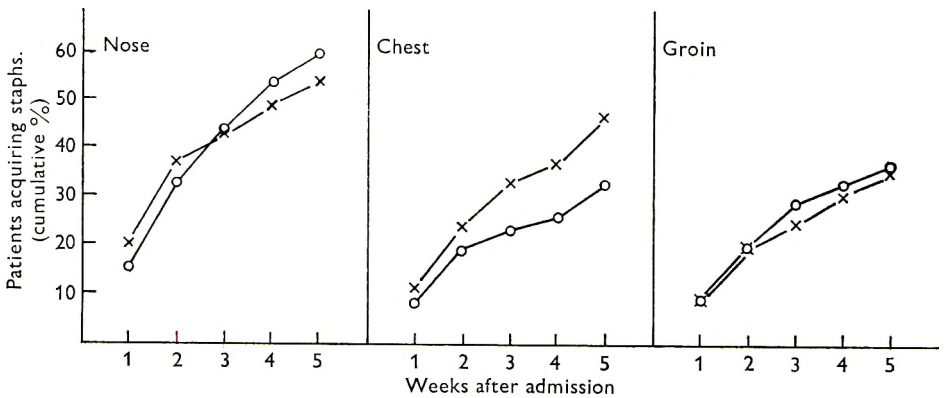

Fig. 2. Acquisition in nose, chest and groin in relation to hospital stay.
○, Psoriasis; ×, eczema.

Table 3. *Acquisitions in relation to antibiotic treatment*

Acquisition site	Antibiotic therapy	Acquisitions per 100 patient-weeks occurring after admission: 1–14 days		15–28 days		29 + days	
Nose	Topical	24	***	15	***	3	†
	Systemic	21		6		6	
	None	16		8		3	
Chest	Topical	17	***	12	***	4	†
	Systemic	15		8		4	
	None	10		5		3	
Groin	Topical	17	***	8	*	5	**
	Systemic	12		8		5	
	None	8		6		2	
Total patient-week contributing	Topical	304		256		170	
	Systemic	446		358		170	
	None	2322		1416		410	

Statistical significance: *** $P < 0{\cdot}01\,\%$; ** $0{\cdot}1\,\% < P < 1\,\%$; * $2\,\% < P < 5\,\%$; † not significant at the 5 % level.

sensitivity patients. An examination of the use of antibiotics has yielded an unexpected result however (Table 3); because it is almost inevitable that patients receiving antibiotics will have a longer stay in hospital than those not receiving antibiotics (the diagnosis and severity of the disease will influence both factors) the results are presented in the form of acquisition for various periods of hospitalization. It is curious that the giving of *topical* as well as systemic antibiotics should lead to increase in the acquisition rate, for topical preparations might be expected to act only locally. However, as shown in Table 4, the giving of any antibiotic increased the likelihood that a tetracycline-resistant staphylococcus would be acquired (45% of the tetracycline-resistant staphylococci were sensitive to penicillin, compared to 65% of the tetracycline-sensitive strains).

Table 4. *Acquisition of tetracycline-resistant staphylococci in relation to antibiotic therapy*

		Antibiotic therapy		
	Strain acquired	Topical	Systemic	None
		Acquisitions (%)		
Nose	S/T	50	46	65
	R/T	50	54	35
Chest	S/T	39	47	62
	R/T	61	53	38
Groin	S/T	37	36	51
	R/T	63	64	49
Total acquisitions		282	404	1038

S/T, Strain sensitive to tetracycline; R/T, strain resistant to tetracycline.

Infections and antibiotics

It has proved exceptionally difficult to define 'infection' or 'sepsis' in skin diseases (e.g. Noble, 1970) and in this paper the word infection refers to the isolation of potentially pathogenic bacteria from a swab submitted to the laboratory under the heading 'infected eczema', 'boil', 'infected ulcer', etc.

In all, *Staphylococcus aureus* was recovered from 64% of the infected lesions, but the lesions frequently yielded more than one 'pathogen'. Only one-third of the streptococcal lesions yielded a pure growth of streptococci, most being mixed with staphylococci, and nearly 10% of all lesions yielded both Gram-positive cocci and Gram-negative rods. However, it can be seen (Table 5) that there was a considerable change in the organisms isolated from these lesions over the short period of the survey. In the first period there was an accent on penicillin-sensitive staphylococci and on staphylococci resistant to tetracycline only. In the second period there was a shift towards penicillin-resistant strains. There was also a marked drop in the isolation of *Pseudomonas aeruginosa* in the second period, this may be attributed to the greater awareness of the problems of *Pseudomonas* infection (Noble & Savin, 1966; P. M. White, in preparation). The change in the staphylococci, however,

may reflect changes in therapeutic preference (Table 6), for whilst there was no major change in the rate of prescribing antibiotics there was a swing to greater use of penicillin (penicillinase-*sensitive*) as a systemic antibiotic. In the topical antibiotics there was a change from neomycin to gentamicin and a reduction in the use of tetracycline, and these changes are reflected in the sensitivity of the bacteria isolated from the lesions.

Table 5. *Distribution of 'infections'*

% patients with lesions yielding:	1st period	2nd period
Staph. aureus		
S/PT	22	10
R/P	4	7
R/T	11	3
R/PT	3	5
β-Haemolytic streptococcus	9	7
Ps. aeruginosa	9	2
Proteus	3	2
Coliform-type organisms	2	3
Total patients	531	1100

S/PT, Sensitive to penicillin and tetracyline; R/P, resistant to penicillin only; R/T, resistant to tetracycline only; R/PT, resistant to penicillin and tetracycline.

Table 6. *Distributions of antibiotic prescriptions*

		No. of prescriptions	
Route	Antibiotic	1st period	2nd period
Systemic	Penicillin	16 (12%)	68 (30%)
	Orbenin + ampicillin	28 (22%)	41 (18%)
	Tetracycline	60 (46%)	100 (44%)
	Other	25 (19%)	18 (8%)
	Total	129	227
Topical	Tetracycline	49 (70%)	42 (45%)
	Neomycin	17 (24%)	1 (1%)
	Gentamicin	0	38 (40)
	Other	4 (6%)	13 (14%)
	Total	70	94
Total patients		531	1017

Epidemic spread of staphylococci

The phage-typing results for the tetracycline-resistant staphylococci were used to study the spread of organisms between and within the wards. As was expected, the two wards for male patients (A and B) shared their epidemic staphylococci – presumably via the treatment room. Each ward had several small episodes of spread of the type familiar from surgical ward studies. Only one strain became widespread in all four wards and this was a penicillin- and tetracycline-resistant strain of phage type 6/47/54/75/83A.

Most of the strains which were observed to spread were of phage group III and

the remainder of phage group I or mixed group I and group III. No phage group II strains (which are the classic 'skin' strains) were observed to spread, although this may be an artifact due to the restriction of typing only the tetracycline-resistant strains. Nevertheless, nine patients carried tetracycline-resistant group II strains; one patient with eczema carried a penicillin- and tetracycline-resistant type 71 in the nose, chest, groin and skin lesions without this strain being recovered from any other patient.

DISCUSSION

The findings reported in this paper are remarkably similar to those published in the past on surgical-ward sepsis (e.g. Shooter *et al.* 1958; Williams *et al.* 1962) in relation to epidemic spread, type of infecting organism, etc., with the exception that many more tetracycline-resistant but penicillin-sensitive strains were encountered. In surgical wards such strains have formed less than 1% of the nasal staphylococci but in the skin hospital they accounted for more than 10% of the strains colonizing patients on admission to the wards. This doubtless reflects the use of topical tetracycline as a therapeutic agent; penicillin is not used for this purpose. Changes in the prescription of antibiotics during the period of study were reflected in the organisms isolated from infected lesions.

Few if any other investigators appear to have considered the skin as a possible site for colonization with staphylococci and indeed it may be that the results reported here apply only to patients with diseases of the skin. Nevertheless, in these wards carriage of staphylococci on areas of 'normal' skin was high and must be considered as a source of infection. It is a moot point whether the clinically uninvolved skin of a patient with extensive eczema or psoriasis can be considered 'normal'.

The definition of 'sepsis' in patients with diseases of the skin is difficult. In a rather subjective survey of clinicians' opinion (Noble, 1970) there was a tendency to equate severity of infection with the isolation of an organism resistant to two or more antibiotics and to regard those patients who suffered cross-infection as more severely infected than those who were self-infected. Since about 42% of the patients had some degree of colonization or sepsis of skin lesions, the problem is a considerable one. Although clearly there are factors other than sepsis which determine the length of hospital stay, in this series eczema patients who had no sepsis had a mean stay of 17 days whilst those with some degree of sepsis stayed 23 days in hospital; for psoriatics the figures were 23·5, and 34·5 respectively. These figures agree broadly with those published by the Public Health Laboratory Service (1960) for surgical patients, where the difference in hospital stay between those developing and those not developing sepsis was about 8 days.

The finding that much of the 'infection' was with phage group III staphylococci was unexpected, for the classic skin strains are those of phage group II. The type 71 strains of *Staph. aureus* in particular have been associated with impetigo (Parker & Williams, 1961) and more recently with toxic epidermal necrolysis (Lyell, Dick & Alexander, 1969). The phage group I has more frequently been associated with skin sepsis, for the classic type 80 strain first appeared in an out-

break of boils and other local sepsis in a general population (e.g. Rountree & Beard, 1958) and was found to be extensive in studies of skin patients in the U.S.A. (Greer, Menard & Livingood, 1961).

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An evaluation of the efficiency of cleaning methods in a bacon factory

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SUMMARY

The germicidal efficiencies of hot water (140–150° F.) under pressure (method 1), hot water + 2 % (w/v) detergent solution (method 2) and hot water + detergent + 200 p.p.m. solution of available chlorine (method 3) were compared at six sites in a bacon factory. Results indicated that sites 1 and 2 (tiled walls) were satisfactorily cleaned by each method. It was therefore considered more economical to clean such surfaces routinely by method 1. However, this method was much less efficient (31 % survival of micro-organisms) on site 3 (wooden surface) than methods 2 (7 % survival) and 3 (1 % survival). Likewise the remaining sites (dehairing machine, black scraper and table) were least efficiently cleaned by method 1. The most satisfactory results were obtained when these surfaces were treated by method 3.

Pig carcasses were shown to be contaminated by an improperly cleaned black scraper. Repeated cleaning and sterilizing (method 3) of this equipment reduced the contamination on carcasses from about 70 % to less than 10 %.

INTRODUCTION

The standard of hygiene in food processing is most important. As pointed out by Goldenberg (1968), 'there is often a real correlation between the cleanliness of a factory and the quality of its goods'. Properly planned and executed, a cleaning programme forms part of the factory's quality control measures and enhances the product's reputation by helping to reduce spoilage and cross-contamination by pathogenic micro-organisms. As recently as 1967 opinions were expressed that not enough is known about cleaning and sterilizing materials, and even when the facts are known they are not put into practice (Goldenberg & Relf, 1967). That hygienic control in food processing and distribution is vitally important is made clear from the report of the Aberdeen typhoid outbreak (Report, 1964).

There are available today many types of cleaning and sterilizing materials suitable for use in the food industry (Thomas, 1969). However, efficient cleaning is an expensive operation. It should be the responsibility of the control laboratory to ensure that it is done as efficiently and economically as possible (Dyett, 1963).

The slaughter, butchery and dressing of a bacon pig (Wiltshire process) involves the following stages. The animal is stunned, shackled, and exsanguinated in an

area remote from the remainder of the killing line. The carcass is then immersed in a tank of hot water (*c.* 140° F.) in which it is scalded. In this tank the surface hairs are softened for subsequent removal in the dehairing machine. After dehairing, the carcass passes into the singeing furnace (*c.* 1400° F.) for about 15 sec. The burnt skin is removed by a black scraper. This equipment consists of a tunnel in which rotating blades scrape off the charred tissue. After scraping, the carcass is eviscerated, split down the backbone into sides which are chilled overnight before being cured. This whole process results in much debris (blood, skin, hair, etc.) collecting on floors, walls and equipment. The work described in this paper was undertaken to devise a reliable method of cleaning such an area. The germicidal efficiency of a number of cleaning methods was compared at various sites along the killing line. Because of its construction, the black scraper soon builds up hardened residues of skin and hair on the blades unless it is regularly cleaned after use. The scraper was repeatedly cleaned by one of the test methods and the effect of this on the surface bacterial content of carcasses was investigated.

MATERIALS AND METHODS

The following cleaning procedures were selected for comparison: (1) hot water (140–150° F.) high-pressure spray; (2) method 1 followed by brushing with a 2% (w/v) solution of hydrated sodium silicate at 150–160° F. and rinsing with cold water; (3) method 2 followed by spraying with a working solution of 'Chloros' containing 200 p.p.m. of available chlorine. The stock solution of Chloros contained 12% active constituent. A working solution was made by adding about 7·5 ml. of stock solution to 1 gal. of cold water. The Chloros was applied with a knapsack sprayer and allowed to act for 20 min. before being rinsed off.

Six areas were chosen for treatment as follows: tiled wall of sticking pen (wall A), tiled wall of bleeding passage (wall B), wooden barrier rail of scalding tank, metal platform of dehairing machine, blades of black scraper and the surface of an evisceration table. These areas were selected because of the build-up of contamination known to occur at these sites. The allocation of cleaning method to area was randomized to prevent bias in favour of any one method. Each area was treated by each method on five separate occasions to give a total of 90 results (6 areas × 3 methods × 5 replications). Each area was swabbed as follows; the tiled walls, dehairer platform and the table were swabbed on two adjacent 100 cm.^{2} areas with a metal template; the rail of the scald tank was swabbed on duplicate areas with a 25 cm.^{2} template; and four adjacent blades of the scraping machine were swabbed with the same 25 cm.^{2} template.

The swabs consisted of cotton gauze wound on $5\frac{1}{2} \times \frac{3}{8}$ in. wooden spatulas. The swabs were transferred to 20 ml. quarter-strength Ringer's solution in Universal bottles and dispatched to the laboratory within 1 hr. Sodium thiosulphate (0·5%, w/v) was added to the Ringer's solution to inactivate any chlorine carried over when the areas were swabbed after cleaning by method 3. Serial decimal dilutions were prepared in Ringer's solution to which 0·1% of peptone was added (Straka & Stokes, 1957), plated on Plate Count Agar (Oxoid) and the plates in-

cubated for 5 days at 25° C. The results were expressed as a percentage survival. The F ratio and standard error of the mean percentage survival were calculated.

The effect of cleaning and sterilizing the scraping machine by method 3 on the surface bacterial load of pig carcasses was studied. Observation had shown that the undersides of the scraping blades were heavily contaminated particularly at the point where they are bolted to the horizontal shaft. The most effective method of cleaning was firstly to remove gross dirt with the high-pressure water spray. The detergent was then applied to 6 sets of blades at a time (upper and lower surfaces) with a soft-haired brush. The remaining blades were brought into position in sets of six and treated likewise. After rinsing with cold water, Chloros solution was applied and allowed to act for 20 min. before being rinsed off. Ten carcasses, randomly selected, were examined before and after passage through the scraping machine on two occasions before the machine was cleaned by the test method and on nine occasions after the test method had been introduced. The method used to determine surface contamination was the agar-sausage technique (ten Cate, 1965) The speed of throughput on the killing line precluded the use of the swab-rinse method. The agar sausages consisted of (%, w/v) peptone 1·0; lab-lemco 1·0; NaCl 0·5; agar 2·5 in distilled water and were made by filling 200 ml. of melted medium into 80 cm. lengths (40 mm. diam.) of Nalophane casing (Kalle Aktien Geselleschaft, Weisbaden, Germany). They were sterilized at 121° C. for 15 min. Agar impressions were taken at 10 points on the surface of each carcass and the samples incubated at 25° C. for 3 days. The counts were plotted as a log distribution on probability paper by the method of Hansen (1962) and recorded as the logarithm of the mean count per 10 cm.^{2}. The results were expressed as a percentage survival as before.

RESULTS

The bacterial counts of the six areas before and after cleaning are shown in Tables 1–3.

In Table 1 it is seen that all three cleaning methods produced a satisfactory reduction in numbers of micro-organisms on tiled wall A; 1·32 % of the population survived treatment by method 1 and less than 1 % survived treatment by methods 2 and 3. There was no significant difference between the efficiencies of the last two methods, but there was between these and method 1 ($P < 0{\cdot}01$). Similarly, all the methods were efficient in cleaning tiled wall B, but no significant difference was found between the different methods on this surface.

Table 2 shows that cleaning by method 1 was grossly inefficient for the rail of the scalding tank (31 % survival). Treatment by methods 2 and 3 resulted in 7 % and 1 % survival respectively. The metal platform of the dehairer likewise was least efficiently cleaned by method 1; there was no significant difference between methods 2 and 3 for this surface.

Table 3 shows that there was a highly significant difference between methods 2 or 3 and method 1 for the cleaning of the scraping machine ($P < 0{\cdot}001$). Of the population 35 % survived treatment by method 1 whereas 9 % and 3 % respectively survived after cleaning by methods 2 and 3. In this instance the

action of the hot water/detergent/sterilizer (method 3) would not be efficient. Cavett (1969) considered that an effective sanitizer was one which when tested at half user concentration reduced the microbial population by 99·9–99·99 % (i.e. 3–4 log. cycles) in 10 min. The present results may have been partly due to the very poor condition of this equipment at the beginning of the experiment. A

Table 1. *Total plate counts at 25° C. from swabbed areas of tiled walls before and after cleaning*

	Site swabbed					
	Tiled wall A			Tiled wall B		
	Count/cm.²			Count/cm.²		
Cleaning method	Before cleaning	After cleaning	Survival (%)	Before cleaning	After cleaning	Survival (%)
1	13,080	170	1·29	7,570	22	0·28
	113,000	888	0·78	16,080	191	1·18
	27,900	729	2·61	96,700	15	0·01
	39,800	219	0·55	48,290	30	0·06
	22,400	308	1·37	399,000	46	0·011
			1·32†			0·308†
2	197,600	284	0·14	5,880	7	0·11
	58,800	42	0·07	22,000	440	2·00
	165,000	10	0·006	10,800	2	0·01
	192,000	1,860	0·96	1,740	1	0·05
	188,800	17	0·009	644,000	5,200	0·80
			0·237†			0·594†
3	25,000	24	0·09	11,790	11	0·09
	85,000	402	0·47	3,800	24	0·63
	900,000	1,020	0·11	33,300	40	0·12
	373,000	350	0·094	11,280	32	0·28
	596,000	6	0·001	83,300	2	0·002
			0·153†			0·224†
	F = 7·58** (P < 0·01). S.E. of mean % survival = 0·236.			F = 0·54 (N.S.) S.E. = 0·262.		

† Mean.

similar result was obtained for the surface of the evisceration table. Sixteen, 5 and 2 % of organisms survived after treatment by methods 1, 2 and 3 respectively, although no significant difference was found between methods 2 and 3 (P < 0·01) (Table 3).

In Fig. 1 are shown the results of cleaning and sterilizing the black scraper by method 3 on the bacterial content of pig carcasses. Before cleaning the scraper by this method, the number of surface organisms on carcasses was about 70 % of the number present on the carcasses after dehairing. Repeated sterilizing of the scraping blades during a 4-week period reduced the contamination to less than 10 %.

Table 2. *Total plate counts at 25° C. from swabbed areas of scalding tank rail and dehairer platform*

	Site swabbed					
	Scald tank rail			Dehairer platform		
	Count/cm.²			Count/cm.²		
Cleaning method	Before cleaning	After cleaning	Survival (%)	Before cleaning	After cleaning	Survival (%)
1	4,020	1,040	25·87	284,400	860	0·30
	1,353	538	39·70	88,400	2,100	2·37
	48,000	2,980	6·20	151,800	4,580	3·01
	21,030	4,020	19·11	249,800	13,456	5·38
	39,600	25,000	63·11	93,573	7,904	8·44
			30·802†			3·90†
2	39,490	31	0·07	536,000	10,400	1·94
	3,450	449	13·02	177,200	3,540	1·99
	350	1	0·28	9,360	72	0·76
	1,490	22	1·48	57,600	144	0·25
	232	44	18·96	198,400	1,847	0·92
			6·76†			1·172†
3	2,310	32	1·389	124,000	392	0·31
	3,800	31	0·82	82,400	144	0·17
	8,550	4	0·04	360,000	24	0·006
	3,020	75	2·48	272,000	19	0·006
	65,800	156	0·237	130,400	1,931	1·48
			0·993†			0·396†
	F = 6·83* ($P < 0·05$)			$F = 4·75$* ($P < 0·05$)		
	S.E. = 6·05			S.E. = 0·844.		

† Mean.

DISCUSSION

The choice of cleaning materials for use in a food-processing establishment is governed by the following factors: (*a*) they must be efficient, (*b*) they must not affect those who use them, (*c*) they must not damage equipment; (*d*) they must not affect the colour or flavour of food in contact with equipment cleaned by their use, (*e*) they must be easily rinsed away, (*f*) they must be easy to handle, and (*g*) they must be cost efficient, i.e. the relative costs of producing the desired effect must be considered (Gilbert, 1960; Thomas, 1967, 1969).

Since the chief concern of the plant hygienist is the protection of the product from contamination it is essential to establish which points in the processing line constitute 'direct' and 'remote' product contamination. Those surfaces which routinely contact the product require immediate and efficient cleaning. Walls would normally be 'remote' contact points and therefore unlikely to constitute a direct hazard. The present results, Table 1, indicate that tiled walls can be as efficiently cleaned by a high-pressure hot-water spray as by treatment with detergent or detergent + sterilizer. It is suggested that the reduction in bacterial numbers on these walls by method 1 was effected by mechanical removal of organisms since the water temperature never exceeded 150° F. Since it would be

Table 3. *Total plate counts at 25° C. from swabbed areas of black scraper and evisceration table*

	Site swabbed					
	Black scraper			Evisceration table		
	Count/cm.²			Count/cm.²		
Cleaning method	Before cleaning	After cleaning	Survival (%)	Before cleaning	After cleaning	Survival (%)
1	81,460	37,760	46·35	10,600	2,420	22·83
	3,760	800	21·27	21,100	3,320	16·51
	4,420	1,800	40·72	8,400	1,510	17·97
	19,200	9,040	46·50	14,000	2,860	20·42
	60,400	14,400	22·18	18,200	268	1·47
			35·404†			15·84†
2	242,060	70,960	29·31	6,600,000	576,000	8·72
	71,200	6,160	8·65	9,200	168	1·82
	35,250	1,704	4·83	24,400	490	2·00
	86,800	552	0·63	19,380	2,420	12·48
	30,080,000	80,900	0·26	19,800	184	0·92
			8·736†			5·188†
3	54,400	656	1·20	530	9	1·69
	704,460	16,520	2·34	154,000	28	0·01
	240,215	1,046	0·43	1,240	45	3·62
	25,600	536	2·09	800	18	2·25
	21,900	1,832	8·36	1,420	16	0·40
			2·884†			1·594†
	F = 14·29*** (P < 0·001)			F = 8·33** (P < 0·01)		
	S.E. = 4·585.			S.E. = 2·566.		

† Mean

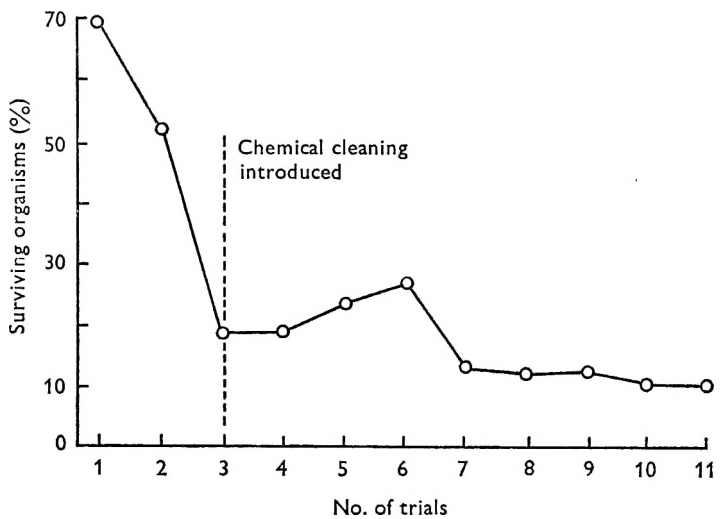

Fig. 1. The effect of sterilizing the blades of the scraping machine on the surface bacterial load of pig carcasses.

cheaper to use hot water only on such surfaces both from a materials cost and time point of view, such a procedure could be adopted in routine cleaning. Weekly bacteriological tests, e.g. the agar-sausage technique (ten Cate, 1965) would indicate the efficiency of the hot-water method. Only when the results proved unsatisfactory would such surfaces be treated with detergent or detergent and sterilizer. In this way much valuable time would be saved and the rationalization of a cleaning schedule effected.

Wooden surfaces with which food comes in contact are most undesirable because they are difficult to clean (Cooper & Dyett, 1967). This has been confirmed in the present investigation. Only after a three-stage treatment (method 3) was a satisfactory reduction in contamination realized. Wood expands when wet and any cracks allow the entry of contamination. Blood soaks in and solid particles of meat, fat, hair and other debris form reservoirs of infection. Working surfaces and containers should preferably be made of stainless steel (Hobbs, 1967).

The dehairing machine is a difficult piece of equipment to clean. Much hair becomes trapped on the drums and at the attachments of the dehairing flails to the drums. Although the hot-water treatment (method 1) removed most of the contamination, effective sterilization was only realized after the surfaces were treated with detergent or detergent and sterilizer (methods 2 or 3). Since the dehairer is a direct contact point it would be necessary to ensure sterilization of this equipment. Galton, Smith, McElrath & Hardy (1954) have shown that extra cleaning of such equipment reduced positive cultures of *Salmonella* on pig carcasses from 69 % to 10 %. Similarly the black scraper requires to be effectively sterilized after use. In fact, this equipment is one of the most serious sources of contamination in a killing line. The carcasses enter it after passing through the singeing furnace in which the temperature is about 1400° F. and are therefore virtually free of surface-contamination at this point. Much transfer of infection can take place from the blades of a poorly cleaned scraper. Since every precaution must be taken to prevent contamination of carcasses during the butchering and dressing operation (Patterson, 1968), extra special care should be taken in the cleaning of the black scraper.

The results for the cleaning of the table confirms observations of other workers (Spencer, 1965; Chalmers, 1961) that efficient cleaning (detergent action) removes most of the contaminating microflora from a surface and therefore paves the way for subsequent disinfection. However, the surface must be clean before it is treated with a sterilizing agent.

Mention has already been made of the necessity for thoroughly cleaning the scraping machine. The results presented in Fig. 1 emphasize the importance of this. Although certain areas of a pig carcass remain unsinged after passing through the singeing furnace (Gardner & Patton, 1969), substantial numbers of organisms on the skin after scraping is indicative of recontamination. As the present results suggest, an improperly cleaned black scraper will cause this recontamination. The results confirm earlier observations that where the bacterial load on equipment is higher than on food, the latter will be contaminated (Shotts, Martin & Galton, 1962; Ølgaard, 1964; Gilbert & Maurer, 1968; Gilbert, 1969). The present results also show that within a short time recontamination of carcasses was substantially

reduced when the scraping machine was effectively cleaned and sterilized. Hansen (1962) similarly demonstrated that the average number of bacteria on dressed carcasses was reduced by about 90 % when equipment was satisfactorily cleaned and sterilized.

I wish to record my appreciation of the skilful technical assistance of Mr S. N. Reid, F.I.M.L.T., Mr B. Lynch and Miss C. Murphy. Thanks are also due to Mr A. Kinsella for the statistical interpretation of results. The co-operation of the management of the factory is also noted.

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Recoveries of *Salmonella ndolo* from desiccates exposed to 100° C under various conditions with respect to desiccant, desiccant temperature and atmosphere

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SUMMARY

Desiccates of *Salmonella ndolo* dried in sodium glutamate have been exposed to 100° C. in air or *in vacuo*; in the presence of wet or dry P_2O_5; and with desiccant maintained at either 25° or 100° C. Of the eight treatments thus compared, rapid killing occurred where the desiccate was heated *in vacuo* and in the presence of dry P_2O_5 maintained at 100° C. There was little difference among the death-rates of the other seven treatments. Sublimation of the P_2O_5 with resultant attack on the desiccate would appear to be responsible for the rapid killing in the treatment where it was observed.

INTRODUCTION

In a study on the survival of dried bacteria at 100° C. (Annear & Bottomley, 1965) it was shown that recoveries were much modified by the condition of the phosphorus pentoxide which was used as a desiccant in the ampoules to produce dry atmospheres. *In vacuo* and where the P_2O_5 was maintained in the dry state, a relatively rapid killing occurred, associated with browning and bubbling of the desiccate. *In vacuo* and with the surface of the P_2O_5 wetted to generate phosphoric acid, these events did not occur, nor did they occur in air with either wet or dry P_2O_5.

It seemed highly probable that the effects obtained with dry P_2O_5 *in vacuo* were due to sublimation of that compound and that they might be reduced if the P_2O_5 was kept cool during the heating of the desiccates. A comparision of survival was therefore made between two sets of desiccates; in one set the P_2O_5 was exposed to the same temperature as the desiccates (100° C.), while in the other the P_2O_5 was maintained near ambient temperature (*ca.* 25° C.). In each set the effects of wet and dry P_2O_5 were tested and also the effects of storage in air and storage *in vacuo*. In all, therefore, eight treatments were compared.

METHODS

A suspension of the test organism *Salmonella ndolo* (NCTC 8700) was made in 20% sodium glutamate and single-drop volumes of it were dried *in vacuo*. Small tubes of wet or dry P_2O_5 were included in the ampoules which were sealed either

in vacuo or in air. Details of these procedures have been described previously (Annear & Bottomley, 1965). The desiccates were heated in an atmosphere of free steam and Fig. 1 shows the arrangement by which the two temperatures for the P_2O_5 were obtained and that by which the desiccates were held at the one temperature (100° C.). The constrictions in the ampoules were flattened so that in the upper set the P_2O_5 tubes were prevented from occluding the orifice between the body and neck of the ampoule.

Viable counts were made on blood-agar plates by methods also previously described (Annear, 1965).

RESULTS AND DISCUSSION

The results of treatments 5–8 (Table 1) confirmed those previously obtained, namely that with dry P_2O_5 *in vacuo* (treatment 8) a relatively rapid killing associated with browning of the desiccates was obtained, and that between the

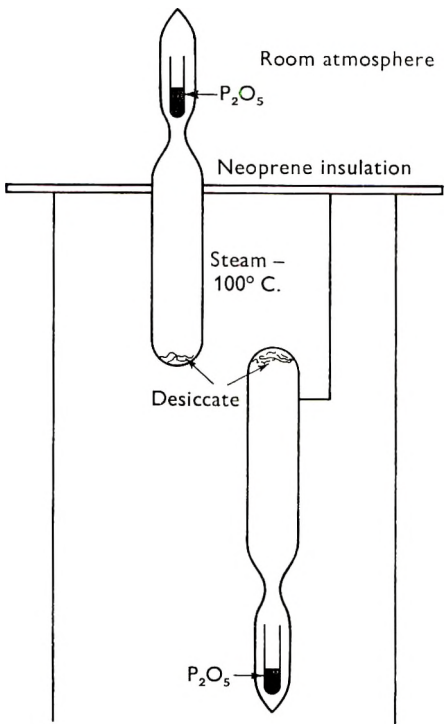

Fig. 1. Arrangement for maintaining desiccates at 100° C. and desiccant at either 100° or 25° C.

recoveries in the other three treatments there was little difference. Where the P_2O_5 was maintained at 25° C. (treatments 1–4) there were no large differences, although the recoveries from desiccates held *in vacuo* were somewhat higher than from those held in air.

The comparison of most interest in this investigation is that between treatments 4 and 8, and the results strongly indicate, as suggested, that the more rapid

killing of the organism in treatment 8 is due to P_2O_5, which sublimes more freely *in vacuo* than it does in air and more freely at the higher temperature than the lower.

In blank experiments it was shown by colorimetric methods that in conditions obtaining in treatments 1–7 only traces of phosphate were detected on the walls of the ampoules, whereas for conditions obtaining in treatment 8 tests for phosphate were strongly positive.

Table 1. *Recoveries of* Salmonella ndolo *from desiccates exposed to* 100° *C. under eight sets of conditions with respect to desiccant condition, desiccant temperature and atmosphere*

	P_2O_5 at 25° C.				P_2O_5 at 100° C			
	Wet P_2O_5		Dry P_2O_5		Wet P_2O_5		Dry P_2O_5	
Treatment	Air	Vac.	Air	Vac.	Air	Vac.	Air	Vac.
no...	1	2	3	4	5	6	7	8
Days at 100° C.								
3	6·1*	6·5	6·2	6·6	6·0	5·9	6·1	2·2
5	4·8	5·1	4·8	5·5	4·8	4·4	4·8	< 1
7	4·0	4·8	4·0	4·7	3·8	4·0	3·7	< 1
9	2·9	4·0	2·9	4·1	2·3	2·6	2·6	< 1

Initial number of organisms per tube = $6{\cdot}0 \times 10^9$. Recovery immediately after drying = $5{\cdot}9 \times 10^9$.

* Recovery expressed as $\log_{10}$.

It seems most unlikely from a consideration of the results as a whole that P_2O_5 plays any role other than that of a desiccant at ambient temperature. However, it would be of interest to make some critical comparisons between this compound and other systems suitable for controlling low humidity levels in bacterial desiccates.

The highest recoveries obtained in these experiments reveal the remarkable heat-tolerance that can be conferred on some vegetative bacteria when dried and held under conditions of extreme desiccation.

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The production of pneumonia with or without pleurisy in gnotobiotic piglets with pure cultures of strain TR 32 of *Mycoplasma hyorhinis*

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SUMMARY

Nine gnotobiotic piglets, when 8–9 days old, were exposed to an aerosol of strain TR 32 of *Mycoplasma hyorhinis* and killed at intervals from 14 to 37 days after infection. The aerosol of *M. hyorhinis* caused bronchopneumonia in 1 pig, pleurisy alone in 2 pigs, pleuropneumonia in 2 pigs and no lung changes in 4 pigs. *M. hyorhinis* was re-isolated from the lungs of all infected pigs, irrespective of the presence or absence of lesions, though it was only isolated from the serosa and joints when lesions were present. Four control pigs exposed to aerosols of mycoplasma medium had no lesions.

INTRODUCTION

'Purified' cultures of *Mycoplasma hyopneumoniae* (*M. suipneumoniae*) have been shown to cause pneumonia in gnotobiotic piglets (Hodges, Betts & Jennings, 1969), and the view has been expressed that it is the only recognized mycoplasma causing pneumonia in pigs. Differences of opinion exist as to the role played by *Mycoplasma hyorhinis* in porcine pneumonia.

Up to 1965, *M. hyorhinis* was considered to be the cause of a disease of young piglets characterized by polyserositis and arthritis but not by pneumonia (Roberts, Switzer & Ramsey, 1963*a*, *b*). However, *M. hyorhinis* is a common inhabitant of the nasal cavities and lungs of both healthy and diseased pigs. Switzer (1955) isolated from 48 % and 67 % of the nasal cavities of two batches of normal pigs a 'pleuropneumonia-like organism' which he named *M. hyorhinis*, whilst Ross, Switzer & Mare (1963) isolated *M. hyorhinis* from at least 30 % of normal pigs. Hartwich & Niggeschulze (1966) recovered the organism from 75 % of normal lungs, and Scheer, Hartwich & Sandersleben (1969) found that 69 % of normal pigs harboured the organism in their lungs or nasal cavities.

However, several workers have reported that *M. hyorhinis* occurs more frequently in the nasal cavities and lungs of pigs with pneumonia than in normal pigs. Thus L'Ecuyer, Switzer & Roberts (1961), in a survey of pulmonary microflora, found that 51 % of pneumonic lungs but only 6·6 % of normal lungs revealed organisms believed to be *M. hyorhinis*. Estola & Schulman (1966) found that 14 of 22 lungs and 7 of 22 nasal swabs from pigs in Finland with enzootic pneumonia yielded this organism whilst none of 9 lungs or 9 nasal swabs from normal pigs

did so. Gois, Cerny, Roskosny & Sovadina (1969) found that none of 202 nasal swabs or 107 lungs from a herd free from enzootic pneumonia yielded *M. hyorhinis*, whereas this organism could be recovered from 56 of 61 nasal swabs and from at least 15 of 33 lungs from a herd with pneumonia.

Nevertheless, in spite of these observations it has been generally considered that *M. hyorhinis* is either only a secondary invader in porcine pneumonia or merely present in pneumonic lesions (Switzer, 1964; Goodwin, Pomeroy & Whittlestone, 1968). Contrary evidence was produced by Gois and his colleagues working in Czechoslovakia, who induced pneumonia, sometimes without pleurisy, in both conventional and gnotobiotic pigs (Gois, Valicek & Sovadina, 1968; Gois *et al.* 1970). In this country Gois (unpublished observations) found that 20 of 30 lungs and 28 of 30 nasal swabs from a herd with a pneumonia problem yielded *M. hyorhinis* in circumstances which suggested that it might have played a primary pathogenic role in the pneumonia.

Accordingly, the present work was undertaken to confirm the potentiality of the TR 32 strain of *M. hyorhinis* to produce pneumonia and to extend the work of Gois *et al.* (1968). Gnotobiotic piglets were used in the experiment so that no potentially pathogenic bacteria or viruses could be incriminated as having a synergistic action with *M. hyorhinis*, and to avoid the risk of accidental infection with micro-organisms of human origin.

MATERIALS AND METHODS

Mycoplasma culture

The *M. hyorhinis* strain used in this experiment has been designated TR 32 and was isolated originally from the lungs of a pig from a fattening station in Czechoslovakia. It has been classified as *M. hyorhinis* on the basis of growth inhibition (Gois *et al.* 1969) and of metabolism inhibition and colonial growth (Gois, Cerny & Veznikova, 1970).

Strain TR 32 used in these experiments was 'cloned' three times by culturing from single colonies into liquid medium and inoculating the tenfold dilutions of the resulting 48 hr. culture on agar plates. It was then inoculated into a gnotobiotic piglet which was killed 14 days later. The strain was re-isolated from the pneumonic lungs of this piglet, and further 'purified' by plating out from single colonies three times. It was then grown in liquid medium suitable for inoculation into pigs. The medium comprised 20% pig serum, 70% Difco P.P.L.O. broth, 10% of a 25% yeast extract (D.C.L. Dried Yeast), 0·5% glucose, and 0·002% phenol red. The pH of the inoculum was adjusted to 7·4 and the number of mycoplasmas titrated according to the number of colour changing units (C.C.U.)/ml. After infection of the piglets in the isolators, the remains of the inocula were removed from the isolators, tested for bacteriological sterility and titrated again.

Experimental pigs

The experimental pigs used were two litters (42 and 46) of Large White gnotobiotic piglets produced and reared by the techniques described by Betts & Trexler

(1969). Each plastic isolator contained within it a stainless-steel cage which housed either one or two pigs. The pigs were fed on a sterilized proprietary milk product with added minerals and vitamins. The pigs were maintained under these sterile conditions throughout the course of the experiment. The first litter of three pigs was infected by aerosol when 8 days old, and the second litter of ten pigs was infected by aerosol when 9 days old.

Microbiological tests of pigs before inoculation

Swabs were taken from the anus and mouth of each pig 4 days after birth and tested for bacteria and fungi aerobically and anaerobically by the methods of Betts & Trexler (1969).

Inoculation of pigs

Each group of pigs was exposed to an aerosol of *M. hyorhinis* in culture medium. The aerosol apparatus* was placed inside the animals' cage, which was covered with a plastic canopy that exactly covered the top and four sides. The two pigs in the isolator in the first experiment were exposed for 2 hr. to a total of 12 ml. of a nebulized culture containing 10^8 C.C.U./ml. The pigs in each of the isolators in the second experiment were exposed for $1\frac{1}{4}$ hr. to a total of 8–10 ml. of a nebulized culture containing 10^8–10^9 C.C.U./ml., and these pigs also either ingested or insufflated a further 2 ml. of culture (due to the frothing-over of culture from the aerosol apparatus). No anti-foam agent, as described by Wright, Bailey & Hatch (1968), was included in the medium since frothing was not a problem in the first litter. The particle size of the droplets was approximately 5–8 μ. Control pigs were exposed to similar volumes of nebulized uninoculated medium for the same lengths of time.

Necropsy procedure

The pigs were anaesthetized with pentobarbitone sodium B.P. inside the isolators, removed from the isolators, exsanguinated and necropsied. In the first litter the 2 infected pigs were killed 15 and 30 days post inoculation (p.i.) and the control at 30 days. In the second litter 3 pigs (1 control and 2 infected) were killed at 14 days and at 23 days p.i.; 4 pigs (1 control and 3 infected) were killed between 33 and 37 days p.i.

Examination of materials collected at necropsy

Re-isolation of mycoplasmas

Materials were collected from 30 sites. Direct culture to solid media was carried out from the following sites: turbinates, tonsil, trachea, bronchus, pleural fluid, pericardial fluid, peritoneal fluid, cerebrospinal fluid, lumen of the large intestine, and joints (left and right elbow, carpus, stifle and hock).

Pieces of tissue from the lung, heart, liver, spleen, kidney, brain and lymph nodes were stored at $-70°$ C. and later ground up in liquid medium before inoculation into liquid medium and onto solid medium.

Liquid medium was the same as that used for growth of cultures for inoculation,

* The Midhurst Nebuliser, Aerosol Products (Colchester) Ltd.

except that horse serum replaced pig serum, and 10% of pig lung extract, prepared according to the method of Hodges (1969), was added. Solid medium contained 0·85% Oxoid Ion Agar No. 2.

Microbiology

Materials from the same 30 sites were tested for micro-organisms capable of anaerobic growth by direct culture on heated blood agar with additional yeast extract (1%) in the case of the first ten sites listed above, and by culture after grinding up in the mycoplasma liquid medium in the case of the viscera, brain and lymph nodes. Since the solid mycoplasma medium contained neither penicillin nor thallium acetate, it was also used for aerobic growth of micro-organisms.

Serology

All sera were tested by means of the metabolism inhibition test against *M. hyopneumoniae* strain J and *M. hyorhinis* strain TR 32. The medium used for all tests had a pH of 7·8 and comprised 20% fresh horse-serum, 10% of a 25% yeast extract (DCL Dried Yeast), 40% Hanks's solution, 30%-Hartley digest broth,* 0·5% glucose, 0·0028% phenol red, 0·05% thallium acetate and 1000 units/ml. of penicillin G. In addition, the medium used for tests against *M. hyorhinis* contained 2% fresh guinea-pig serum. The wells of Lucite† trays were filled with 0·05 ml. of each serum dilution, 0·05 ml. of antigen dilution (1000 C.C.U./ml.) and 0·1 ml. of medium, and then sealed with liquid paraffin. Inhibition of glucose metabolism was considered to have occurred in those wells where the pH remained higher than 7·4 when the pH of antigen control wells had fallen to 6·8 or less.

Histopathology

Pieces of tissue were taken from representative areas of all the 30 sites mentioned above, fixed in 10% formol saline and embedded in paraffin, and 5 μ sections were stained with Mayer's haematoxylin and eosin. In addition, selected sections were stained with methyl green pyronin, by the Picro-Mallory method for fibrin, and the Gordon and Sweet method for reticulin.

RESULTS

Microbiological examination of pigs before inoculation

No micro-organisms were recovered from any of the pigs with the exception of the control pig 42C, which was contaminated with *Staphylococcus epidermidis* and a *Streptococcus* sp. in the turbinates and tonsil.

Clinical signs

Pigs 46P and 46C became clinically ill 6 days after inoculation, especially 46P, which was lethargic, weak on its back legs, and breathing very rapidly on the 8th day p.i. However, it seemed to recover about a week later.

* Oxoid Limited, London, S.E.1.

† Linbro 1S MRC 96, Cooke Engineering Co., Alexandria, Virginia, U.S.A.

Table 1. *The location of lesions and re-isolation of* Mycoplasma hyorhinis *from gnotobiotic pigs inoculated with strain TR*32 *of* M. hyorhinis

Pig no.	Inoculum	Time p.i. (days)	Lesions					Re-isolations of *M. hyorhinis*					Bacteriological findings at necropsy
			Lung	Pleura	Peri-cardium	Peri-toneum	Joints	Lung	Pleura	Peri-cardium	Peri-toneum	Joints	
46N	Medium	14	–	–	–	–	–	–	–	–	–	–	–
46B		23	–	–	–	–	–	–	–	–	–	–	–
42C		30	–	–	–	–	–	–	–	–	–	–	*Staph. epidermidis* + *Streptococcus* sp.
46I		34	–	–	–	–	–	–	–	–	–	–	–
42D	*M. hyorhinis*	14	–	–	–	–	+ +	+	–	–	–	+	–
46P		14	–	+ +	+ +	+ +	+ +	+	+	+	+	+	–
46L		14	+ +	–	–	–	–	+	–	–	–	–	Clostridium sp.
46F		23	–	–	–	–	+ +	+	–	–	–	+	–
46H		23	–	–	–	–	–	+	–	–	–	–	–
42E		30	+ +	+ +	+ +	–	+ +	+	+	+	–	+	–
46Q		33	–	–	–	–	+ +	+	–	–	–	+	*Clostridium* sp.
46C		33	+ +	+ +	+ +	+ +	+ +	+	+	+	+	+	–
46A		37	–	+ +	+ +	+ +	+ +	+	+	+	+	+	–

+, Microscopic lesion; + +, macroscopic + microscopic lesion.

Lameness was difficult to detect even when gross joint lesions were evident (see Table 1), since movement is restricted in isolators. However, pig 46Q became obviously lame 30 days p.i.

All the pigs in the other five isolators appeared clinically healthy.

Re-isolations of Mycoplasma hyorhinis

Table 1 indicates sites from which organisms were re-isolated. *M. hyorhinis* was re-isolated from the turbinates, tonsil, trachea and bronchi of all the inoculated pigs, although no lesions were observed in these tissues. *M. hyorhinis* was also isolated from the ground-up lungs of all the inoculated pigs, whether the lungs had lesions or not. Otherwise mycoplasmas were only re-isolated from tissues with lesions.

Microbiological examination of pigs after necropsy

No micro-organisms other than *M. hyorhinis* were recovered from any of the pigs, with the exception of pig 42C which was contaminated with the same organisms as were found before inoculation, and pigs 46L and 46Q which were contaminated with a *Clostridium* sp. in the turbinates, tonsil, trachea, bronchus and intestines (this *Clostridium* sp. was presumed to have entered with the plastic canopy just before inoculation).

Serology

No antibodies to *M. hyopneumoniae* were revealed in the metabolism inhibition test, but antibodies to *M. hyorhinis* were detected at titres of at least 1/4 in all the infected pigs except the three killed 14 or 15 days p.i. and pig 46H which was one of the pigs killed 23 days p.i.

Pathology

The incidence of lesions in the tissues typically affected by *M. hyorhinis* (the serosa and joints) and in the lung are recorded in Table 1. The changes seen in the serosa and joints were very similar to those described by Roberts *et al.* (1963*a*, *b*). They occurred in all ages of pigs.

Macroscopically, affected joints were swollen and hyperaemic. The increased synovial fluid in these joints was cloudy and occasionally contained fibrino-purulent flakes. Histologically there was an extensive cellular infiltration of the tissue underlying the synovial membrane, which showed degenerative changes with desquamating cells being shed into the joint cavity and inflammatory cells lying between and on the inner surface of the serosal cells. The infiltrating cells were predominantly mononuclear, but in the joints in which neutrophils were seen they occurred in foci outnumbering the mononuclear cells. It was not possible to differentiate histologically between the lesions produced 14, 23 and 30 days p.i.

In the abdomen the most characteristic lesion was a creamy yellow, fibrino-purulent exudate on the margin of the liver and spleen. Fibrino-purulent flakes were also seen on the omentum and lying free in the peritoneal cavity. Histological examination confirmed that the lesions did not invade the parenchyma of the liver and spleen, but affected only the mesothelial cells. The exudate which accumulated around these degenerating serosal cells contained mainly lymphocytes and plasma

cells; macrophages and neutrophils were present in smaller numbers. Strands of fibrin lay immediately around the serosal cells while capillary invasion from the parenchyma was evidence of early organization occurring in some of the pigs.

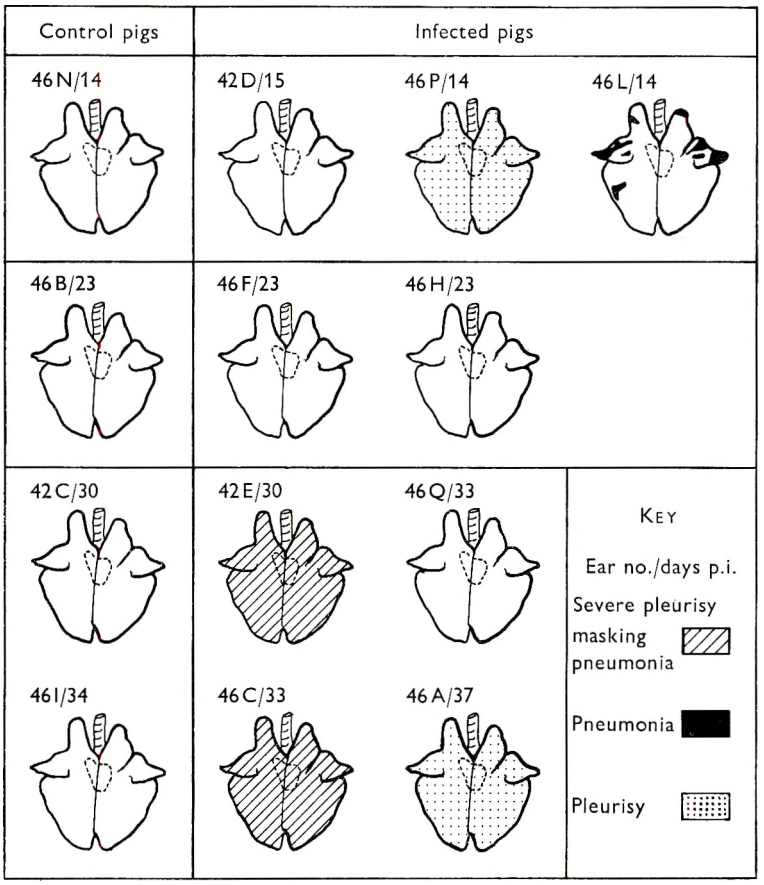


Fig. 1. The incidence of macroscopic lesions in the lungs and pleura of gnotobiotic pigs inoculated with strain TR 32 of *Mycoplasma hyorhinis*.

The incidence and distribution of pneumonia and pleurisy in pigs inoculated with *M. hyorhinis* are summarized in Fig. 1. Of the three pigs killed 14 days p.i., one (46L) had extensive macroscopic lesions in the lungs involving the apical and cardiac lobes of both lungs; one (46P) had developed a diffuse fibrino-purulent pleurisy and pericarditis, and the third pig (42D) did not show any changes macroscopically or microscopically in the lungs or pleura.

The pneumonic lesions in pig 46L were discrete, firm, dark-red areas; microscopically they showed congestion and collapse of the alveolar tissue with considerable exudation of neutrophils and, to a lesser extent, of macrophages. A few mononuclear cells were observed in the lumina of the alveoli and of the bronchioles (Plate 1A). Tissue surrounding the pneumonic areas was emphysematous.

In pig 46P pneumonic lesions were only seen histologically where inflammatory cells spread from the pleurisy into subpleural alveoli. Here some of the alveoli were collapsed and packed with both mononuclear cells and neutrophils (Plate

1B). The pleurisy varied in its severity. In its mildest forms the mesothelial cells of the visceral pleura were still recognizable, although disrupted and hyperchromatic, and were underlaid by congested capillaries and clumps of neutrophils, while in more severe areas infiltration and proliferation of fibroblasts and mononuclear cells occurred underneath the neutrophils. In the most severe form of the pleurisy the mesothelial cells, where visible, were necrotic and the inflammatory tissue contained a fibrinous exudate which stained positively with Picro-Mallory.

The two pigs killed 23 days after inoculation (46F and 46H) showed no lesions in the lungs or pleura.

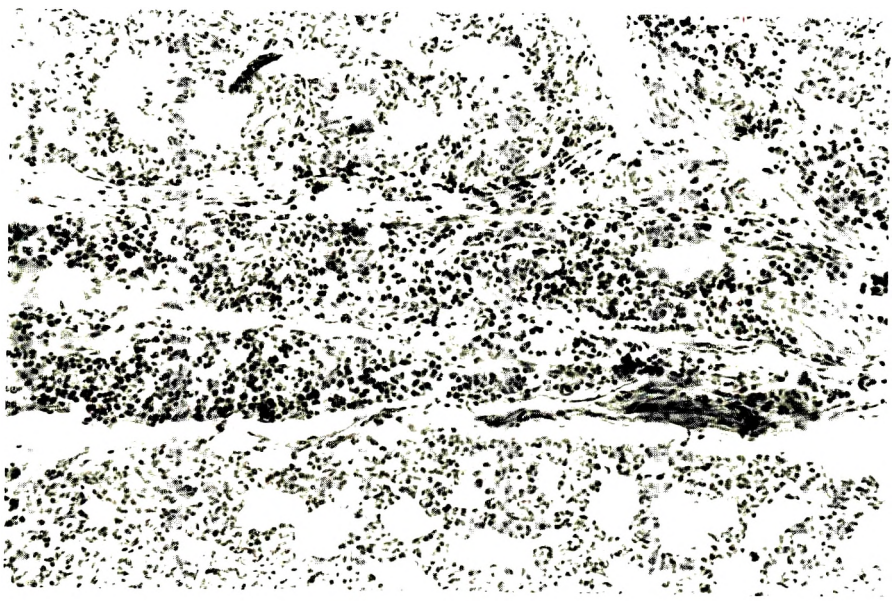
Three of the pigs killed between 30 and 37 days after inoculation had severe fibrinopurulent pleurisy and pericarditis. The opacity of the inflamed visceral pleura, with adhesions to pericardium, parietal pleura and diaphragm, made it difficult to assess the extent of the pneumonia macroscopically. However, in two of the pigs sections of lung from apical, cardiac and diaphragmatic lobes all showed engorged capillaries and lymphatics in the inter- and perilobular septa. Mononuclear cells, a few neutrophils and proliferating reticular fibres surrounded these dilated vessels. The material in the lymphatics was eosinophilic and homogeneous but did not stain positively for fibrin. The alveolar tissue round these foci was often collapsed and invaded by mononuclear cells from the interstitial foci (Plate 2A). No cellular exudation was seen in the bronchioles but some peribronchial mononuclear hyperplasia was seen when the surrounding alveolar tissue was totally collapsed and infiltrated with mononuclear cells. The goblet cells of the bronchial epithelium were hyperactive in comparison with control pigs. Perilobular septa connecting the deeper pneumonic foci with the inflamed pleura also had congested capillaries and lymphatics (Plate 2B). The pleurisy in these two pigs showed a considerable degree of organization with capillary invasion, proliferation and orientation of fibroblasts, very few neutrophils and many mononuclear cells.

No lesions were seen macroscopically or histologically in control pigs.

DISCUSSION

The only published work that associates pneumonia with the inoculation of pigs with 'cloned' cultures of *M. hyorhinis* is that of Gois *et al.* (1968). In the present investigation it was felt necessary to avoid gaseous anaesthesia at the time of inoculation because of increased bronchial secretion of mucus, particularly with ether (Lewis's Pharmacology, 1970), and because of the decreased ciliary activity that occurs with some gaseous anaesthetics (Rivera, 1962). The intranasal route was chosen because it simulates 'natural' infection; control pigs were given the same volume of mycoplasma medium as the infected pigs since medium alone has been recorded by Jericho (1968) as causing lymphoid proliferation in the connective tissue of bronchioles in a gnotobiotic pig inoculated 3 weeks earlier both intratracheally and intranasally. Roberts *et al.* (1963*a*), in their extensive investigation, inoculated pigs intraperitoneally and established that *M. hyorhinis* usually attacks mesothelial tissues, causing pleurisy, pericarditis, peritonitis, and synovitis. This classical pattern of response to *M. hyorhinis* was seen in our experiment following the intranasal inoculation of unanaesthetized pigs with TR32 strain of *M. hyo-*

A



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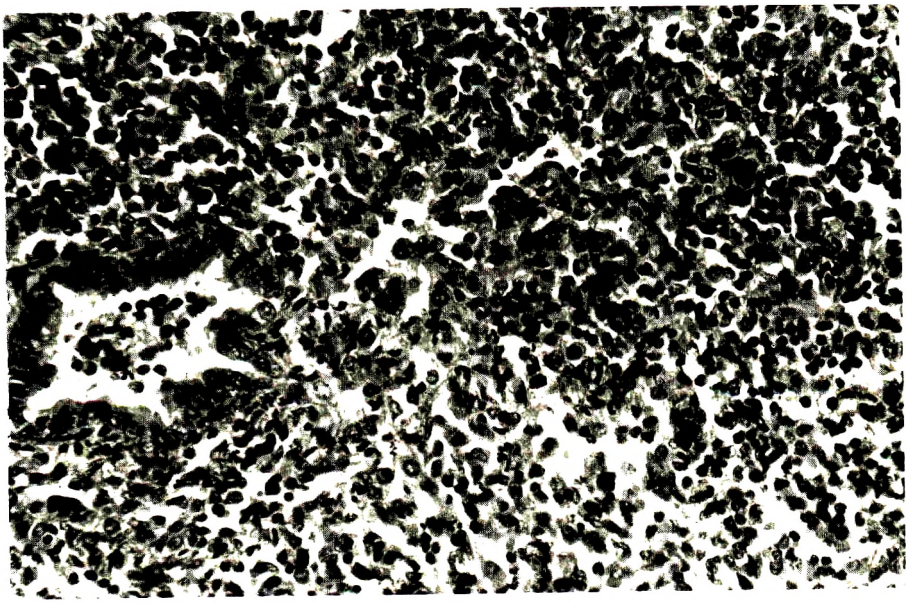

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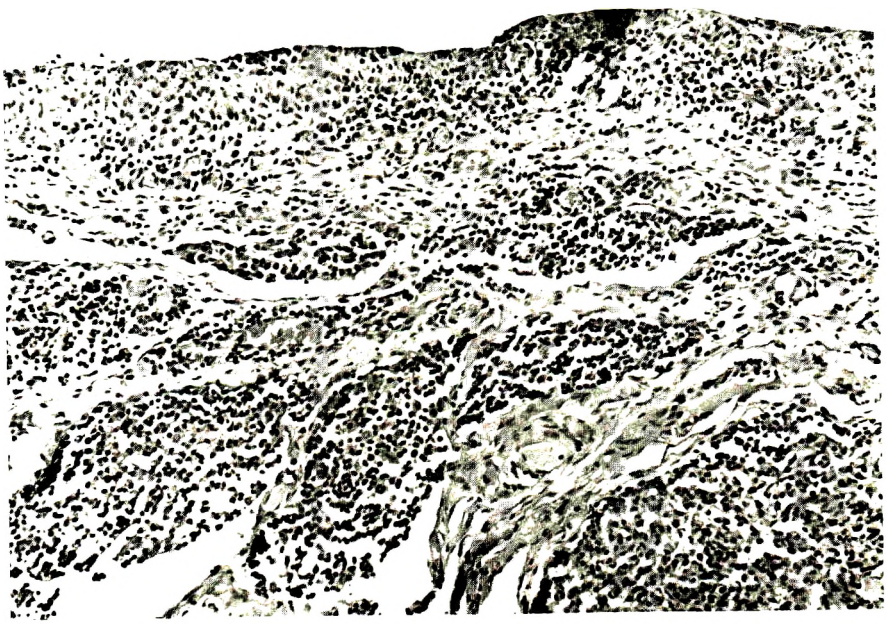

B

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(*Facing p.* 154)

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EXPLANATION OF PLATES

PLATE 1

(A) Lung 14 days after inoculation with *Mycoplasma hyorhinis*, showing collapse of the alveoli with exudation of neutrophils and some mononuclear cells. Cells can also be seen in the lumen of the bronchiole. H. and E., × 100.

(B) Pleura 14 days after inoculation with *Mycoplasma hyorhinis* showing fibrino-purulent pleurisy with inflammatory cells from the pleurisy invading the underlying alveolar tissues. H. and E., × 40.

PLATE 2

(A) Lung 32 days after inoculation with *Mycoplasma hyorhinis*. The interlobular septa are dilated with mononuclear cells and a few neutrophils while the lymphatics are distended with an eosinophilic homogeneous exudate. The surrounding alveolar tissue is collapsed and on the left inflammatory cells can be seen spreading from the interlobular connective tissue into the alveolar tissue. H. and E., × 40.

(B) Lung 30 days after inoculation with *Mycoplasma hyorhinis*. The interlobular septum is engorged by inflammatory cells and both capillaries and lymphatics are dilated. There is no involvement of the surrounding alveolar tissue. This inflamed septum ran from the inflamed pleura (as in Plate 1B) to a deeper focus of pneumonia (as in A). H. and E., × 40.

rhinis, but three of the nine pigs also had pneumonic lesions. The pneumonia seen in a single pig killed 14 days after inoculation occurred without pleurisy and was typical of an early bronchopneumonia, although in contrast to the lesions described in 'enzootic pneumonia' (Pattison, 1956) there was little peribronchiolar or perivascular reaction. The pneumonia in two of the four pigs killed between 30 and 37 days after inoculation, however, was quite different in that a severe pleurisy was present in both cases and reaction in the lung occurred around the capillaries and lymphatics in the inter- and perilobular interstitial tissue, spreading from the interstitial tissue into the alveoli without affecting the bronchioles. A similar pattern of change was seen in association with *Haemophilus pleuropneumoniae* infection in pigs (Shope, 1964), although the changes in the pigs due to strain TR 32 of *M. hyorhinis* were not as severe as the changes recorded by Shope.

It thus seems that strain TR 32 of *M. hyorhinis* is sufficiently pathogenic to induce a primary bronchopneumonia as does *M. hyopneumoniae* (Hodges *et al.* 1969), or a pleuropneumonia in which infection spreads from the pleura into the lung via the lymphatics or capillaries of the interlobular septa.

It is interesting that whenever *M. hyorhinis* was re-isolated from pleura, pericardium, peritoneum or joints a lesion had developed at the site of isolation, and if *M. hyorhinis* was not isolated there was no lesion, but in the lungs *M. hyorhinis* was re-isolated from all infected pigs, whilst pneumonic and/or pleural lesions were seen in only five of the nine pairs of lungs. It is suggested that strain TR 32, like other strains of *M. hyorhinis*, usually survives in the trachea and bronchus (therefore in any sample of lung) as well as in the turbinates and tonsil, without stimulating an inflammatory reaction. However, if it survives or localizes in mesothelial tissues it produces there a marked inflammatory reaction, which in the case of the pleura may spread to involve the lung.

This work indicates that at least one strain of *M. hyorhinis* can produce pneumonia in pigs, so that *M. hyopneumoniae* is not the only mycoplasma able to cause pneumonia. This has implications in the diagnosis of enzootic pneumonia. More work is needed, however, to determine the pathogenicity of British strains of *M. hyorhinis* for gnotobiotic and older conventional pigs.

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