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Salmonellae in abattoirs, butchers' shops and home-produced meat, and their relation to human infection

Report of a Working Party of the PUBLIC HEALTH LABORATORY SERVICE*

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INTRODUCTION

Meat was first implicated as a source of human salmonella infection by Gaertner in 1888. Since then there have been many improvements in the handling, inspection and hygiene of meat but even today salmonella infection may often be traced to infected or contaminated meat. Meat and its products were the commonest known vehicle of salmonella infection in England and Wales between 1949 and 1961 (Galbraith, 1961; Reports, 1961, 1962*a*). The part played by home-produced meat in outbreaks in Great Britain is not known, although it has been proved to be the vehicle of infection in some of them. Some outbreaks have been related to a local abattoir (Camps, 1947) whereas others, such as those recorded by Anderson, Galbraith & Taylor (1961) and Galbraith, Archer & Tee (1961), have been more widespread. The source of infection in smaller outbreaks or in sporadic cases is often not found, but McDonagh & Smith (1958) in Bradford, and Harvey & Phillips (1961) in Cardiff noted a close relation between the types of salmonellae isolated from local abattoirs and from human cases in their districts. Anderson (1960) compared the phage-type distribution of strains of *Salmonella typhimurium* from human infections with that of strains from animals; of the six types encountered most frequently in man and animals, five were common to both groups.

A Working Party of the Public Health Laboratory Service was formed in 1960 to ascertain the degree of infection of meat and meat products from the abattoir, and the route of infection through the food factory and the butcher's shop, to the public. This report of the Working Party records the results of a study of the incidence of salmonellae in thirty-two abattoirs in 1961 and 1962, and of the relation of these findings to human salmonella infection. The distribution of the laboratories which participated in the work is shown in Fig. 1.

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Fig. 1. Distribution of laboratories which participated in the field work.

MATERIALS AND METHODS

The abattoir

(1) Abattoir drains were examined by Moore's gauze-swab technique (Moore, 1948). Swabs were generally left in position for at least 2 days and often for a longer period.

(2) Tissue specimens were collected from animals immediately after slaughter at the abattoir and placed in sterile containers for transport to the laboratory. The tissues selected were mainly spleen, liver and mesenteric lymph nodes. Other material, such as caecal swabs and faeces, was also examined but such specimens were too few to warrant inclusion in this report.

Retail butchers' shops

Gauze swabs from drains or gulleys of retail butchers' shops and markets were examined by the swab method as for abattoir drains.

Meat and meat products

Materials examined included pork and beef sausages, raw meat, raw pie meat, potted meat and minced meat.

Laboratory technique

Laboratory methods were not standardized and each member used the methods for isolation of salmonellae with which he was most familiar. Selenite F broth was used as an enrichment medium except in one laboratory where tetrathionate was used. Enrichment media were incubated at either 37 or 43° C. and subcultured daily for 2, 3 or 4 days. Most laboratories used a modification of Wilson and Blair's bismuth sulphite agar for subculture and many used additional selective media such as deoxycholate-citrate agar or brilliant-green MacConkey agar. The selective media were examined after varying times of incubation at 37° C. and colonies suspected of being salmonellae were examined in the usual manner by serological and biochemical tests. The phage-typing of *S. typhimurium* was carried out by the Enteric Reference Laboratory and the serotyping of the majority of other salmonellae by the Salmonella Reference Laboratory.

RESULTS

Abattoir investigation

(*a*) *Drain swabs*

Drain swabs from thirty-two slaughterhouses were examined. Twenty-eight of the abattoirs killed cattle, sheep and pigs; two killed only pigs; one, cattle and sheep; and another, pigs and sheep. Details of the animals killed and of the results of examination of drain swabs for salmonellae from each of these abattoirs are shown in Table 1. Drain swabs were examined regularly during 1961–62 from twenty-six abattoirs, from one abattoir during 1961 only, and from five abattoirs during 1962 only. Salmonellae were isolated from 930 (20·7 %) of 4496 swabs examined during the investigation. From two abattoirs in which only pigs were killed, isolations of salmonellae were made from 57 and 91 % of drain swabs examined. From abattoirs in which pigs, cattle and sheep were killed, isolations of salmonellae ranged from 73 % of swabs examined to zero.

Factors which may have influenced the isolations of salmonellae from drain swabs from the twenty-four abattoirs which killed pigs, cattle and sheep (numbers 1–24 in Table 1), and which were examined regularly during both 1961 and 1962, have been analysed. To study the influence of the numbers of animals killed abattoirs were divided into three groups of equal size according to the annual slaughter rate. Table 2 shows the number of animals killed in 1961 and 1962 and the percentage of swabs from which salmonellae were isolated; no correlation is evident. In each group there were considerable differences in salmonella isolations between abattoirs killing similar numbers of animals, as may be seen in Table 1.

The figures in Table 1 also suggest that the proportion of different animal species killed is of importance. To study this suggestion the twenty-four abattoirs were

Table 1. *Results of the examination of abattoir drain swabs during* 1961 *and* 1962

No.	Laboratory	Abattoir	Animals slaughtered in a year: Total	Pigs (%)	Cattle (%)	Sheep (%)	Drain swabs, 1961: Positive/ examined	Positive (%)	Drain swabs, 1962: Positive/ examined	Positive (%)	Drain swabs, 1961–62 Positive (%)
1	Ipswich	Beccles, 1	13,347	6	61	33	63/78	81	47/72	65	73
2	Winchester	Winchester	31,000	55	33	11	70/106	66	15/20	75	67
3	Preston	Walton le Dale	16,203	0	59	41	42/77	55	22/84	26	40
4	Bournemouth	Wimborne	61,704	37	35	28	70/175	40	67/209	32	36
5	Cardiff	Barry	28,242	16	14	70	9/51	18	36/85	42	33
6	Ipswich	Ipswich	21,500	59	30	11	8/20	40	7/29	24	31
7	Ipswich	Beccles, 2	1,464	47	20	33	21/69	30	13/88	15	22
8	Bedford	Bedford, 1	15,200	30	18	52	4/47	9	12/34	35	20
9	Cardiff	Cardiff	149,000	18	16	66	11/98	11	21/84	25	18
10	Preston	Poulton le Fylde, 1	3,144	22	25	53	15/61	25	4/68	6	15
11	Leeds	York	48,737	61	17	22	21/162	13	21/150	14	13
12	Northallerton	Darlington	65,534	23	19	58	12/75	16	0/42	0	10
13	Salisbury	Salisbury	87,000	33	19	47	13/115	11	9/120	8	9
14	Guildford	Guildford	59,500	34	16	50	7/110	6	15/158	9	8
15	Carlisle	Carlisle	52,500	11	12	76	1/160	2	6/33	18	8
16	Bedford	Bedford, 3	8,400	36	17	47	1/57	2	3/39	8	4
17	Preston	Poulton le Fylde, 2	7,148	29	5	65	6/65	9	0/70	0	4
18	Preston	Lytham St Annes	14,699	15	10	75	3/78	4	4/92	4	4
19	Ipswich	Beccles, 3	342	67	33	0	3/79	4	2/88	2	3
20	Worcester	Worcester	11,233	52	35	12	2/59	3	0/17	0	3
21	Southend	Hadleigh	21,537	35	19	46	2/35	6	0/33	0	3
22	Conway	Llandudno	18,492	10	8	82	2/38	5	0/33	0	3
23	Plymouth	Modbury, Street	11,180	9	11	80	1/239	< 1	0/208	0	< 1
24	Bradford	Keighley	46,418	21	19	60	0/25	0	0/52	0	0
25	Hull	Bacon factory	125,000	100	—	—	37/38	97	35/41	85	91
26	Ipswich	Bacon factory	125,000	100	—	—	21/44	48	28/42	67	57
27	Bedford	Bedford, 2	650	29	—	71	0/34	0	—	—	—
28	Leeds	Leeds, 1	236,549	11	24	64	—	—	63/150	42	—
29	Leeds	Leeds, 2	31,662	58	7	34	—	—	28/83	34	—
30	Dorchester	Dorchester	56,000	20	42	38	—	—	26/117	22	—
31	Southend	Rayleigh	5,164	58	15	27	—	—	1/26	4	—
32	Bradford	Bradford	172,680	23	18	59	—	—	0/34	0	—
						Totals	445/2,095	(21·2%)	485/2,401	(20·2%)	
							930/4,496 (20·7%)				

divided into three groups of eight according to the percentage of sheep killed. Table 3 reveals an inverse relation between the percentage of sheep killed at abattoirs and isolations of salmonellae from drain swabs. This observation is in keeping with the known infrequency of salmonellae in sheep in the British Isles. The relation goes some way towards explaining the differences in isolation rates of salmonellae between pairs of abattoirs which slaughtered approximately the same numbers of animals, for example 1 and 18, 6 and 21, and 4 and 14 in Table 1.

Table 2. *Salmonella isolations from abattoir drain swabs and the number of animals killed:* 1961 *and* 1962

No. of abattoirs	Animals slaughtered (thousands)	Drain swabs		
		Examined	Positive	Positive (%)
8	0–12	1377	78	5·7
8	13–36	842	339	40·3
8	46–149	1668	274	16·4

Table 3. *Drain swab isolations of salmonellae from abattoirs killing approximately the same percentage of sheep, or pigs, or cattle*

No. of abattoirs	Animals slaughtered	% of all animals	Drain swabs		
			Examined	Positive	Positive (%)
8	Sheep	0–33	1421	430	30·3
8		41–58	1155	161	13·9
8		60–82	1311	100	7·6
8	Pigs	0–18	1410	268	19·0
8		21–35	1110	99	8·9
8		36–61	1367	324	23·7
8	Cattle	5–16	1502	122	8·1
8		17–20	1143	132	11·5
8		25–61	1242	437	35·2

Similar subdivisions of the twenty-four abattoirs were made in order to analyse the effect of the proportions of pigs and cattle on the isolation rate (Table 3). Little can be said about the effect of the number of pigs, but the increased frequency of the isolation of salmonellae in abattoirs dealing with a high proportion of cattle might explain the differences between the results obtained at Ipswich and Preston (see Table 4), where each laboratory examined by the same technique swabs from two abattoirs in the same area. Results from all abattoirs did not, however, conform to this pattern as may be seen if the following pairs of abattoirs killing approximately the same number of cattle are compared: 6 and 20, 2 and 24, and 5 and 23 (Table 1).

Isolations of salmonellae from abattoirs were thus related to some extent to the predominant animal species killed. In general, a high rate of isolation of salmonellae from abattoirs was associated with a high proportion of cattle; a low rate with a low proportion. The lowest rates of isolation of salmonellae occurred in abattoirs

in which a low proportion of cattle and a high proportion of sheep were killed. Among abattoirs where a large proportion of cattle was killed, the highest rate of isolation of salmonellae occurred at Beccles, 1 abattoir (73 % of swabs positive), where most of the cattle killed were aged dairy cows.

Table 4. *The results of laboratories which examined by an identical technique drain swabs from two abattoirs*

		Animals slaughtered				Drain swabs		
			Percentages of					
Laboratory	Abattoir	Total	Pigs	Cattle	Sheep	Examined	Positive	Positive (%)
Ipswich	Beccles, 1	13,347	6	61	33	150	110	73
	Ipswich	21,500	59	30	11	49	15	31
Preston	Poulton le Fylde, 1	3,144	22	25	53	129	19	15
	Poulton le Fylde, 2	7,148	29	5	65	135	6	4

Table 5 presents the frequency distribution of the serotypes and strains of salmonellae isolated from all the abattoir drain swabs examined.

In swabs from abattoirs slaughtering cattle, pigs and sheep, *S. typhimurium* was the dominant serotype, 228 (26 %) of 883 strains, followed by *S. dublin*, 139 (16 %), and *S. heidelberg*, 105 (12 %). Six other serotypes, *S. menston*, *S. senftenberg*, *S. livingstone*, *S. anatum*, *S. meleagridis* and *S. give* accounted for a further 150 (17 %) of the 883 strains. Eighteen strains of *S. paratyphi B* were isolated.

In swabs from the abattoirs which dealt only in pigs, *S. typhimurium* was again the dominant serotype, accounting for 47 (23 %) of 208 strains isolated. Six serotypes, *S. bredeney*, *S. livingstone*, *S. thompson*, *S. menston*, *S. meleagridis* and *S. anatum* were responsible for 82 further isolations (39 %). Four strains (2 %) of *S. heidelberg* and a single strain of *S. paratyphi B* (0·5 %) were also isolated.

In swabs from the Beccles, 1 abattoir, which chiefly slaughtered old dairy cattle, *S. typhimurium* was the dominant serotype, accounting for 30 (26 %) of the 110 strains isolated. Four other serotypes, *S. livingstone*, *S. menston*, *S. kiambu* and *S. schwarzengrund* made up 35 (32 %) of the 110 strains isolated. *S. dublin* was not isolated.

(*b*) *Tissue specimens*

A number of laboratories examined abattoir tissue specimens, usually spleen, liver and mesenteric lymph nodes. Only a few, however, examined specimens consistently throughout 1961 and 1962 and, therefore, the results shown in Table 6 are not as widely representative as are the results from abattoir drain swabs. The pig was the main animal species examined. From 9351 specimens there were 180 isolations of salmonellae (1·9 %). The serotype most frequently isolated was *S. menston*, 41 (23 %), followed by *S. typhimurium*, 36 (20 %), *S. heidelberg*, 22 (12 %), and *S. livingstone*, 11 (6 %).

Table 5. *Salmonella isolations from abattoir drain swabs in* 1961–62

Abattoirs dealing with cattle, pigs and sheep (numbers 2 to 24). Drain swabs			Abattoirs killing pigs only (numbers 25 and 26). Drain swabs			An abattoir chiefly killing aged dairy cattle (number 1). Drain swabs		
Examd.	Pos.	Pos. (%)	Examd.	Pos.	Pos. (%)	Examd.	Pos.	Pos. (%)
4181	699	16·7	165	121	73·3	150	110	73·3
Serotypes and number of strains			Serotypes and no. of strains			Serotypes and no. of strains		
adelaide	*meleagridis*, 20		*agama*			*anatum*, 4		
agama, 12	*menston*, 39		*anatum*, 10			*bovis-morbificans*		
anatum, 22	*mikawasima*, 2		*bonariensis*			*corvallis*		
bere	*millesi*		*brancaster*, 9			*cubana*, 3		
blockley	*montevideo*, 8		*brandenburg*, 2			*derby*, 4		
bovis-morbificans, 5	*muenchen*, 6		*bredeney*, 17			*give*, 3		
brancaster, 6	*muenster*		*cubana*, 2			*heidelberg*, 4		
brandenburg, 18	*newington*, 5		*dublin*, 5			*kiambu*, 8		
bredeney, 17	*newport*, 15		*enteritidis*, 5			*kinshasa*		
canastel	*oranienberg*, 4		*frintrop*			*livingstone*, 11		
chailey, 3	*orion*		*give*, 6			*manchester*, 4		
coleypark	*panama*, 2		*heidelberg*, 4			*meleagridis*		
cubana, 2	*paratyphi B*, 18		*kentucky*, 2			*menston*, 9		
derby, 14	*poona*		*kiambu*, 4			*montevideo*, 4		
dublin, 139	*reading*		*liverpool*, 2			*newington*, 5		
enteritidis, 12	*san-diego*, 2		*livingstone*, 16			*paratyphi B*, 3		
fayed	*saint-paul*, 13		*manhattan*			*reading*		
fresno, 2	*schwarzengrund*, 2		*meleagridis*, 11			*schwarzengrund*, 7		
frintrop, 4	*seegefeld*		*menston*, 12			*tennessee*		
gallinarum	*senftenberg*, 26		*mgulani*			*typhimurium*, 30		
give, 19	*stanley*, 5		*newington*, 2			*uganda*		
godesberg	*taksony*, 6		*newport*			*virchow*, 3		
havana, 2	*teddington*		*norton*, 2*			unidentified		
heidelberg, 105	*tennessee*, 2		*oranienberg*					
infantis, 5	*thompson*, 12		*paratyphi B*					
kentucky, 4	*typhimurium*, 228		*roodepoort*					
kiambu, 6	*uppsala*		*saint-paul*, 4					
lexington	*vejle*, 9		*senftenberg*, 7					
liverpool, 6	*westerstede*, 2		*stanley*, 2					
livingstone, 24	*worthington*, 3		*thompson*, 16					
luke	unidentified, 3		*typhimurium*, 47					
mbandaka, 6			*umbilo*					
			vejle, 4					
			worthington, 2					
			unidentified, 5					

* New serotype: 6,7 : i–lw.

Some laboratories examined both bovine and porcine tissues, including liver, spleen, mesenteric lymph nodes, kidneys, muscle tissue, etc. These miscellaneous specimens were not classified under the animal species of origin and form a separate group in Table 6. From 1996 specimens there were 38 isolations of salmonellae

(1·9%). The serotype most frequently isolated was *S. heidelberg*, 21 (55%), followed by *S. dublin*, 6 (16%) and *S. senftenberg*, 4 (11%).

It is evident from these results that offals, and indeed carcass meat, contaminated with salmonellae must have been conveyed to butchers' shops and meat-processing establishments during the period of the investigation.

Table 6. *Salmonella isolations in* 1961–62 *from animal tissues, specimens from butchers' shops and meat products*

Abattoirs									Retail butchers' shops					
Pig tissues (liver, spleen and mesenteric lymph nodes)			Tissues other than those exclusively from pigs			Meat factory Drain swabs			Drain swabs			Meat and meat products		
Examd.	Pos.	Pos. (%)	Examd.	Pos.	Pos. (%)	Examd.	Pos.	Pos. (%)	Examd.	Pos.	Pos. (%)	Examd.	Pos.	Pos. (%)
9351	180	1·9	1996	38	1·9	96	11	11	1117	73†	6·5	4127	33	0·8
Serotypes			Serotypes			Serotypes			Serotypes			Serotypes		
brancaster, 2			*agama*, 2			*bredeney*, 4			*alachua*			*agama*		
bredeney, 5			*bredeney*			*give*, 3			*anatum*, 3			*bredeney*, 2		
cubana, 5			*dublin*, 6			*lomita*			*brancaster*			*dublin*		
derby			*entebbe*			*menston*			*brandenburg*, 3			*gatuni*, 3		
dublin, 7			*heidelberg*, 21*			*typhimurium*, 2			*derby*, 7			*heidelberg*, 2		
enteritidis, 6			*meleagridis*						*give*, 2			*mbandaka*		
frintrop, 4			*muenchen*						*heidelberg*, 3			*meleagridis*, 2		
give, 10			*senftenberg*, 4						*infantis*, 4			*newport*, 7		
heidelberg, 22			*typhimurium*						*livingstone*, 2			*saint-paul*		
kentucky, 3									*manchester*			*senftenberg*, 3		
kiambu, 2									*meleagridis*			*typhimurium*, 10		
livingstone, 11									*menston*					
menston, 41									*newport*					
*newport**, 7									*paratyphi B*					
*paratyphi B**, 5									*senftenberg*, 2					
saint-paul, 2									*stanley*					
stanley									*tennessee*, 5					
typhimurium, 36									*thompson*, 4					
unidentified, 10									*typhimurium*, 32					

* Related to human infections.

† Two serotypes were isolated from two of these swabs.

The abattoirs studied varied widely in respects other than the numbers and types of animals slaughtered. Some were old and obsolete, others new, well designed and well equipped: differences also existed in the length of time animals were held in lairages before slaughter, and in the conduct and hygiene of slaughtering. Of these differences, the most important is probably the length of the holding time in lairages (McDonagh & Smith, 1958). Holding times varied from less than 6 hr. to 7 days in the present study; variations were too great to allow any conclusions to be drawn on the relation of this factor to salmonella isolation.

Drain swabs from butchers' shops

The results are summarized in Table 6.

From the drains of retail butchers' shops the serotype most frequently isolated was *S. typhimurium*, 32 (43 %) of 75 strains, followed by *S. derby*, 7 strains (9 %), *S. tennessee*, 5 strains (7 %), *S. infantis* and *S. thompson*, each 4 strains (5 %) and *S. anatum*, *S. brandenburg* and *S. heidelberg*, each 3 strains (4 %).

The results of drain swabs from a meat products factory are also shown in Table 6.

Meat samples from retail shops and food factories

The examination of 4127 specimens of a variety of meat and meat products including raw pork, uncooked meat products such as pie meat, and also potted meat and pies, yielded 33 salmonella isolations (0·8 %). Of these samples, 331 were cooked products which all gave negative results. The serotype most frequently isolated was *S. typhimurium*, 10 strains (30 %), followed by *S. newport*, 7 (21 %), *S. gatuni* and *S. senftenberg*, each 3 (9 %), and *S. bredeney*, *S. heidelberg* and *S. meleagridis*, each 2 (6 %).

The relation between serotypes isolated from abattoirs and from human infections

Table 7 lists the serotypes and strains of salmonellae isolated from abattoirs and from human infections in the areas of the abattoirs. Thirty-eight serotypes were isolated only from abattoirs; thirty-five from both abattoirs and human infections; and twenty from human infections only.

Table 8 compares the serotypes most frequently isolated from abattoirs with those most often isolated from human infections in the areas of the abattoirs. The abattoirs examined were not evenly distributed over England and Wales. No abattoirs were examined in the London area or in the Midlands (Fig. 1).

Three groups of serotypes can be distinguished: one of serotypes isolated frequently from abattoirs but infrequently from human infections (*S. dublin*, *S. livingstone*, *S. senftenberg*, *S. give*, *S. brandenburg* and *S. kiambu*); a second isolated about as often from abattoirs as from human infections (*S. meleagridis*, *S. bredeney*, *S. heidelberg*, *S. menston* and *S. thompson*); and a third group isolated less frequently from abattoirs than from man (*S. typhimurium*, *S. newport* and *S. enteritidis*).

S. typhimurium was the serotype most commonly isolated from both sources. *S. dublin*, a specific bovine pathogen and the second commonest serotype isolated from abattoirs, was rarely isolated from human infections. *S. heidelberg*, the third commonest serotype isolated from abattoirs, was the fourth commonest from human infections.

S. paratyphi B (including *S. paratyphi B* var. *java* and *S. java*) is considered separately from the other serotypes. Between September 1961 and March 1962 *S. paratyphi B* phage-type Beccles var. 5 was isolated on seven occasions from abattoir drain swabs. Five isolations were made from a York abattoir between

Table 7. *Salmonella serotypes isolated from abattoirs (drain swabs and tissue specimens) and from human infections in the area of the abattoirs*, 1961–62

Abattoir but not human	Abattoir and human*	Human but not abattoir
adelaide	*anatum*, 36, 9	*abony*, 3
agama, 15	*bovis-morbificans*, 6, 1	*bareilly*, 5
bere	*blockley*, 1, 3	*bleadon*
bonariensis	*brandenburg*, 20, 8	*cambridge*
brancaster, 17	*bredeney*, 40, 31	*chester*, 2
canastel	*cubana*, 12, 2	*cholerae-suis*, 2
chailey, 3	*derby*, 19, 4	*coeln*, 3
coleypark	*dublin*, 157, 9	*drypool*
corvallis	*enteritidis*, 23, 29	*essex*, 3
entebbe	*give*, 38, 6	*haifa*
fayed	*havana*, 2, 1	*hvittingfoss*, 4
fresno, 2	*heidelberg*, 156, 65	*litchfield*
frintrop, 9	*infantis*, 5, 9	*london*
gallinarum	*kentucky*, 9, 1	*paratyphi A*
godesberg	*livingstone*, 62, 2	*richmond*
kiambu, 20	*manhattan*, 1, 1	*rublislaw*
kinshasa	*meleagridis*, 33, 31	*saarbruecken*
lexington	*menston*, 101, 25	*teshie*
liverpool, 8	*montevideo*, 12, 11	*typhi*, 19
lomita	*muenchen*, 7, 1	*weltevreden*, 3
luke	*newington*, 12, 3	
manchester, 4	*newport*, 23, 122	
mbandaka, 6	*oranienberg*, 5, 2	
mgulani	*panama*, 2, 4	
mikawasima, 2	*paratyphi B*, 27, 49	
millesi	*reading*, 2, 3	
muenster	*saint-paul*, 19, 20	
norton, 2	*senftenberg*, 37, 2	
orion	*stanley*, 8, 17	
poona	*tennessee*, 3, 1	
roodepoort	*thompson*, 28, 67	
san-diego, 2	*typhimurium*, 342, 737	
schwarzengrund, 9	*uppsala*, 1, 1	
seegefeld	*vejle*, 13, 3	
taksony, 6	*virchow*, 3, 2	
teddington		
uganda		
westerstede, 2		
worthington, 5		
unidentified, 19		

* First figure indicates number of strains isolated from abattoirs and tissue specimens; second figure from human incidents.

November and March. Investigations revealed that pigs from a farm attached to a mental hospital were the source of the organism and that an outbreak of infection due to this phage-type had occurred at the hospital concerned between October and December 1961 (Ludlam & Payne, personal communication). A few human infections due to this strain also occurred in various parts of Yorkshire between September and December 1961 but no connexion with meat from York abattoir could be established in these instances. Two other isolations of *S. paratyphi B*

phage-type Beccles var. 5 were made from the drain of an abattoir in Beccles, Suffolk, in September 1961. No human infections were recorded in East Anglia at this time and the animal species responsible for the organism could not be traced. Most of the animals killed during this month at the abattoir concerned were cattle or sheep, but also eighty-two pigs were killed during September. *S. paratyphi B* phage-type Battersea was isolated from the drains from the pig slaughter area at Cardiff abattoir in January and July 1962. No cases due to this type occurred in the immediate locality during the relevant times but a human infection was reported in south-west England during the summer of 1962.

Table 8. *Number of isolations of the serotypes found most commonly in abattoirs and in human infections in the areas of the abattoirs*

(The ten most common serotypes from each source are in bold type.)

Serotypes	Abattoirs		Human infections in area of abattoirs
	Drain swabs	Tissues	
S. typhimurium	**300** (23·9%)	**37** (17·0%)	**737** (59·6%)
S. dublin	**138** (11·0%)	**13** (6·0%)	9 (0·7%)
S. heidelberg	**113** (9·0%)	**43** (19·7%)	**65** (5·3%)
S. menston	**59** (4·7%)	**41** (18·8%)	**25** (2·0%)
S. livingstone	**51** (4·1%)	**11** (5·0%)	2 (0·2%)
S. anatum	**34** (2·7%)	—	9 (0·7%)
S. senftenberg	**33** (2·6%)	**4** (1·8%)	2 (0·2%)
S. meleagridis	**32** (2·5%)	1 (0·5%)	**31** (2·5%)
S. bredeney	**31** (2·5%)	**6** (2·8%)	**31** (2·5%)
S. give	**31** (2·5%)	**10** (4·6%)	6 (0·5%)
S. thompson	28 (2·2%)	—	**67** (5·4%)
S. brandenburg	20 (1·6%)	—	8 (0·6%)
S. kiambu	18 (1·4%)	2 (0·9%)	—
S. saint-paul	17 (1·4%)	2 (0·9%)	**20** (1·6%)
S. stanley	17 (1·4%)	1 (0·5%)	**17** (1·4%)
S. newport	16 (1·3%)	**7** (3·2%)	**122** (9·9%)
S. enteritidis	16 (1·3%)	**6** (2·8%)	**29** (2·3%)
S. manhattan	1 (0·1%)	—	1 (0·1%)

All the strains of *S. paratyphi B* belonging to phage-types Beccles var. 5 and Battersea isolated during the survey were slime-wall negative and *d*-tartrate positive; they were, therefore, *S. paratyphi B* var. *java* or *S. java*. Human infections due to this organism usually take the form of food-poisoning and not enteric fever.

Table 9 shows the isolations of phage-types of *S. typhimurium* from abattoirs and from human infections in the area of the abattoirs. Seventeen phage-types (24 strains) were isolated from abattoirs alone: 21 phage-types were isolated from abattoirs and from human infections (265 strains from abattoirs, 593 from human infections in the areas of the abattoirs; there were in addition 45 untypable strains, 20 from abattoirs and 25 from human infections). Fourteen phage-types (55 strains) were isolated from human infections in the area of the abattoirs, but not from abattoirs. Of 648 strains of *S. typhimurium* isolated from human infections in the area of abattoirs and phage-typed, 593 (91·5%) belonged to 21 phage-types which were also isolated from local abattoirs.

Table 9. *Relationship between phage-types of* Salmonella typhimurium *isolated in* 1961–62 *from abattoirs and from human infections in area of abattoirs*

Abattoir only			Abattoir and human				Human alone		
Phage-type			Phage-type				Phage-type		
Old designation	Prov. new designation	No. of strains	Old designation	Prov. new designation	No. of strains: Abatt.	No. of strains: Human	Old designation	Prov. new designation	No. of strains
1 var. 4	U100	1	1	1	44	41	1b	4a	1
2	10	1	1 var. 2	U41	5	3	1b	U59	1
2	U80	5	1 var. 5	U9	6	95	2	U72	10
2b	Untypable	1	1a	2	53	120	2b	14a	3
2c	15a	2	1a var. 1	3 (an.)	14	25	2b	18	5
2d	20a	2	1a var. 2	3 (aer.)	2	29		5a	1
4	U38	2	1a var. 3	U57	2	4		22	4
5	26	1	2	9	2	9		80	1
	U11	1	2	12	7	3		U25	1
	U17	1	2	12a	62	79		U59	1
	U24	1	2a	13	24	11		U70	1
	U77	1	2b	16	4	5		U80	8
	U101	1	2b	17	3	74		U122	1
	U118	1	2b	20	1	4		U125	17
	U119	1	2b	U107	2	4			
	U127	1	2c	14	7	55			
	U131	1	2c	28	2	12			
			4	8	13	15			
				24	4	1			
				32	2	2			
				U40	6	2			

Note: Forty-five strains were untypable: twenty from abattoirs and twenty-five from human infections.

Table 10 compares the phage-types most frequently isolated from abattoirs with those most frequently isolated from human infections in the areas of the same abattoirs. The pattern noted above in connexion with the distribution of serotypes in abattoirs and human infections in the areas of abattoirs again emerges. Certain phage-types were common either in abattoirs or in human infections but not in both; a larger number of phage-types were common to both abattoirs and human infections. In this latter group the association between abattoir isolations and human isolations in the regions of the abattoir was reasonably close.

Abattoir isolations and human infections

The results were analysed to study the relation between abattoir isolations and human infections occurring locally within specified periods of time.

Table 11 presents the figures for abattoir isolations and isolations from human infections in the same area within the same 4-week period, and within the 4-week period immediately preceding or following, for serotypes other than *S. typhimurium* and *S. paratyphi B* during 1961. In seventy-nine (30%) of 263 incidents of salmonella infection listed in Table 11, at least one abattoir isolation was made of

Table 10. *Frequency of isolation of phage-types of* Salmonella typhimurium *from abattoirs and from human infections in the areas of the abattoirs*

(The ten most common phage-types from each source are in bold type.)

Phage-types			
Old designation	Provisional new designation	Abattoir isolations	Human isolations*
2	12a	**62** (21·7%)	**79** (12·6%)
1a	2	**53** (18·5%)	**120** (19·2%)
1	1	**44** (15·4%)	**41** (6·6%)
2a	13	**24** (8·4%)	11 (1·8%)
1a var. 1	3 (an.)	**14** (4·9%)	**25** (4·0%)
4	8	**13** (4·5%)	**15** (2·4%)
2	12	**7** (2·4%)	3 (0·5%)
2c	14	**7** (2·4%)	**55** (8·8%)
	U 40	**6** (2·1%)	2 (0·3%)
1 var. 5	U 9	**6** (2·1%)	**95** (15·2%)
1 var. 2	U 41	5 (1·7%)	3 (0·5%)
2b	17	3 (1·0%)	**74** (11·8%)
1a var. 2	3 (aer.)	2 (0·7%)	**29** (4·6%)
2c	28	2 (0·7%)	**12** (1·9%)
2b	29	2 (0·7%)	11 (1·8%)
6 other types		16 (5·6%)	25 (4·0%)
	Total	266	600

* 112 strains of *S. typhimurium* from human sources were not phage-typed.

Note: Twenty strains isolated from abattoirs and twenty-five strains from human infections were untypable.

the same serotype causing the human infection in the area of the abattoir within 4 weeks, and 53% of the human infections could be related to local abattoir isolations within a period of 12 weeks. Delay in identification of organisms and recording of results must have often considerably increased the time interval between reporting of a human case and the corresponding abattoir isolation.

Table 12 lists thirty-one incidents of human infections due to serotypes which were also isolated from local abattoirs at the relevant time. The incidents are classified according to the likelihood of their being caused by contaminated meat and meat products. In eight incidents, involving a total of 281 infected persons, it was probable that infection was conveyed by meat or meat products. In twenty-three further incidents there may have been a connexion with the isolation of the serotype concerned from the local abattoir or meat product.

DISCUSSION

In sporadic cases of human salmonella infection, the sources and vehicles of infection can rarely be traced. Parker (1954) found that many apparently sporadic infections with *S. paratyphi B* in an urban population could be associated with a few 'prevalences', each of which consisted of a group of cases occurring simultaneously though no common food could be identified. A 'prevalence' is the

Table 11. *Associations in* 1961 *between abattoir isolations and human infections occurring in the same area*

(*S. typhimurium* and *S. paratyphi B* are excluded from this table.)

Serotype	No. of incidents of human infection	No. of times there was association between a human infection and an abattoir isolation: In the same 4-week period	In the preceding or following 4-week period	Total
S. agama	3	2	1	3
S. anatum	16	3	3	6
S. brandenburg	20	11	2	13
S. bredeney	6	2	1	3
S. derby	6	2	1	3
S. dublin	17	9	2	11
S. enteritidis	24	5	6	11
S. give	8	4	2	6
S. heidelberg	26	10	8	18
S. kiambu	2	1	0	1
S. livingstone	3	1	0	1
S. manhattan	8	0	1	1
S. meleagridis	26	7	8	15
S. menston	22	5	3	8
S. montevideo	9	2	3	5
S. muenchen	1	1	0	1
S. newport	19	2	2	4
S. saint-paul	17	4	7	11
S. san-diego	1	1	0	1
S. senftenberg	4	3	1	4
S. stanley	2	0	1	1
S. tennessee	1	1	0	1
S. thompson	22	3	8	11
Totals	263	79	60	139

An abattoir isolation of the same serotype was made in 53% of twelve-weekly periods in which an incident of human infection occurred. Serotypes with no such correlation are omitted from this table.

occurrence within a period of time of a number of cases of infection with the same salmonella serotype or phage-type. McDonagh & Smith (unpublished observations) investigated sporadic cases of salmonella infection in Bradford where children under 5 years of age made up 52% of sporadic infections between 1954 and 1956. They found that, although direct investigation was fruitless, correlation in time of outbreaks and sporadic cases with isolations of salmonella serotypes and phage-types from a local abattoir yielded good evidence that many outbreaks and sporadic cases had their origin in home-produced meat. Harvey & Phillips (1961) made similar observations in Cardiff. Jones, Bennett & Ellis (1961), however, in Coventry found little similarity between serotypes and phage-types isolated from a city abattoir and from human infections in the city over a 12-month period. As independent local investigations might not necessarily reflect the general relation

Table 12. *Incidents of human salmonella infection in which the same organism was isolated locally from meat, butcher's shop or abattoir specimens*

A. *Incidents of human infection in which there was strong evidence that contaminated meat was involved*

(1)	Bradford	Aug. 1960–June 1961	111 cases	*S. typhimurium* type 17 isolated from abattoir drain swabs and tissue specimens, the drain of a retail market, pie meat and raw pork
(2)	Hull	Apr. 1961	16 cases	*S. meleagridis* in potted meat used in three old people's homes where cases occurred
(3)	Hull	Apr. 1961–Sept. 1961	31 cases	*S. typhimurium* type 1 var. 5 in potted meat and in pig tissues from an abattoir
(4)	Northallerton	May 1961–June 1961	9 cases	*S. heidelberg* isolated from twenty-three of forty-six abattoir specimens, two drains of butchers' shops and from sausage meat
(5)	Dorchester	Oct. 1961	6 cases	*S. saint-paul* in sausage meat in kitchen of hospital where cases occurred and in drain of food factory where the meat was processed
(6)	Ipswich	Nov. 1961	4 cases	*S. typhimurium* type 1a var. 1 isolated on five occasions from drain of butcher's shop from which the families purchased pork and sausages
(7)	Northallerton	June 1962–July 1962	65 cases (+35 excreters)	*S. newport* isolated from pork sausage meat and pie meat from a retail shop and associated factory. Seven of thirty-seven abattoir drain swabs from abattoir and factory were positive
(8)	Winchester	July 1962	4 cases	*S. typhimurium* type 12a isolated from drain of shop from which two patients purchased cooked meat. Abattoir drain also infected

B. *Incidents of human infection in which isolations of the same type were made from abattoirs or meat in the locality but in which no direct connexion could be established*

(1)	Winchester	Feb. 1961	1 case	*S. brandenburg*—abattoir drain isolation
(2)	Plymouth	Feb. 1961	1 case	*S. dublin* (anaerogenic)—abattoir drain isolation
(3)	Hull	Mar. 1961	1 case	*S. saint-paul*—isolated from drain of local bacon factory
(4)	Winchester	May 1961–June 1961	2 cases	*S. agama*—abattoir drain positive on five occasions
(5)	Northallerton	June 1961–Sept. 1961	37 cases	*S. manhattan*—isolated from drain of bacon factory
(6)	Hull	July 1961	1 case	*S. meleagridis*—isolated from drain of bacon factory
(7)	Ipswich	July 1961–Sept. 1961	4 cases	*S. typhimurium* type 1a var. 1—butcher's shop and abattoir drains positive
(8)	Winchester	Aug. 1961	3 cases	*S. heidelberg*—abattoir drain isolation
(9)	Bradford	July 1961–Oct. 1961	13 cases	*S. typhimurium* type 12a—isolated from pig tissues at abattoir
(10)	Bradford	Sept. 1961	1 case	*S. give*—isolated from pig tissues at abattoir
(11)	Hull	May 1961	4 cases	*S. brandenburg*—isolated from bacon factory
(12)	Guildford	Sept. 1961–Nov. 1961	8 cases	*S. typhimurium* type 2c—isolated from pork and from abattoir drains
(13)	Bradford	Oct. 1961–Nov. 1961	2 cases	*S. typhimurium* type 1 var. 2—isolated from raw meat and pig tissues at abattoirs
(14)	Cardiff	Oct. 1961–Dec. 1961	6 cases	*S. heidelberg* (serological variant)—abattoir drain isolations on five occasions
(15)	Ipswich	Nov. 1961	1 case	*S. give*—isolated from pig tissues at bacon factory
(16)	Leeds	Nov. 1961–Dec. 1961	5 cases	*S. menston*—two incidents; abattoirs positive on thirteen occasions
(17)	Winchester	July 1961–Sept. 1961	4 cases	*S. montevideo*—abattoir drain isolations on two occasions
(18)	Hull	May 1962–June 1962	3 cases	*S. saint-paul*—isolated from bacon factory drain
(19)	Bedford	May 1962–July 1962	1 case (+2 excreters)	*S. typhimurium* type 1a isolated on three occasions from abattoir supplying butcher's shop used by infected family
(20)	Dorchester	July 1962	3 cases	*S. heidelberg*—isolated repeatedly from drain of abattoir supplying butcher's shop used by family concerned
(21)	Bournemouth	July 1962	3 cases	*S. typhimurium* type 12a found on twelve occasions in local abbatoir drain during July
(22)	Ipswich	Aug. 1962	2 cases	*S. infantis* isolated on four occasions from drain of an abattoir in the area
(23)	Bournemouth	Aug. 1962–Oct. 1962	7 cases	*S. typhimurium*, untypable by phage, found in five abattoir drain swabs

Note: The names of the towns are those in which the investigating laboratories are situated.

between home-killed meat and human salmonella infection, it was hoped that a number of laboratories working in different parts of England and Wales might provide a more general picture.

Salmonellae were isolated from twenty-nine of the thirty-two abattoirs examined. The percentage of positive drain swabs varied widely between abattoirs. This was evident not only between abattoirs in the same area examined by one laboratory, but also in the same abattoir examined by the same laboratory in different years. Variations in the isolation of salmonellae from different abattoirs were not likely, therefore, to be due to individual differences in either the frequency of examination of swabs or the laboratory techniques of collection and examination of swabs.

The differences in salmonella isolations from abattoirs did, however, appear to be related to the species of animals killed. In general, salmonellae were isolated more often from abattoirs killing a large proportion of cattle and less often from abattoirs where few cattle were killed in proportion to sheep. The importance of cattle as a source of *S. typhimurium* infection was first pointed out by Anderson (1960); he analysed the distribution of phage-types in animals and man and concluded that cattle and fowls were the main animal sources of human infection during the period of his survey. The influence of the proportion of pigs killed on salmonella isolations from abattoirs proved more difficult to assess. High isolations of salmonellae were recorded at the two bacon factories examined (91 and 57 % of swabs positive), but the number of pigs killed each week at these factories was greater than the number killed annually at many of the other abattoirs. One laboratory (Ipswich), however, examined swabs from an abattoir (no. 1) killing annually approximately 8000 aged dairy cows and from a bacon factory (no. 26) killing annually 125,000 pigs. Salmonella isolations from drain swabs were 73 and 57 %, respectively (Table 1). On balance it appears that the proportion of pigs killed influenced the salmonella isolations from abattoirs much less than the proportion of cattle.

In connexion with pigs, it is noteworthy that no isolations of *S. cholerae-suis* were made during the survey. The significance of this observation is difficult to assess, because selenite F broth was used as an enrichment medium for many of the specimens; Smith (1952) reported that selenite broth was not a suitable culture medium for *S. cholerae-suis*. Another possibility is that intestinal carriers of *S. cholerae-suis* may be rare among healthy pigs which are sent for slaughter.

Culture of drain swabs from abattoirs provides ample evidence of the presence of salmonellae in the intestines of animals, but the presence of salmonellae in animal tissues is a more reliable index of carcass contamination and provides a link between abattoirs and butchers' shops. If tissue samples are regarded as representative of carcass meat, which some may question, approximately 2 % of carcasses at abattoirs immediately after killing were probably contaminated with salmonellae.

A direct link is thus established between the abattoir and the butcher's shop. Eighteen of thirty-five serotypes isolated from abattoir drain swabs and from human infections (Table 7) were isolated from animal tissues at the abattoir.

Fourteen of these eighteen serotypes were isolated also from butchers' shop drains or from meat products. *S. heidelberg*, *S. meleagridis*, *S. newport*, *S. senftenberg* and *S. typhimurium* were isolated from abattoirs, animal tissues at the abattoirs, butchers' shop drains, and meat or meat products.

Seven of thirty-eight serotypes isolated from abattoir drain swabs but not from human infections were isolated from animal tissues. Four of these seven serotypes were also isolated from butchers' shop drains or from meat and meat products. Of the serotypes isolated from abattoir drain swabs, those which were also found in animal tissues were more common than those which were not. There may therefore be a quantitative variation in the contamination of carcass meat with salmonellae, and this suggestion is supported by the analyses of human infections discussed in the next paragraph. Two serotypes, *S. alachua* and *S. gatuni*, were isolated from a butcher's shop drain and from butcher's meat, but not from tissues or abattoir drains. It is possible that these strains were derived from sources other than home-killed meat, such as fowls or imported meat.

The relation between contaminated meat and human infections must now be examined. Perfect agreement between contaminated meat from abattoirs and human infections is difficult to show, since meat from an abattoir is often distributed to and consumed in distant areas. For example, carcass trimmings from an East Yorkshire bacon factory were sent for processing and distribution to West Yorkshire, and pie meat was sent to the London area. A relation between abattoir isolations and human infections might be expected more frequently in large outbreaks than in sporadic cases not only because outbreaks are generally investigated more thoroughly, but also because it is likely that contamination is then widespread in abattoirs and food premises and is thus more likely to be detected. Table 12 lists eight outbreaks in which the chain of infection could be traced back through meat or butchers' shops to abattoirs, and twenty-three outbreaks or sporadic cases in which a close relation in time with abattoir isolations was shown. Most human salmonella infections, in England and Wales are however, sporadic cases in which the vehicle of infection is rarely identified. Table 11 shows that many sporadic cases had a close association in time with the isolation of the infecting serotype from an abattoir, and could be classed as 'prevalences' (Parker, 1954); 30 % of sporadic human infections occurred within the same 4-week period as the isolation of the infecting serotype from the abattoir. If the period of time be extended to 12 weeks, to cover the inevitable delays in the identification of serotypes and notification of human infections, 53 % of sporadic human infections could be related to an abattoir isolation.

In addition, many of the serotypes isolated most frequently from abattoirs were also the serotypes most commonly isolated from human infections. Thirty-five serotypes were isolated both from abattoirs (drain swabs and tissue specimens) and from human infections (Table 7). Eighteen of the serotypes were also isolated from animal tissues, and these eighteen serotypes were responsible for 1153 (90 %) of 1282 human infections. The five serotypes isolated from abattoirs, tissues, butchers' drains, and meat products (*S. heidelberg*, *S. meleagridis*, *S. newport*, *S. senftenberg* and *S. typhimurium*) were isolated from 956 (75 %) of 1282 human

infections. The seventeen serotypes isolated from abattoirs and human infections but not from tissues were found in only 129 (10%) of 1282 human infections. *S. thompson*, which is commonly isolated from fowls and egg products, accounted for 67 of these 129 human infections.

These findings would seem to establish evidence of a chain of infection from abattoirs via retail butchers' shops and meat and meat products to man. Healthy animals are known to harbour salmonellae in the intestine (Field, 1948; Smith, 1959, 1960) and after leaving the farm there are many opportunities for cross-infection of animals before reaching the abattoir (Anderson *et al.* 1961). The salmonellae isolated from animal tissues at the abattoir are probably the result of surface contamination from the intestinal contents of apparently healthy animals, but it is possible that invasion of deep tissues may occur post-mortem, or ante-mortem in animals exhausted through physical stress (Jepsen, 1957). The factors leading to severe abattoir contamination may be manifold, however, and probably include the numbers of infected animals entering the abattoir, the length of holding time before slaughter, and the general hygiene of the abattoir. With reference to the numbers of infected animals entering the abattoir, it may be noted that continuous observation of a herd of sixty calves in 1960 showed the occurrence of a sudden brief outbreak of infection with *S. typhimurium* phage-type 1a in which at least 50% of animals were excreting enormous numbers of salmonellae but showed only mild evidence of constitutional upset (Wormald, personal communication). It seems likely that routine slaughter of such a group of animals during the period of symptomless infection could initiate a widespread outbreak of human infection; contamination of the abattoir might persist for some time after the infected animals were killed, and an outbreak could be prolonged in this way.

As to the role of home-produced meat in salmonella infection in England and Wales, it has already been noted that only on two occasions were serotypes isolated from butchers' shop drains or meat which were not isolated from abattoirs. In this investigation 2% of animal tissues from abattoirs were found contaminated with salmonellae. This figure is regarded as indicative of carcass contamination and is to be compared with 4·3% of imported chilled carcasses and 10·3% of imported frozen boneless meat found contaminated in 1956–59 (Hobbs & Wilson, 1959); during this period home-produced meat formed 65%, imported chilled and frozen meat 35%, and boneless meat 0·3% of meat consumed (Report, 1962*b*). Both home-produced and imported carcass meat are sources of human salmonella infection. The amount of imported boneless meat consumed is small compared with that of carcass meat, but a much higher proportion of samples are found to be contaminated; furthermore, this material is used in manufacturing establishments and for large-scale catering.

As to the prevention of human infection from home-killed meat, the greatest return may be expected by concentrating on the prevention of cross-contamination in the abattoir, which serves as a centre both for the collection of animals for slaughter and for the distribution of meat. Attention should also be directed to markets and dealers' premises as the places where cross-infection of animals is most likely to occur in transit from farm to abattoir.

Attention should finally be directed to infection of individual animals on the farm. Field (1959) has described a system of control measures under which clinical salmonellosis of adult cattle would be a notifiable disease, leading to the identification of infected or excreting animals in herds.

SUMMARY

In 1961 and 1962 a Working Party of the Public Health Laboratory Service, in which twenty-two laboratories participated, investigated the occurrence of salmonellae in abattoirs, meat factories, butchers' shops and meat products, and their association with human infections.

Thirty-two abattoirs were studied. Salmonellae were isolated from 930 (21 %) of 4496 swabs of abattoir drains. There was great variation between different abattoirs, but in general salmonellae were found most frequently in those which slaughtered a high proportion of cattle and a low proportion of sheep; more serotypes were isolated from bacon factories than from abattoirs which slaughtered more than one species of animal. Of 11,347 tissue specimens collected at abattoirs, 218 (1·92 %) yielded salmonellae.

Drain swabs from butchers' shops were examined and 73 (6·5 %) of 1117 swabs were positive. Meat and meat products were less commonly contaminated but 0·8 % of 4127 samples yielded salmonellae.

Salmonella typhimurium was the serotype isolated most frequently from all sources. It was often shown that the same serotypes or phage-types were occurring in abattoirs and in human cases in an area at the same time. In eight food-poisoning incidents, involving a total of 281 cases and excreters, there was convincing evidence that meat or a meat product was the vehicle of infection; in a further twenty-three incidents the organisms causing disease were isolated from sources which suggested that infection might have been meat-borne.

The evidence collected suggests that cattle introduce salmonellae into abattoirs more often than other species of animals. The importance of pigs as a source of human infection is confirmed. Sheep are not a source of salmonella infection in man from meat and meat products, whereas meat from pigs, cattle and calves is a source of infection and is responsible for both sporadic cases and outbreaks of disease.

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Survival of shigellae in food*

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INTRODUCTION

Various food preparations may harbour and transmit *Shigella* to humans, which may result in clinical bacillary dysentery. The longevity of shigellae in food materials is therefore of fundamental importance in the epidemiology of shigellosis. Some data are available on the survival of *Shigella* species in a variety of environments (Wilson & Tanner, 1945; Hartsell, 1951*a*; Shrewsbury & Barson, 1957; Talayeva, 1960; Nakamura, 1962). In the present investigation we determined the survival of *S. flexneri* and *S. sonnei* in various foods and food products, namely milk, orange juice, whole egg, egg white, oyster, clam, shrimp, flour, tomato juice, root beer and cooking oil. Furthermore, the survival and persistence of the shigellae in these food preparations were studied at several temperatures.

MATERIALS AND METHODS

Cultures employed in these experiments were maintained on nutrient agar, stored at 0·5° C., transferred every 4 weeks, and tested serologically for purity with Bacto-Shigella Antisera (Difco). *S. flexneri* serotype 2a was supplied by Dr W. H. Ewing, Laboratory Branch, Communicable Disease Center, Atlanta, Georgia; *S. sonnei* strain 13327 was obtained from Frank P. Pauls, South Central Regional Laboratory, Department of Health and Welfare, Anchorage, Alaska; strain B-2569-2 of *S. sonnei* was sent to us by Commander T. M. Floyd, U.S. Naval Medical Center, Bethesda, Maryland.

Cells for survival studies were harvested from the third consecutive transfer of actively growing cultures. Inocula were prepared from each of the cultures used by washing 24 hr. nutrient broth cultures in sterile physiological saline and resuspending the cells in saline. Suspensions of cells consisting of 1·5 to 3·5 $\times$ 10^8 cells/ml. were added in 1 ml. volumes to tubes containing various sterile menstrua. These suspensions were standardized by measurement of optical density, and plate counts were made at the time the menstrua were inoculated for determination of the initial cell concentration. We have taken the liberty of using the term menstruum to designate the food preparation in which survival of shigellae was determined. We are aware of the fact that this is a demarcation from the definition of the term, namely, a solvent. Each menstruum was tubed and sterilized; in some

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cases the food preparation was dispensed aseptically into sterile containers and survival studies performed without sterilization of the menstruum.

Preparation of menstrua

Milk. Pasteurized, homogenized whole milk containing vitamin D was obtained from a commercial source, dispensed in screw-capped test tubes (16 × 150 mm.) in 9·0 ml. amounts, and autoclaved.

Orange juice. Frozen concentrated orange juice (Old South) was reconstituted. The pH of the juice was 3·7; the juice was tubed in 9·0 ml. amounts and autoclaved. In a second series of experiments the orange juice was adjusted to pH 7·0 with NaOH.

Whole egg. One-day-old eggs were obtained from a farm, soaked in 60% ethyl alcohol for 5 min., allowed to drain dry on sterile gauze pads, broken open aseptically in an ultra-violet light chamber and blended in a Waring Blendor for about 3 sec. Samples of the blended egg were dispensed into sterile test tubes, sterility tests being put up at the same time.

Egg white. This was prepared similarly to the whole egg except that the white was separated aseptically from the yolk of each egg before blending.

Oyster, clam and shrimp. Commercially canned sea foods were packaged in metal-capped tubes and autoclaved. Whole oysters (Finer Foods) with salt added were cut into pieces weighing approximately 1 g. each. Minced sea clams (Sonny Boy) with salt and monosodium glutamate added were tubed in approximately 1 g. amounts. Whole small shrimps (Blue Plate Shor-Pak) with salt added were found to weigh approximately 1 g. each and were tubed individually.

Carbonated beverages. Root beer (Hires) and ginger ale (Clicquot Club) were inoculated with organisms directly into their commercial glass containers. In preparation, the bottles were washed with soap and water and opened with a bottle opener which had been flamed.

Sampling procedures

At various intervals the food products containing the shigellae were sampled. Liquid preparations were decimally diluted and plate-counted. Dilutions of 10^{-3}, 10^{-5}, and 10^{-7} were simultaneously plated in duplicate. Towards the end of the survival period when the population declined considerably, 0·1 ml. of the undiluted menstruum was also plated.

Whole objects including oysters, clams, etc., were blended in sterile Waring Blendor cups for 3 sec. and the suspension decimally diluted for the plate counts.

At least ten samples were studied in duplicate for each food preparation at each holding temperature. Standard plate count techniques were used to determine the number of viable cells. Surviving fraction curves were determined by dividing the number of cells surviving by the number in the initial population.

RESULTS

The survival periods of the shigellae in various food preparations at different temperatures are illustrated in Figs. 1 and 2. In general, the survival rates of *S. sonnei* and *S. flexneri* in various menstrua were comparable. However, there

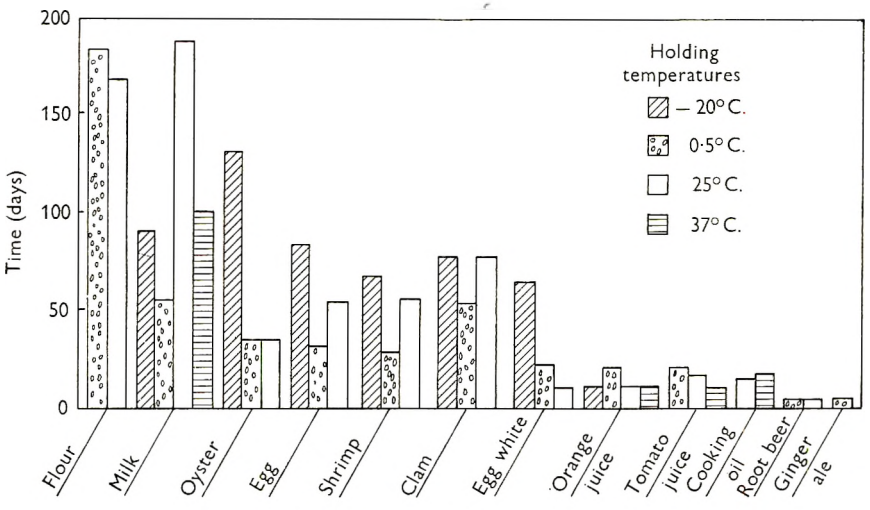


Fig. 1. Survival of *Shigella flexneri* in foods.

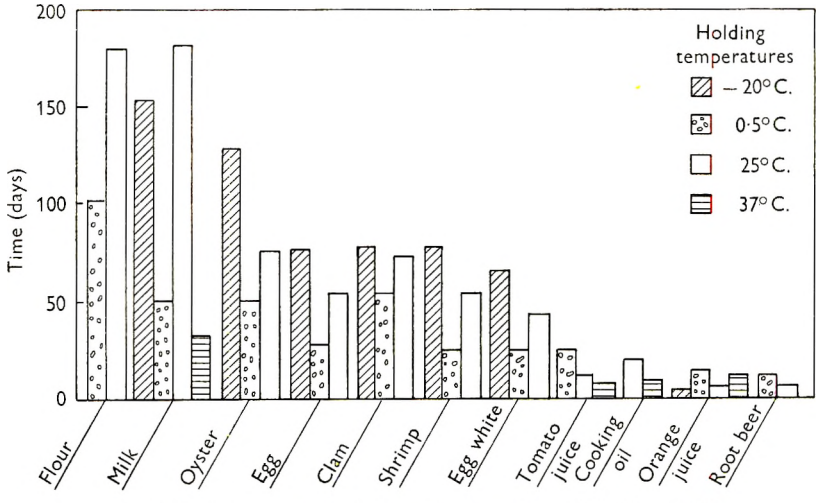


Fig. 2. Survival of *Shigella sonnei* B-2569-2 in foods.

was a considerable difference in the survival rates at the different holding temperatures. The survival periods were longest when the holding temperature was 25° C. or lower. The length of time the shigellae survived varied also with the menstrua. For example, the organisms survived for over 170 days in flour and milk and for lesser periods in oyster, egg, shrimp, clam, and egg white, and for relatively short periods in orange juice, tomato juice, cooking oil, root beer, and ginger ale. Factors which may have influenced these differences will be discussed later.

The survival of *Shigella* in milk at different temperatures is illustrated in Fig. 3. The surviving fractions decreased slowly and persisted for long periods; in fact, in some cases the numbers increased, possibly owing to multiplication of the organisms

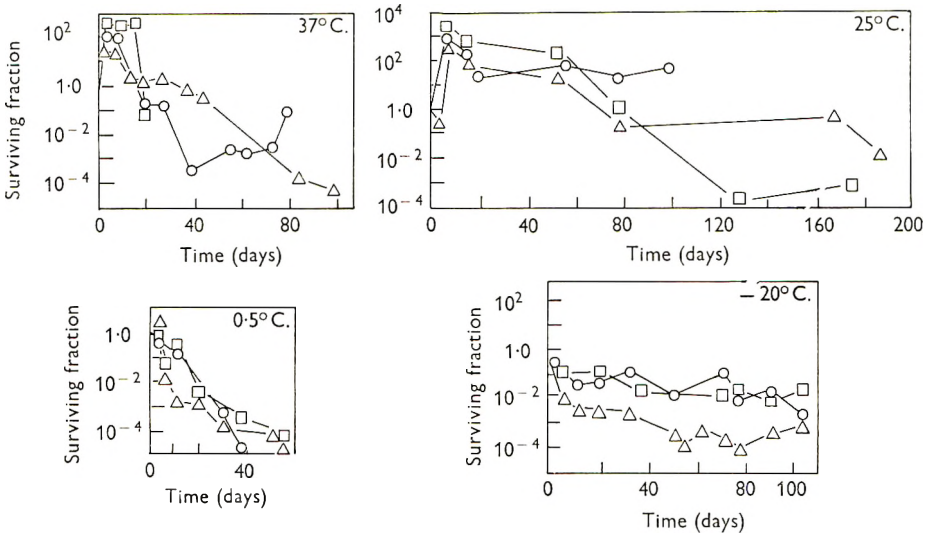


Fig. 3. Survival of *Shigella* spp. in milk: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

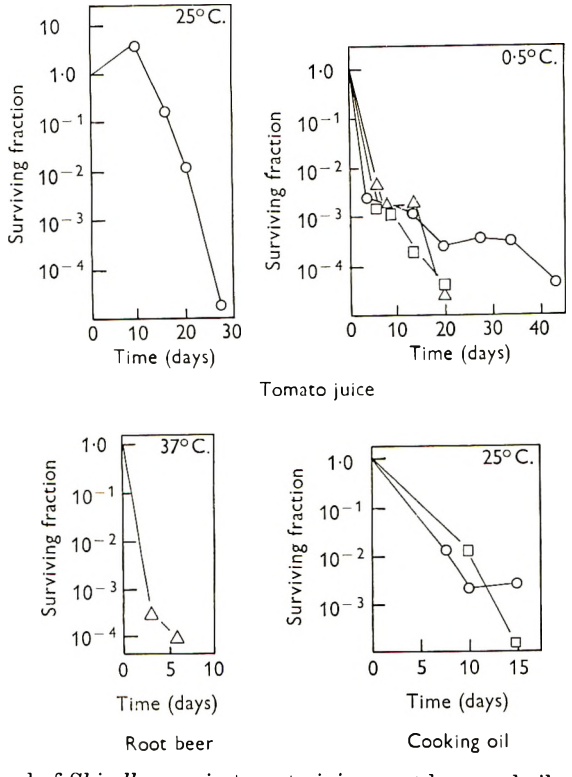


Fig. 4. Survival of *Shigella* spp. in tomato juice, root beer and oil: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

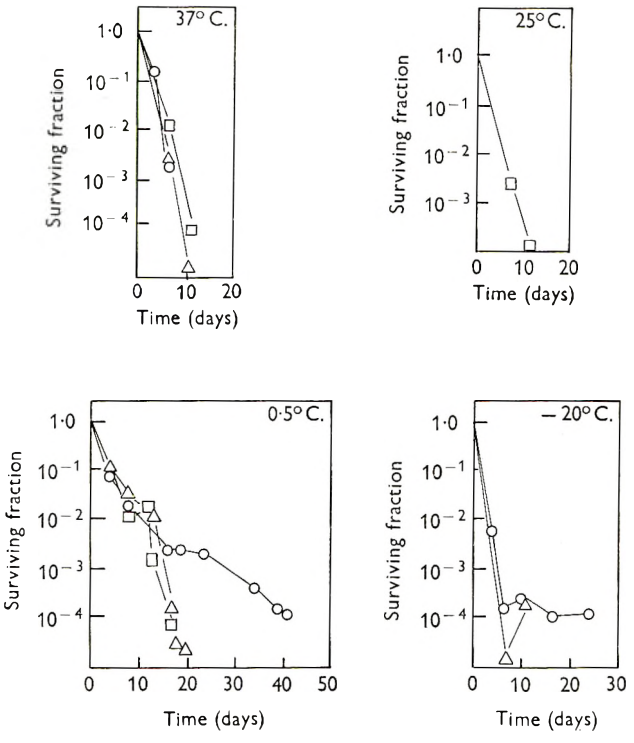


Fig. 5. Survival of *Shigella* spp. in orange juice: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

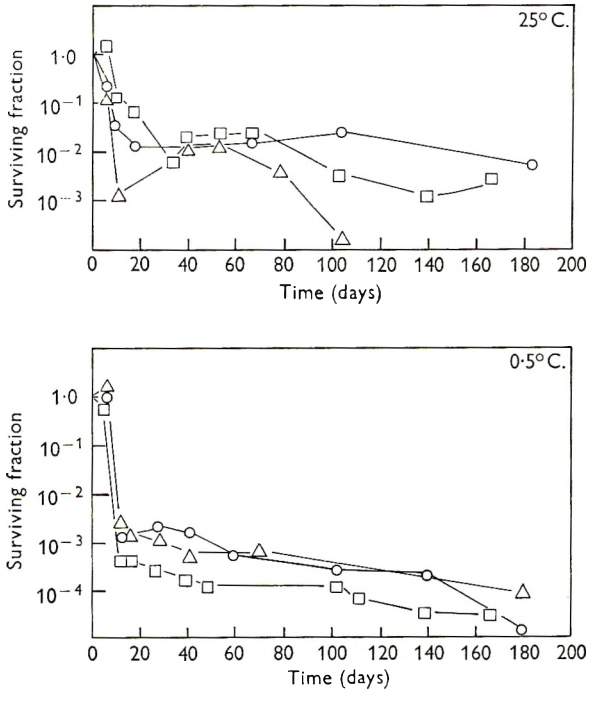


Fig. 6. Survival of *Shigella* spp. in flour: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

in the milk at the higher temperatures. By contrast, the surviving fractions declined rapidly when the shigellae were placed in tomato juice, root beer, cooking oil, and orange juice (see Figs. 4 and 5).

The longevity and persistence of shigellae in flour, egg white, oyster, clams, shrimp, and whole egg are illustrated in Figs. 6–11.

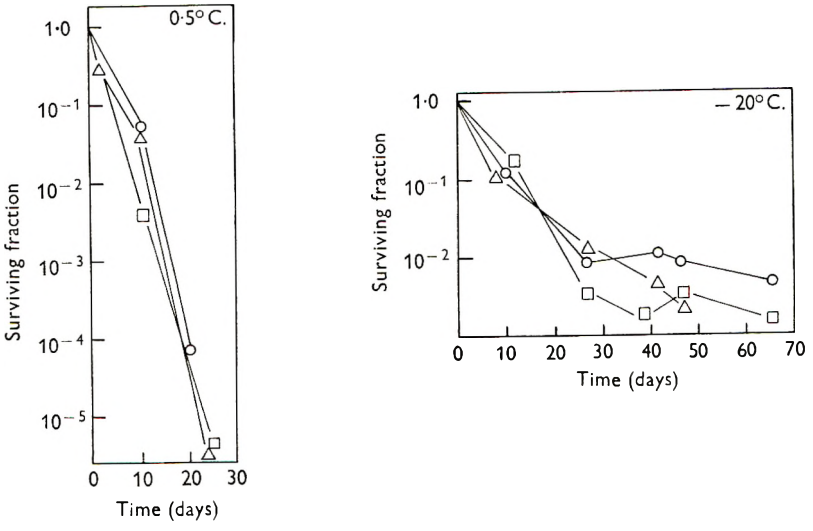


Fig. 7. Survival of *Shigella* spp. in egg white: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

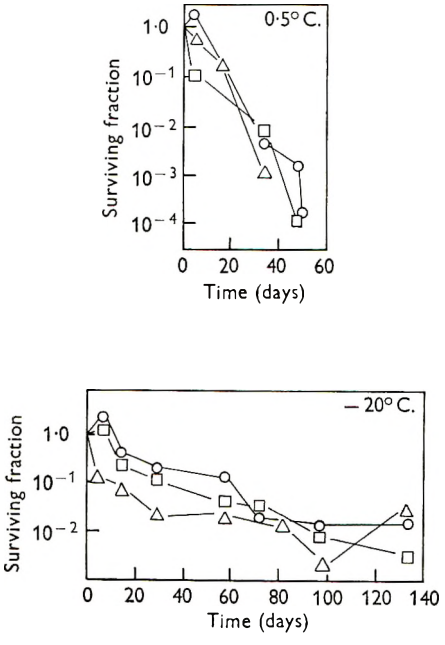


Fig. 8. Survival of *Shigella* spp. in oyster: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

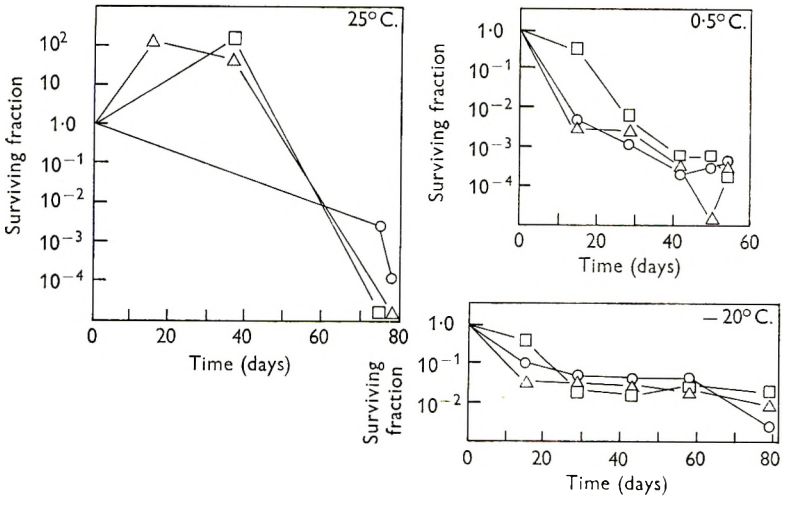
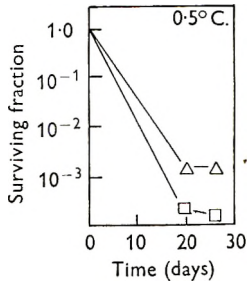


Fig. 9. Survival of *Shigella* spp. in clams: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.



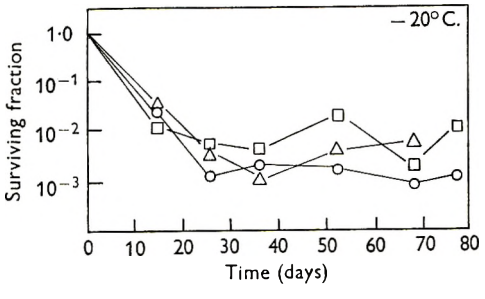


Fig. 10. Survival of *Shigella* spp. in shrimp: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

DISCUSSION

The recovery of shigellae from the various menstrua is an indication of biologically undamaged and viable cells. Although the recovery medium used was a complex medium it is conceivable that some viable cells were not recovered. Hartsell (1951*a*, *b*) and Nakamura & Dawson (1962) reported that highly nutritive media were necessary to recover *Shigella* species from the frozen state. In our

experiments it appeared that some menstrua propagated the organism, whereas others protected the cells from damage (extended survival in certain substances). In addition, it appeared that some menstrua were toxic to shigellae as noted by the sharp drop in the surviving fractions. Since Naylor & Smith (1946), Meynell (1958), and Mead, Wessman, Higuchi & Surgalla (1960) found that the survival of micro-organisms was optimal in cultures during the early stationary growth phase, it is very probable that the organisms used in these experiments possessed the capabilities of maximal survival.

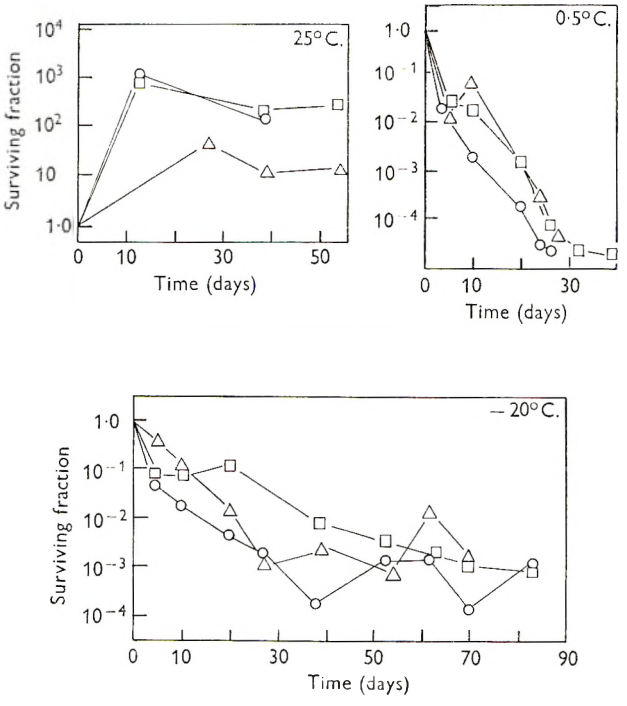


Fig. 11. Survival of *Shigella* spp. in whole egg: —△—△— *S. flexneri*; —○—○— *S. sonnei* 13327; —□—□— *S. sonnei* B-2569-2.

The survival curves of *Shigella* in milk, flour, oyster, clams, shrimp and whole egg showed an initial steep decline which was followed by decreased slopes as time increased and later the slopes became almost negligible. This pattern is in agreement with the results obtained by others (Hartsell, 1951*b*; Major, McDougal & Harrison, 1955; Clement, 1961).

The shigellae did not survive very long in orange juice. Orange juice is acidic and contains citric acid which was reported to be lethal to some bacteria (Erickson & Fabian, 1942; Hahn & Appleman, 1952*a*, *b*). When we neutralized the orange juice before the addition of the inocula the survival was double that in the unneutralized orange juice. The total survival period was not very extensive, however. The limited survival of the organisms in tomato juice was probably also due to the low pH (4·5) of the juice. Johnston & Kaake (1935) found that *S. dysenteriae* cells could not be recovered from tomatoes after 6 days.

The shigellae survived for only brief periods in the carbonated beverages studied. Low pH was probably also a factor in limiting survival (Ramadan & Abd-Elnaby, 1962). Eagon & Green (1957) concluded that carbonation increased the bactericidal activity of acid environments. Cooking oil provided poor conditions for the survival of shigellae; this was in agreement with the work of Pivnick, Englehard & Thompson (1954), who reported on the limited survival of enteric pathogens in soluble oil emulsions at 20° C.

SUMMARY

The survival and persistence of *Shigella sonnei* and *S. flexneri* in various food preparations were studied. The length of survival and the rate of decrease in surviving fractions varied considerably depending upon the menstruum and the holding temperature. This study points out the potential hazards involved in consuming food products that may be contaminated by shigellae.

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An outbreak of *Salmonella typhi-murium* food poisoning

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(*Received* 29 *January* 1964)

This paper describes an outbreak of food poisoning in the North London area due to *Salmonella typhi-murium* which accounted for at least twenty-one cases and sixteen symptomless infections from 23 June to 17 July 1963.

In the literature there are many accounts of food poisoning due to *S. typhi-murium* involving large numbers of individuals who had eaten the same infected meal and subsequently become ill (Harvey, Price, Davis & Morley-Davies, 1961; Lennox, Harvey & Thomson, 1954). This incident differed in that the outbreak and the subsequent investigations extended over a period of 25 days. Not only were cases reported day by day, but from different parts of a large borough. Seven infections were diagnosed during the first week, twenty-two during the second and a further eight found among family contacts of cases during the third and fourth weeks.

CLINICAL FINDINGS

All patients had fever and diarrhoea, some had headache, nausea and abdominal pain, and others also complained of malaise and weakness in the limbs. Approximately half the patients were severely ill—an infant of 11 months was admitted to hospital with pyrexia and nuchal rigidity, and a 7-year-old boy became delirious.

INVESTIGATION

An intensive investigation was begun as soon as word was received from the local public health laboratory that there appeared to be an increase in the number of isolations of *S. typhi-murium* from cases of gastro-enteritis in the district. The investigation was conducted along the lines set out by Savage (1956). Faecal specimens were taken from the patients, from their families and from other close relatives (Cockburn, 1960).

As the initial phase of the investigation got under way, two common factors emerged. The public health laboratory continued to report the isolation of *S. typhi-murium* phage-type 1 a from all cases of gastro-enteritis. It was also found that in all but two of the cases, cakes and (or) bread had been eaten which had originated from a particular bakery firm's bakehouse or from one of its branch shops in the borough. This firm distributed its bread, cake and pastry products to shops, restaurants, cafés, public houses and industrial undertakings in the area.

With suspicion falling on the firm referred to, a preliminary inspection of the parent bakery was carried out. This revealed an abominable lack of hygiene; with filthy sinks, grimy towels, and a total absence of soap. All baking equipment and trays were swabbed for pathogens and a number of very questionable looking bench

washing cloths (rags) removed for laboratory examination. Concurrently, faecal specimens were procured from all available members of the staff both there and from a branch-shop nearby.

INITIAL RESULTS—EXTENSION OF INVESTIGATION

At the bakehouse, an apprentice baker of fifteen (who figures largely in the investigation) and a female packer of cakes and bread, were found to be infected with *S. typhi-murium* phage-type 1a; both were symptom-free. A female assistant from the branch shop, though likewise asymptomatic, was found to be excreting an identical organism in her faeces. A profuse mixed growth of bacteria was obtained from several mixing bowls, a beater, from the surface of a baking bench and from two sinks. The filthy washing cloths (rags) habitually used for 'cleaning' bakery benches and equipment, similarly provided a mixed growth of bacteria, but *S. typhi-murium* was not isolated.

The investigation was immediately extended to the other branch shops and arrangements were made to collect faecal specimens from all members of the firm including the vanmen who effected deliveries. Two part-time counter-hands (a mother and daughter) employed at another branch shop were found to be infected with *S. typhi-murium* phage-type 1a. They admitted having had symptoms of vomiting and diarrhoea a week or so previously.

Of the twenty-two *S. typhi-murium* food poisoning cases diagnosed during the second week of the outbreak, fourteen occurred among pupils and staff at a residential school for cripples. Six of the children had symptoms of acute gastro-enteritis between 2 July and 4 July, seven others were subsequently found to be symptomless excretors as was a young nurse on the staff. Although the school did not receive cakes from the firm, bread eaten by staff and patients came from the bakery.

A number of recommendations were immediately put into force at the bakery whereby the hygiene conditions were improved. These consisted in the destruction of all existing scrubbing brushes and washing materials and their replacement by new equipment. The entire bakery premises were scrubbed out and a thorough cleaning of benches, bakery equipment and trays carried out. 'Cidal' toilet soap was provided in plentiful supply outside the male and female W.C.'s, with paper towels replacing the existing roller towelling. All staff were given copies of *The Guide to the Food Hygiene (General) Regulations*, 1960, and it was recommended that all newcomers to the firm be given copies. The extreme importance of scrubbing the hands in hot soapy water and of then immersing them in 'Roccal' for 60 sec. after use of the W.C. was impressed upon all members of the staff. It was also suggested that before starting each new baking process the employee in the bake-house should routinely immerse the hands for 60 sec. in 'Roccal'.

All infected food-handlers were excluded from work in the usual way. The submission by cases (and symptomless excretors) of three negative stools taken at intervals of 3 days was taken as indicating post-treatment freedom from infection.

A foreman baker and his family who had been on holiday (29 June–13 July) were investigated on their return. The baker's faecal specimen was negative but his 7-year-old daughter was found to be a symptomless excretor of *S. typhi-murium* phage-type 1a. The father had experienced pruritus ani on or about 29 June.

The apprentice baker previously referred to, and who was a symptomless excretor, had joined the firm on 10 June, 12 days before the onset of symptoms in the first case. One month before joining the staff (whilst employed in a clothing factory) he had been incapacitated for a whole day with a severe febrile headache which had not been accompanied by enteritis. On interrogation by one of us (T.F.M.J.) his description of his duties and personal habits appeared to be of significance in the possible chain of infection.

From the first few days of his new employment, he was given cakes to pack in trays, then he graduated to the filling and decorating of confectionery. On arriving at work, it was his responsibility to prepare cream doughnuts by cutting down the centre of each with a knife, then by hand squirting in artificial cream from a porous bag which he had previously filled from the mixer. He also decorated the fairy cakes by taking cherries from a jar, splitting each cherry in half with his finger nails and placing a half cherry on top of the icing on each cake. He sugared ring doughnuts by immersing them in a bin of sugar, moving them to and fro by hand. He prepared cream slices as follows: jam was transferred from a spoon to his fingers which he then trailed along the surface of a length of pastry. On top of this, artificial cream was squirted followed by a covering of pastry on the surface of which fondant was spread by means of a knife. Coralettes were finally sprinkled over the fondant. His duties were also concerned with the packing of these and other confectionery into trays, either to be sold subsequently in the front shop, or to await collection for distribution to branch shops and elsewhere. His daily routine included the washing down of the baking benches and the 'cleaning' of the mixing bowls and equipment which he effected by using any one of a number of cloths (rags) which hung over the side of a sink close to the W.C. Following a bowel evacuation, it was his practice to 'run his hands' under a tap, and then wash them at the sink using one of the self same rags used previously (and later) for the so-called cleaning of benches and equipment!

DISCUSSION

Twenty-one cases of gastro-enteritis due to *S. typhi-murium* phage type 1a together with a further sixteen symptomless excretors, were revealed during the investigation.

All but two of the thirty-seven persons involved had eaten cakes and (or) bread obtained either directly or indirectly from the bakery firm. Considering the firm's wide distribution of its products (and human forgetfulness) it is possible that the two others had also eaten bread or pastries produced by the bakery firm in question.

The school for cripples did not receive cakes from the firm, but bread eaten by staff and patients came from the bakery. Hence, though it was not established that

the bread eaten had been infected, it is possible that it could have been contaminated with the floury dusts and debris from trays which had been used for infected pastries (Taylor, 1960); alternatively an infected food handler or infected equipment undetected at the stage of investigation might have been responsible.

The evidence pointed to the bakery firm as the likely source of infection, and in particular to its headquarters bakehouse; for it was on the premises of the latter that the pastries, confectionery and bread were baked, and it was from there that the branch-shops and all catering and other establishments were supplied. Preliminary investigation of the bakery revealed no pathogenic organisms in the raw materials used in the preparation of bread and confectionery; nevertheless, some of the equipment, and all the washing cloths, grew heavy mixed growths of organisms on culture, and the factory hygiene was of a very low standard.

If the cream cakes and other confectionery and bread did cause food poisoning, how had they been infected with *S. typhi-murium* if the raw materials from which they had been prepared were free from pathogens? According to Taylor (1960), food found to be the vehicle need not necessarily contain an infected ingredient, but may become infected through infected bakery dust contaminating it by settling on it, or by the use of infected equipment. Also, cream confectionery has a reputation for acting as a suitable medium for the proliferation of salmonellae (Reports, 1958 *a*, *b*) and the working temperature of the bakehouse would favour multiplication of organisms. The foreman baker who used the mixer experienced pruritus ani on or about 29 June when he left on holiday. It is possible, despite the fact that he was subsequently found to be negative, that he might have had a fleeting infection before going on leave and that he was the source of the trouble. His daughter was found to have a latent infection when the family returned home on 13 July.

On the other hand, the apprentice baker, who was excreting organisms and who was so intimately concerned with the cream cakes and pastries, had only recently joined the firm, having suffered from a particularly severe febrile headache one month before this. He had no symptoms of food poisoning but his faeces were positive for *S. typhi-murium* phage-type 1 a. It is generally accepted that an attack of salmonella food poisoning can begin with a headache and chills (Dack, 1956), hence it is tempting to regard his previous febrile headache as having been associated with a salmonella infection. It cannot be said with certainty that this teenage apprentice baker was the source of the outbreak. However, when it is considered that he worked with and handled the cakes and pastries as he did, that he was completely ignorant of food hygiene and had poor personal standards, at the same time as he was excreting *S. typhi-murium* in his faeces, the likelihood of his having at least spread the infection cannot be denied. If in fact he was the source of the infection, it can readily be seen how food prepared on the premises became contaminated. Outbreaks such as that described will continue to arise where dirty working conditions are permitted and where untrained adolescents are allowed to do such work.

SUMMARY

1. An outbreak of food poisoning due to *Salmonella typhi-murium* phage-type 1a is described.

2. Bread and (or) bakery confectionery eaten by all but two of the victims, had originated from a particular bakery firm.

3. *S. typhi-murium* phage-type 1a was found in the stools of an apprentice baker, a female packer of cakes and bread, and in three female counter-hands. The apprentice baker was responsible for the preparation and decoration of the confectionery which was distributed to the branch-shops and other establishments throughout the area.

4. *S. typhi-murium* was not found in any of the raw materials used at the bakehouse.

5. The cases which arose at a residential school for cripples gave a ratio of six cases to eight symptomless excretors.

6. Seven weeks after the start of the outbreak the last of the infected food-handlers was considered to be free from infection and fit to return to work.

Twelve weeks later the last member of the public was declared to be negative.

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A rapid immunofluorescence technique for detecting salmonellae in raw meat

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INTRODUCTION

The sporadic presence of salmonellae in a wide range of food raw materials (Report, 1958; Hobbs & Wilson, 1959; Galbraith, Hobbs, Smith & Tomlinson, 1960; Kampelmacher, 1963) causes concern among food manufacturers and public health workers. Early detection of the contamination in such materials is one of the most important steps in preventing the spread of contamination and possible infection of the consumer. However, with existing techniques for salmonella detection the raw material could possibly be processed and distributed by the time the test answer is known. In addition, for many laboratories the existing techniques are so laborious that it is difficult to attain an adequate sampling rate. These difficulties have caused us to investigate means of radically shortening the salmonella test.

A salmonella detection technique should be able to detect contamination at least as low as one salmonella organism per 50 g. of sample (Hobbs, 1962). For this reason we have accepted that at this stage it is impossible to dispense with the liquid enrichment procedure, which is required to raise the salmonella numbers to a detectable level. However, 1 or 2 days would be saved if the salmonellae in enrichment broths could be demonstrated by direct examination, rather than by further culture on solid diagnostic media. On the basis of existing knowledge, the immunofluorescence technique (Beutner, 1961; Nairn, 1962) offered the best hope of such direct demonstration of salmonellae in enrichment cultures. Though the immunofluorescence technique by itself has not proved successful in detecting salmonellae in human stools (Thomason, Cherry & Edwards, 1959) success has been claimed for the technique in detecting *Salmonella dublin* in artificially contaminated milk (Arkhangel'skii & Kartashova, 1962). We hoped that with some adaptation it would prove suitable for the examination of naturally contaminated foods, particularly meats.

In early tests with pure cultures we found that salmonellae could be stained by the indirect immunofluorescence technique after culture in selenite broth at 43° C. (Plate 1A). We had already established experimentally that, as with faeces (Harvey & Thomson, 1953), enrichment at 43° C. in selenite F broth was very suitable for isolating salmonellae from foods (Georgala, 1963). Practical experience in our laboratory had further confirmed that 43° C. selenite enrichment was an excellent selective procedure for the examination of carcass and frozen boneless meats (to be published). We therefore decided that a rapid salmonella test could

possibly be based on the use of selenite enrichment for 18 hr. at 43° C., followed by immunofluorescence examination of the enrichment culture for the presence of salmonella cells. This communication describes our experiences with such a technique, during the examination of frozen imported meat samples.

The immunofluorescence examinations of the enrichments involved demonstrating O antigens only, as flagellar H antigens are very poorly developed in selenite broth at 43° C. The O antigens as demonstrated by immunofluorescence techniques are located in the cell wall, making the cell outline clearly visible (Thomason, Cherry & Moody, 1957). The indirect technique was used in our investigation because of the many different O antigens in the *Salmonella* group. In this way, only one fluorescent antiserum conjugate was required (e.g. a goat anti-rabbit globulin antiserum), which was then used in conjunction with various polyvalent or pure factor salmonella antisera produced in rabbits (Nairn, 1962). The indirect technique offered an added advantage, in that the first stage of the staining procedure could be performed with ordinary diagnostic salmonella agglutinating antisera.

The direct technique was also tried on a few occasions, and provided excellent staining of salmonellae grown in selenite broth. The staining procedure itself is shorter than the indirect technique, but the major drawback was the need for each polyvalent or single factor salmonella antiserum to be conjugated with fluorochrome.

METHODS AND MATERIALS

Meat samples

The frozen meat samples were obtained from the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale. The samples were taken from shipments of imported frozen meat which had been shown at Colindale to be fairly heavily contaminated with salmonellae. In this way we hoped to avoid running into a long series of negative samples.

Reagents for indirect immunofluorescence testing

Salmonella antisera. Ordinary somatic diagnostic agglutinating antisera were used throughout as the first stage in the indirect immunofluorescence technique. These were obtained from the Standards Laboratory for Serological Reagents, Colindale, and when received had agglutination titres of around 1/250. For use in the indirect immunofluorescence technique the sera could be diluted up to one-quarter with phosphate buffered saline (pH 7·4). Most of the testing was done with the polyvalent O antiserum. However, the Standards Laboratory polyvalent antiserum contains only the most important O factors (Memorandum, 1961). In certain cases salmonellae were encountered which were not agglutinated or stained by this serum. The main example was *Salm. minnesota* (O:21) and to assist detection of this organism Burroughs Wellcome Salmonella O factor 21 serum was usually included in the staining procedure, as described later.

Preparation of fluorescent goat anti-rabbit serum. Rabbit globulin was prepared as follows. The blood of a number of exsanguinated rabbits was pooled, allowed to

stand overnight, and the serum collected and clarified by centrifuging. Using a saturated solution of ammonium sulphate, the serum was brought up to 40% ammonium sulphate saturation and left at room temperature for 1 hr. The precipitated globulins were centrifuged at 10,000 rev./min. for 20 min., and washed by centrifuging three times in fresh 40% saturated ammonium sulphate solution. The final sediment was redissolved in 20 ml. phosphate buffered saline (pH 7·4). This solution was then dialysed for 3 days at 4° C., against three changes of buffered saline. After dialysis the globulin solution was freeze dried, and the solid globulin stored at 4° C. The yield of dry globulins was 4·5 g. from 480 ml. serum.

To obtain an anti-rabbit antiserum, the rabbit globulin preparation was injected into a goat as follows:

	Period after 1st injection	Antigen and injection route
1st injection	0 weeks	500 mg. rabbit globulin dissolved in 8 ml. saline and mixed with 8 ml. complete Freund's adjuvant (Difco). Four intramuscular injections of 4 ml. each, one in each quarter
2nd injection	6 weeks	As above
3rd–7th injections	14 weeks	50 mg. rabbit globulin in 2 ml. saline intramuscularly on each of 5 consecutive days.
Bleeding	15 weeks	About 300 ml. blood drawn. Held at 4° C., and serum separated.

Conjugation of goat serum with fluorochrome. The methods used were identical with those described by Nairn (1962), and derived originally from Chadwick, McEntegart & Nairn (1958*a*, *b*) and Riggs *et al.* (1958). Lissamine rhodamine RB 200 (George Gurr Ltd) was normally used as fluorochrome, but an excellent fluorescent conjugate was also obtained using fluorescein isothiocyanate (Baltimore Biological Laboratories). In the latter case, the globulins in the serum were first precipitated with ammonium sulphate before conjugating them with the dye, thereby reducing the amount of the very expensive fluorochrome required. In addition fluorescein conjugates were purified by gel filtration through G. 25 Sephadex, instead of by treatment with activated charcoal as used for rhodamine conjugates (Nairn, 1962).

The goat anti-rabbit serum was prepared and conjugated in our laboratory so as to provide a large quantity of standardized reagent. However, similar anti-rabbit fluorescent sera are now available commercially and we have found that the Difco (U.S.A.) goat anti-rabbit fluorescein conjugate performs as well as our own serum.

Immunofluorescence testing of meat samples

The frozen meat samples were thawed, and then approximately 25 g. of each was cut into about a dozen small chunks and dropped into 100 ml. single strength selenite F broth (Leifson, 1936). If desired the sample could readily be increased to 50 g. in 200 ml. selenite broth. The enrichments were incubated for 18–24 hr.

in a 43° C. water-bath. A sample of each selenite enrichment was then withdrawn for immunofluorescence examination.

In the early stages of the work (first series of 158 samples) small quantities of the selenite enrichments were transferred with a small platinum loop to clean glass slides. The drops were smeared over about 0·25 cm.^{2}, air dried, and then fixed in formol saline (1 part formalin + 9 parts phosphate buffered saline pH 7·4) for at least 10 min. It was later found preferable to centrifuge (15 min. at 4000 rev./min.) 10 ml. quantities of the selenite broths, and resuspend the sediment in 0·5 ml. sterile distilled water (second series of 128 samples). Smears were then prepared from this suspension. The centrifuging had two advantages—smears of the distilled water suspensions were far easier to fix on the slides, and showed a concentration effect due to the change in total volume.

Fixed smears were washed in buffered saline, and freed of excess saline by passing rapidly through two baths of absolute alcohol, one bath of a 50:50 mixture of alcohol and xylene, and finally through a xylene bath before being air-dried. The dried fixed smears were covered with polyvalent salmonella O antiserum (duplicate smears were tested with O factor 21 antiserum if *Salm. minnesota* was expected) and held at room temperature in a Petri dish containing moistened filter paper to prevent evaporation of the serum. After 30 min. the excess salmonella antiserum was removed by washing in buffered saline, and the slides were dried as before. The smears were then covered with the goat anti-rabbit fluorescent conjugate, and held at room temperature for approximately 20 min. The slides were then washed in buffered saline, dried as before, and the smears mounted in D.P.X. mountant (G. Gurr Ltd) when rhodamine conjugate was used, or pH 7·4 buffered glycerol when fluorescein conjugate was used.

Microscopy

The Reichert fluorescence microscope was used, with an Osram HBO 200 mercury discharge lamp as lightsource. Most examinations were by brightfield illumination, with the condenser oiled to the underneath of the slide with liquid paraffin. A BG 12/4 mm. blue/ultra-violet exciter filter was used, in conjunction with an orange barrier filter. All smears were first examined with a × 40 dry fluorite objective, and doubtful cells checked with the × 100 oil immersion objective (Fluorite). Of several types of oil tested Microil (G. Gurr Ltd) was found to be the best liquid for use with the immersion objective. It possessed negligible fluorescence and was only slowly degraded by the intense ultra-violet illumination.

Assessment of stained smears

As the tests described in this report were of a preliminary nature, an arbitrary system of slide assessment was devised, based on the number of fluorescent cells seen in a preparation. Early experience had shown that one or two fluorescent cells in a whole smear did not usually indicate the presence of salmonellae. Such cells could be elements of the original flora, which had not multiplied during enrichment, and which cross-reacted with the fluorescent serum. On the basis of

known microscope field size, and the fairly constant volume/area ratio employed in smear preparation, it was decided that if salmonellae had multiplied significantly in the selenite enrichments at least one or more fluorescent cells would be visible in a field with the ×40 objective and ×8 ocular (Plate 1 B, C). A sample was judged definitely free of salmonellae when its smear showed none or only a very few fluorescent cells over the whole area of the smear (0·25 cm.²). Samples providing smears with a fair number of fluorescent cells, but just not enough to average one or more per microscope field (×40 objective; ×8 ocular) were judged as probably containing salmonellae.

Confirmation of presence of salmonellae

After smears had been prepared from the 18–24 hr. selenite broth enrichments, each enrichment was also streaked on to Difco brilliant green agar (B.G.A.).

The enrichment tubes were incubated a further day at 43° C. and then again streaked on to fresh plates of B.G.A. The B.G.A. plates were incubated 18–24 hr. at 37° C. Suspicious pink colonies were identified as salmonellae or otherwise by biochemical and serological tests.

We have found the following tests most suitable for the rapid screening of colonies from B.G.A. plates:

(*a*) the lysine decarboxylase test of Møller (1955),

(*b*) the urease test on urea agar of Christensen (1946),

(*c*) the β-galactosidase test of Le Minor & Ben Hamida (1962).

Table 1 shows the reactions obtained with organisms which can produce pink colonies on B.G.A. plates.

Table 1. *Reactions used for screening organisms forming pink colonies on brilliant green agar*

	Lysine decarboxylase	Urease	β-Galactosidase
Salmonellae	+	−	− (Arizona +)
Not salmonellae (probably *Proteus*)	−	+	∓
Not salmonellae (probably paracolon)	−	∓	+

+, vigorous positive; ∓, usually negative, very occasionally weak positive.

Suspect salmonellae were typed by slide agglutination with polyvalent and single factor O and H salmonella antisera. Where flagellar phase changes were necessary we used the simple and very effective paper strip technique of Jameson (1961).

RESULTS

The results with the first series of 158 samples (no centrifuging) are shown in Tables 2 and 3. Table 2 shows that the fluorescence and conventional methods agreed exactly in the recording of *total* number of positive and negative samples. Table 3 reveals the actual agreements and discrepancies for individual samples, and shows that 80 % of the salmonella positive samples and 91 % of the salmonella negative samples were correctly identified by the fluorescence technique. There were ten false negative samples (fluorescence negative, conventional method positive) and ten false positives (fluorescence positive, conventional method negative). Serotypes isolated during these tests were: *Salm. minnesota, S. oranienburg, S. anatum, S. meleagridis, S. typhimurium, S. newport, S. paratyphi B, and S. orion.*

Table 2. *Detection of salmonella by fluorescence and conventional techniques in* 158 *frozen meat samples (first series, no centrifuging)*

	Fluorescence technique		Conventional technique
Samples positive	28	Total 50	50
Samples probably positive	22		
Samples negative	108		108

Table 3. *Detailed comparison of results from* 158 *frozen meat samples examined by fluorescence and conventional techniques (first series, no centrifuging)*

Fluorescence technique	Conventional technique	No. of samples
Positive	Positive	24
Probably positive	Positive	16
Positive	Negative	4
Probably positive	Negative	6
Negative	Positive	10
Negative	Negative	98

In the second series of 128 samples (Tables 4 and 5) centrifuging of the selenite enrichment resulted in the detection by immunofluorescence of 87 % of the salmonella containing samples, as against 80 % in the first series of samples, i.e. a drop in the failure rate from 20 to 13 %. However, centrifuging also increased the false positive results from 6 to 12 % of the total samples examined.

Of the 13 % false negatives in the second series of samples, about half coincided with samples that required a full 48 hr. enrichment before salmonellae could be detected by conventional means. There seems to be little possibility that this slow development can be avoided—in our experience it occurs in about 5 or 6 % of the total positives encountered. The remaining false negatives represent definite failings in the immunofluorescence technique itself, e.g. inadequate fixing of the smears or incorrect assessment of doubtful slides. It seems likely that with more experience of the technique and with improved antisera these errors would be eliminated.

Table 4. *Detection of salmonella by fluorescence and conventional techniques in* 128 *meat samples* (*second series, selenite centrifuged*)

	Fluorescence technique	Conventional technique
Samples positive	50	61
Samples probably positive	19 (Total 69)	
Samples negative	59	67

Table 5. *Detailed comparison of results from* 128 *frozen meat samples examined by fluorescence and conventional techniques* (*second series, selenite centrifuged*)

Fluorescence technique	Conventional technique	No. of samples
Positive	Positive	47
Probably positive	Positive	6
Positive	Negative	3
Probably positive	Negative	13
Negative	Positive	8
Negative	Negative	51

DISCUSSION

The performance of the rapid immunofluorescence detection technique described here suggests that the technique might have applications in screening food raw materials for salmonellae. The serological cross-reactions were fewer than expected, possibly due to the selective enrichment procedure employed. Provided a number of samples are taken from each batch of meat there would usually be no difficulty in detecting batches that are considerably contaminated, or those that are completely free of salmonallae. It is lightly contaminated batches which could cause difficulty in interpretation, and here much would depend on the skill of the operator in assessing doubtful smears.

In its present form the 18 hr. immunofluorescence test would probably always miss those contaminated samples which require more than 24 hr. enrichment to be detected by conventional means. In our experience 43° C. selenite enrichment limits these to about 5 % of the total positives recorded, and any laboratory using the immunofluorescence technique would have to assess the importance of this level of failure. Other failures are possible but could probably be reduced by improved fixing of the smears, and consistent assessment of the stained preparations. Centrifuging of the selenite enrichments is preferred because it increased the detection rate, and assisted the fixing of the material on to the microscope slide. However, unless cross-reactions can be reduced by purifying the salmonella antisera, centrifuging would usually increase the rate of false positive results.

The immunofluorescence test described here depends on having a suitable polyvalent salmonella antiserum. Such an antiserum would have to include somatic antibodies for all the salmonella types expected in the materials being tested. In the present tests *Salm. minnesota* could not be detected with the polyvalent antiserum (which lacked O factor 21) and had to be tested for separately

on another smear. However, there should be no special difficulty in producing a range of polyvalent antisera to cover all known salmonella O group antigens.

It seems likely that some of the false positive results we recorded (fluorescence positive, conventional method negative) were due to non-specific cross-reactions, and could have been avoided by absorbing the antisera with selected non-salmonellae. A few suitable organisms were isolated and will be tested as absorbing agents. A few cross-reactions are due to organisms which possess antigens identical with those of salmonellae, and absorption is not likely to help with these.

An important advantage of the immunofluorescence technique described here is that the enrichment procedure is identical with the first stage of the conventional test for salmonellae. When smears have been prepared, the selenite enrichment can be streaked on a solid selective medium and the conventional technique proceeded with. Thus any batches of material recorded as doubtful positive by the immunofluorescence technique could be withheld from processing until this finding is confirmed the following day—the immunofluorescence technique would thus be used as a 'presumptive salmonella' test. Combined with an adequate sampling rate at an early enough stage such a 'presumptive test' could play a valuable role in preventing contaminated raw meats reaching the processing lines in food factories.

SUMMARY

A rapid 18 hr. technique has been developed for detecting salmonella contaminated carcass and boneless meats. It is based on 43° C. selenite enrichment of samples, followed by immunofluorescent detection of salmonella cells in the enrichment. In tests with 286 meat samples the rapid and conventional techniques agreed in the detection of 93 positive and 149 negative samples. The two tests failed to agree for the remaining 44 samples. The rapid technique thus lacks precision, but could be used as a rapid 'presumptive' salmonella test, so that contaminated material could be prevented from reaching the processing lines of food factories.

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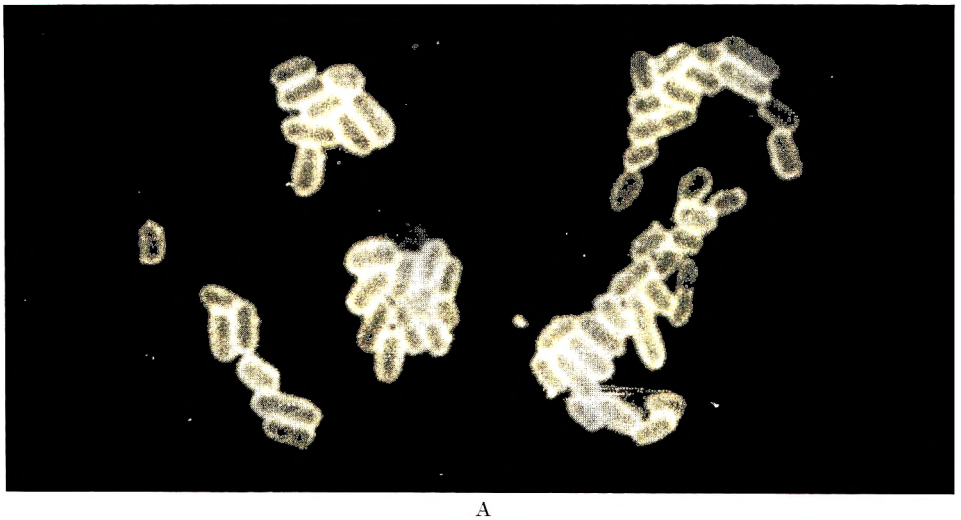

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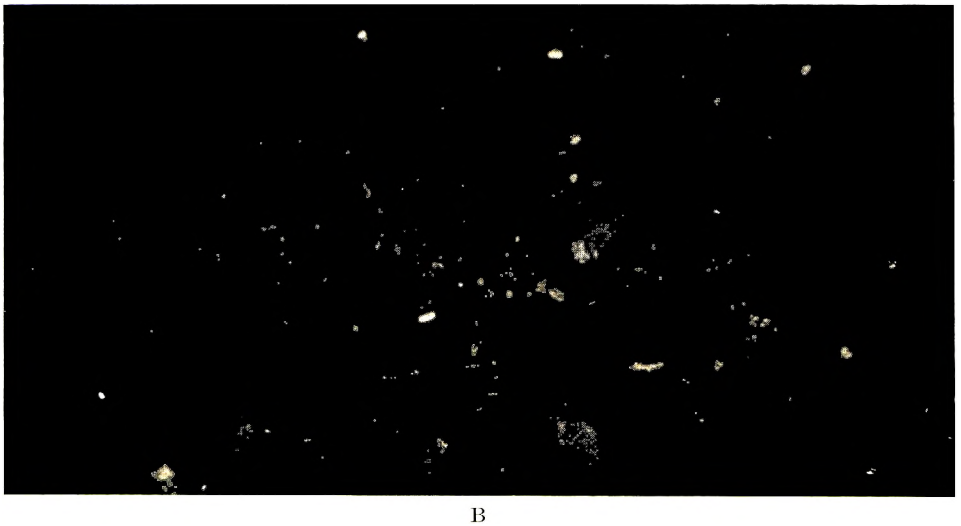

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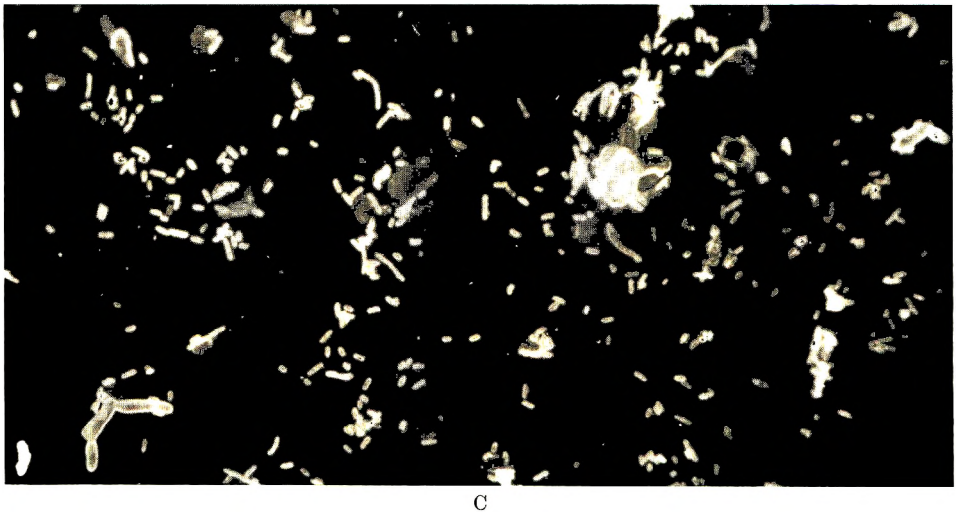

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EXPLANATION OF PLATE 1

A. *Salm. typhimurium* incubated 18 hr. in selenite broth at 43° C., then stained with Standards Laboratory polyvalent salmonella antiserum followed by rhodamine conjugated goat anti-rabbit antiserum. × 4000.

B. Smear prepared from a 18 hr. selenite broth enrichment of a contaminated meat sample; stained as for Plate 1A. Photographed with × 40 objective. A few strongly fluorescent salmonellae cells are visible amongst tissue debris and other bacteria. × 800.

C. Smear of sediment obtained from a centrifuged selenite enrichment of a contaminated meat sample; stained as for Plate 1A. This sample had provided a heavier growth of salmonellae than that shown in Plate 1B. × 800.

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Serological evidence for the susceptibility of the hippopotamus (*Hippopotamus amphibius* Linnaeus) to natural infection with rinderpest virus

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It is well known that a very large number of species of the order Artiodactyla may contract natural infection with rinderpest virus. As noted by Curasson (1932) they include members of the families Bovidae, Giraffidae, Tragulidae and Cervidae, representing the suborder Ruminantia; the same author regarded camels (family Camelidae, suborder Tylopoda) as of proven susceptibility. In the suborder Suiformes many wild species of the family Suidae are certainly frequent victims of the disease and peccaries of the family Tayassuidae (Burton, 1962) were said to have been affected during the 1871 rinderpest outbreak at the *Jardin d'acclimatisation de Paris* (Curasson, 1932). However, we know of no record suggesting that the hippopotamus (*Hippopotamus amphibius* Linnaeus), which belongs to the same suborder, Suiformes (family Hippopotamidae), has ever been seen to be clinically affected by rinderpest. As a result it is generally regarded as insusceptible.

This communication records the finding of rinderpest-neutralizing antibody in serum samples collected from the hippopotamus population of the Queen Elizabeth Park in Uganda. The distribution of this antibody between various age groups supports the contention that it was acquired as a result of infection with rinderpest virus during the course of the recorded epizootics of the disease which occurred in this area during the last 30 or 40 years. The probability that rinderpest-neutralizing antibody persisted in moderately high titre for periods of more than 30 years is an outstanding instance of the stability of such antibodies in an animal species other than man.

MATERIALS AND METHODS

Collection and storage of sera

Hippopotamuses were killed twice weekly, by shooting, during the course of a systematic 'game-cropping' operation to relieve overstocking on the shores of Lake Edward and the Kazinga Channel (see Fig. 1). The animals were towed to land after they had floated to the surface, 1–2 hr. after shooting, under the influence of gases accumulating in their gastro-intestinal tract; they were butchered for meat and also served for general ecological and physiological studies by staff members of the Nuffield Unit of Tropical Animal Ecology.

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They were all weighed and assigned to relative age groups numbered from 3 to 20 (Table 1). In determining these age groups the criteria defined by Longhurst (1958), based on tooth replacement and wear, were adopted with slight modifications. These relative age groups were later transformed to estimated real age groups with a span of $x \pm 1$ or 2 years, ranging from $x = 1$ to $x = 41$; these are the values given

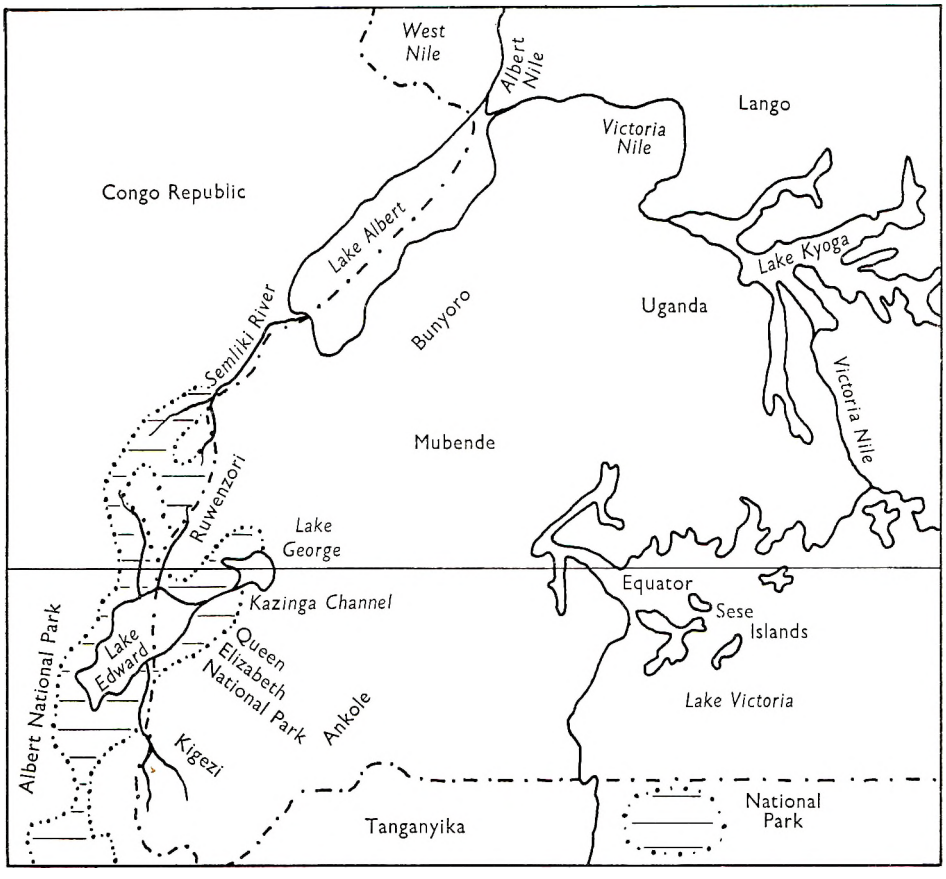

Fig. 1

in Table 1. In assigning real ages arbitrary estimates of the elapsed time between successive relative age groups were made, taking into account tooth growth and wear. The transformation was found to agree reasonably well with the observed tooth replacement and wear in six known-age captive hippopotamuses (aged 15–41 years). The oldest captive animal recorded was over 49 years at death (Burton, 1962), which does not conflict with a maximum longevity of 41 ± 2 years in the present wild sample of 315 animals. A full account of the age criteria and procedure will be given elsewhere.

There were no signs of a generalized disease process in any of the animals. Free-flowing blood was collected from large vessels during the course of the slaughter process; it was allowed to clot in sterile screw-cap bottles and serum was separated

on the following day. All samples were stored at 4° C. until they could be returned to the laboratory—generally speaking by air and within 2–4 days. They were then reclarified and stored at −20° C.

Table 1. *The occurrence of rinderpest-neutralizing antibody in the sera of hippopotamuses shot in the Queen Elizabeth Park, Uganda*

Age group	Estimated age (years)	No. of sera tested	No. of sera positive	Proportion positive (%)	Class figures
3	1	3			
4	3	9			
5	4	11	Nil	Nil	0/70 positive
6	7	7			
7	8	21			
8	11	29			
9	13	35	1	2·9	
10	15	26	1	3·9	
11	18	13	1	7·7	5/120 positive
12	20	9	0	Nil	(4%)
13	22	18	2	11·1	
14	25	19	0	Nil	
15	28	50	13	26·0	
16	31	42	16	38·1	
17	33	16	8	50·0	41/115 positive
18	36	1	0	—	(36%)
19	38	4	3	75·0	
20	41	2	1	—	
Totals		315	46	—	46/315 positive

Virus neutralization tests

Sera were inactivated at 56° C. for 30 min., immediately before setting up the tests. Rinderpest virus neutralization tests were all performed in primary calf kidney tissue cultures using techniques which have already been described in detail (Plowright & Ferris, 1961; Plowright, 1962). Sera were first used undiluted in 'screening' tests, to eliminate all those which had no significant neutralizing activity; the dose of virus employed commonly fell between $10^{2\cdot0}$ and $10^{3\cdot4}$ TCD50 per tube and only two tubes were used per sample. The test dose of virus was thus somewhat higher and more variable than the $10^{1\cdot8}$ to $10^{2\cdot8}$ TCD50 previously advocated (Plowright, 1962) and may have resulted in a failure to detect antibody in a few sera with very low titres.

Quantitative neutralization tests were carried out on all except one of the sera which were positive in the screening tests. For this purpose undiluted serum and 10-fold serum dilutions were mixed with an equal volume of one of two virus stocks. After holding overnight at 4° C., each mixture was inoculated into five tubes of calf kidney cells. Final readings, for cytopathic end-points, were always carried out on the 11th or 12th days following, at which time the dose of virus in different series of tests varied from $10^{2\cdot0}$ to $10^{3\cdot4}$ TCD50 per tube. Log_{10} SN50 titres were calculated by the method of Thompson (1947). When less than half of the five tubes

inoculated with serum at a final dilution of 1/2 were protected, the antibody content was recorded as a 'trace'.

A standard immune cattle serum was included for comparison with each batch of sera from hippopotamuses.

RESULTS

As shown in Table 1, a total of 315 sera were screened for antibody, these being derived from hippopotamuses of eighteen groups, varying in estimated age from 1 to 41 years. The numbers of samples in each group varied considerably, from one to fifty, but it was possible to subdivide all the donor animals into three classes of six age groups each. These classes ranged from 1 to 11, 13 to 25, and 28 to 41 years old, respectively. When this was done it was immediately obvious that marked differences in antibody status characterized the aggregate figures for the three classes (Table 1).

Table 2. *Titrations of rinderpest-neutralizing antibody in the sera of hippopotamuses*

Age group(s)	Number of sera titrated	Titre range ($\log_{10}$ SN 50)	Mean titre ($\log_{10}$ SN 50)	Number with titre $\geqslant$ 1·0
9–14	5	0·4–1·2	0·76	2
15	12	0·3–1·8	1·32	9
16	16	Trace–1·8	1·18	9
17–20	12	Trace–2·0	1·11	6

There were no positives in 70 animals constituting the age class 1–11 years; 4 % (5/120) had antibody in the 13–25 years class, the positives being distributed in small numbers in age groups from nos. 9–13. In the age class 28 years and upwards 36 % of all animals tested possessed antibody and there was some indication of a rising 'immunity' rate between age groups 15–17 inclusive, with the percentage of positives almost doubling from 26 to 50 %.

The overall distribution of positives as between males and females was uneven (13:33) but this was accounted for by a predominance of females in the higher age groups, probably due to the fact that many of the latter were shot from schools of elderly matrons.

The results of quantitative neutralization tests on forty-five positive sera in screening tests are given in Table 2. There was a considerable range of titre in all the larger series, with figures varying from a trace to $10^{1·8}$ or $10^{2·0}$ (final serum dilutions of 1/50 or 1/100). Half to three-quarters of the animals in the age groups 15–20 had titres of 1/10 or above.

DISCUSSION AND INTERPRETATION OF RESULTS

The finding of a heat-stable virus-neutralizing substance in the sera of hippopotamuses immediately raised the question as to whether this appeared as a non-specific concomitant of the ageing processes, as a result of infection with an agent immunologically related to rinderpest virus or as a result of naturally acquired rinderpest infection.

The first possibility would be supported by the figures for a rising percentage of positives in animals of increasing age. However, there were some anomalies, such as absence of positives in age group 14 and the abrupt increase in groups 15–17 inclusive. Such an interpretation would also not be consistent with the results of the quantitative neutralization tests, which showed a slowly decreasing mean titre between groups 15 and 20 (see Table 2).

There were twenty-six animals with titres of $\geqslant 1/10$, and 16 with titres of 1/40 to 1/100, the dose of virus varying, as noted in the section on Materials and Methods, from $10^{2 \cdot 0}$ to as high as $10^{3 \cdot 4}$ TCD 50 per tube. These figures would hardly suggest that a non-specific virus inhibitor was involved. Further, no evidence for such an age-associated factor has come to light in the serum of other animal species which have been investigated (Plowright, unpublished).

The second possibility cannot be dismissed, since it is known that at least two other agents do have immunological relationships with rinderpest virus. These are the viruses of human measles and canine distemper (Warren, 1960; Imagawa, Goret & Adams, 1960; Plowright, 1963), but neither of them is known to infect any species of the order Artiodactyla and the opportunities for them to gain access to populations of hippopotamuses would appear to be somewhat remote.

The third hypothesis, that the hippopotamus is susceptible to natural infection with rinderpest virus, would be made more tenable by evidence that this agent was prevalent in the area under consideration up to 28 years ago, i.e. until 1934, and thereafter recurred in a more restricted form at intervals up to 1950. In the calculations we may ignore the effect of maternally derived antibody, since it is most unlikely that protection due to this factor would last for more than a year and the young born of immune dams in 1949 would have become susceptible by one year later; such animals would still fall in the age group number 9.

The Annual Report of the Uganda Veterinary Department for 1931 described numerous rinderpest deaths in buffalo to the south and west of Lake George in October and November of that year (Poulton, 1932). In 1932 it was noted that 'buffalo were dying along the shores of Lakes George and Edward for a long period' (Poulton, 1933). Rinderpest persisted in game and cattle of the neighbouring Kigezi and Ankole districts at least through 1933 and 1934 but there was no specific report of the disease from the shores of the lakes (Poulton, 1934, 1935).

After 1934 the Annual Reports of the Uganda Veterinary Department do not mention the occurrence of rinderpest in the Western lacustrine region, until the infection was again introduced via the Sudan border in 1942 (Simmons, 1943), spreading southwards to reach the Ankole district, bordering Lakes George and Edward, in 1943/44. Similar, limited incursions occurred in 1948 (Cronly, 1949) and 1955 (Randall, 1956); however, these did not reach the Western Province of Uganda, although the later outbreak was reported by the Belgian Congo authorities to have affected game animals to the West of Lake Albert.

Pitman (1942) in discussing the Uganda Kob, stated that it was 'very susceptible to the ravages of rinderpest, and some 12 years ago suffered disastrously in the Western Rift around Lake George and Lake Edward'. Hubert (1947) in a monograph on the Parc National Albert, to the south of Lake Edward, presented records

of a rinderpest epizootic in 1932, affecting particularly warthogs (phacochères) but possibly also giant forest hogs (hylochères) and topi. In addition, he mentioned a previous well-known and catastrophic outbreak of the disease, in 1920.

According to Bourlière & Verschuren (1960) rinderpest decimated the buffaloes in the area of the Parc National Albert in the epizootic of 1921, and reached them again in April 1932, having arrived from Uganda via the district to the south of Lake George. It reappeared, once more from the vicinity of Lake George, in August 1944 and spread slowly among buffaloes, reaching the plain to the south of Lake Edward in April 1945. Guyaux (1951), who gave an account of the same outbreak, proved the identity of the buffalo disease agent and mentioned that bushpigs, giant forest hogs and bushbuck were also found dead.

In conclusion, from the available records, it can be said with some degree of assurance that the hippopotamus population in the region of Lakes George and Edward was exposed to rinderpest during the epizootics of 1920/21, 1931/33 and 1944/45, but no reliable evidence exists that it has since had contact with the infection. Hence it is possible to account for all the antibody-positive animals with the exception of one each in groups 9 and 10. Apart from the possibilities of confusion in labelling of specimens or minor errors in the estimation of age, it is difficult to explain the possession of antibody by these two individuals.

The much higher 'immunity' rate in animals 28 years of age and over suggests that the intensity of exposure in the earlier epizootics was greater than in 1944/45. The lower rate of serological conversion in the latter episode may also imply that the particular strain of virus involved did not spread so readily among susceptible hippopotamuses, since it would be expected that opportunities for transfer would be optimal in such a gregarious and semi-aquatic species, which is continually fouling its own living medium. The grazing habits of hippopotamuses, including their continual use of the same lake-shore paths, would also appear to favour dissemination of the virus. The same behavioural factors would undoubtedly facilitate the acquisition by the hippopotamus of virus from other species, such as buffalo and waterbuck. These, when suffering from rinderpest, tend to remain near water in order to slake their abnormal thirst; in addition they often die in or near drinking points.

There is apparently no record of disease in the hippopotamus which has been attributed to rinderpest virus. It is, however, well known that a significant local mortality occurs periodically in areas where the species is abundant. Multiple deaths have been observed at intervals over many years and a series of such incidents has been summarized in tabular form by Bourlière & Verschuren (1960). The general opinion is that the mortality results from anthrax (see, for example, Randall, 1951, 1957, 1958 and Cronly, 1952) although Bourlière & Verschuren (1960) make reference only to 'charbon symptomatique', a clostridial infection commonly known as 'blackquarter' in English. The deaths have not ceased in the last 10 years, during which time rinderpest has been absent from the region; nevertheless it is possible that some of the earlier episodes, as in 1930 (Kazinga Channel) or 1933 (Lake George) may have been due, at least partially, to rinderpest infection.

So far as the persistence of rinderpest-neutralizing antibody is concerned, Brown & Raschid (1957) reported that cattle which had received caprinized rinderpest vaccine were still immune and possessed circulating antibody 13 years later, although they had never been re-exposed to virus; quantitative data were not presented. Neutralizing antibody in man, to the closely related measles virus, persists throughout life and the proportion of serological positives among individuals in endemic areas does not change appreciably after the age of 8–9 years (Black, 1959). The mean titre, however, falls gradually in adult life in spite of the fact that there are probably periods of re-exposure, particularly in the 13–14 and 20–40 years age groups (Black, 1959). Black & Rosen (1962) studied measles antibody patterns in residents of Tahiti, where epidemics occurred in the years 1929, 1951 and 1960. There was essentially no change between 1951 and 1959 in the neutralizing-antibody titre of sera from patients infected in the former year, although no re-exposure to measles occurred during this time. Individuals who were infected in 1929 and presumably exposed again in 1951 showed only a very slow decline in serum antibody over a 30-year period. The degree of stability of antibody was regarded as 'almost without precedent'.

The rinderpest titres of sera from hippopotamuses (Table 2) were not appreciably different from those which have been found in other rinderpest-susceptible game species in the Serengeti area of North Tanganyika, where the virus has produced epizootics during the last 3–4 years. Thus forty-two recently-infected yearling wildebeest (*Gorgon taurinus taurinus*) showed log SN 50 titres which ranged from a 'trace' to $10^{2\cdot8}$ but the mean was only $10^{1\cdot15}$, against a dose of virus varying from $10^{1\cdot6}$ to $10^{2\cdot6}$ TCD 50. The wildebeest is a species which reacts clinically, sometimes severely, to rinderpest virus infection and it was all the more surprising, therefore, to find that the mean antibody level in hippopotamuses first infected about 30 years ago ($10^{1\cdot11}$ to $10^{1\cdot32}$) was at least as high as that in yearling wildebeest, infected only a few weeks or months before (Plowright, to be published).

SUMMARY

A survey of rinderpest-neutralizing antibody was carried out on the sera of 315 hippopotamuses which were shot in the Queen Elizabeth Park, Uganda. No serological positives were found in animals aged 11 years or less but about 4 % were detected in the age class 13–25 years. In the 28–41 years age class the proportion of positives rose to about 36 %. All except one of the positive sera were titrated, the figures obtained being comparable to those in other recently infected species of wild ungulates.

It was concluded that the heat-stable antibody in hippopotamuses resulted from inapparent infections with rinderpest virus during epizootics which passed through the areas of Lake George and Lake Albert in the years 1920/21, 1931/33 and 1944/45. No record was available of clinically recognizable rinderpest in the hippopotamus population but deaths due to other causes were mentioned briefly.

Comment was made on the stability of rinderpest-neutralizing antibody over periods of about 30 years.

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Interferon production and sensitivity of Semliki Forest virus variants

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INTRODUCTION

Semliki Forest virus (SFV), a group A arbovirus, grows readily in calf kidney cells in tissue cultures, and has been found to form plaques on these cells under agar. However, SFV which has been grown only in the brains of mice (MB virus) forms plaques which are small and irregular. In an attempt to obtain a variant which might form better plaques, MB virus was passaged serially 10 times in calf kidney cells, but the resultant virus (termed CK) was not more satisfactory. It was however found that the MB and CK strains of SFV formed plaques on L cells which differed significantly in size. In further studies, other differences in the properties of the two strains were noted. The results obtained are presented in this paper. In particular, the relative growth of the two viruses in calf cells, the relative sensitivity of the two strains to added calf interferon, and the relative amounts of interferon which they themselves produce when growing in calf kidney cells were investigated.

MATERIALS AND METHODS

Viruses

MB virus. Semliki Forest virus (SFV) was received from Dr J. S. Porterfield (Medical Research Council, Mill Hill) having had a total of nine passages in the brains of mice since the original isolation (Smithburn & Haddow, 1944). This was inoculated intracerebrally at 10^{-3} into 17–18 g. mice. Fifty-three hours later, their brains were harvested, and made up in 10 % (w/v) suspension in normal horse serum (previously inactivated at 56° C. for 30 min.). This was designated MB (mouse brain) virus. Samples were stored at −20° C.

CK virus. Ten serial passages of SFV were made in primary calf kidney cells, starting with MB virus. In each passage, feeding bottle cultures containing confluent sheets of approximately $10^{7\cdot2}$ cells and 20 ml. of medium were inoculated with $10^{2\cdot0}$–$10^{4\cdot5}$ plaque-forming units (pfu) of virus. After the cultures had been incubated for 24–30 hr., the medium was harvested, clarified by light centrifugation and used, appropriately diluted, as seed for the next passage of the series. Seeds were stored until required as a 1/10 dilution in inactivated horse serum at −20° C. The 24 hr. harvest from the tenth serial passage in calf kidney cells was designated CK virus. Samples were stored at −20° C.

Other viruses. Influenza A (1947) virus, strain Kunz, and Sendai virus were

obtained from Dr D. A. J. Tyrrell (Medical Research Council, Salisbury), and passaged in eggs. Infected allantoic fluid was stored at −20° C., diluted 1/10 in inactivated horse serum.

Tissue cultures

Primary tissue cultures of the kidney cortical tissue of 5 to 7-day-old calves were prepared by trypsinization using standard techniques. The liberated cells were suspended in a growth medium of Hanks's saline with 0·5% lactalbumin hydrolysate, 8% calf serum and 0·05% sodium bicarbonate at a concentration of $0{\cdot}075 \times 10^6$ living cell aggregates (Bishop, Smith & Beale, 1960) per ml. Test tubes, 4 oz. medical flat bottles and Pyrex feeding bottles were seeded with 1, 12 and 20 ml., respectively. Tubes were incubated in sloped racks for 2 days, and then on a roller drum. Medical flat bottles were incubated on a levelled surface. Feeding bottle cultures were rolled throughout. Medium changes were given as necessary. Cultures were used after 6 or 7 days, when confluent cell sheets had formed.

L cells were grown in tubes and medical flats, using medium 199 with 10% calf serum.

All calf serum was inactivated at 56° C. for 30 min. before use. Cultures were incubated at 37–38° C. throughout.

Plaque assays for SFV

Unless otherwise stated, infective virus was assayed on monolayers of L cells in medical flat bottles by a plaque technique. An overlay of medium 199 with 10% calf serum and 1% agar was used. Plaques were counted after 3 days, and titres calculated as plaque-forming units (pfu) per ml. of undiluted virus.

Serum neutralization experiments

Sera were inactivated and diluted appropriately in medium 199 with 2% calf serum and 0·15% bicarbonate. Samples were mixed with equal volumes of virus diluted to contain approximately 200 TCD 50/ml. and held at room temperature for 1 hr.; 1 ml. of each serum–virus mixture was then added to each of five tubes of L cells. These were incubated in sloped racks and observed daily for 5 days. Serum 50% cytopathic-inhibition titres were calculated by the method of Thompson (1947).

Production of calf interferon

Interferon was made from calf kidney cells inoculated with small amounts of influenza A, strain Kunz, essentially as described by Tyrrell (1959). After incubation for 48–60 hr., the tissue culture fluid was harvested, lightly centrifuged and treated at pH 2 for 4 hr. at room temperature in order to destroy the virus present.

Assay of interferon

Interferon titres were measured by a quantitative haemadsorption-inhibition technique to be described fully elsewhere using tube cultures of calf kidney cells and Sendai virus. The titre of an interferon sample was the reciprocal of the log

dilution at which the amount of virus growth was reduced to 50 % of the level in control tubes. Statistical analysis showed that, with the technique used, fourfold differences in the interferon content of two preparations could be discriminated with 99 % certainty, if three tubes were used to test each interferon dilution. By using six to nine tubes for each dilution, twofold differences could be distinguished.

Growth curve experiments

Calf kidney cell cultures in tubes, medical flat bottles or feeding bottles were inoculated with SFV diluted in medium 199 with 0·5 % lactalbumen hydrolysate, 1 % chick embryo extract and sodium bicarbonate at 0·09–0·14 % as appropriate. At the time of harvest, medium was collected from groups of three to five replicate cultures and pooled. Inactivated horse serum was added to give a final 20 % concentration, and samples were stored at −20° C. until titrations could be performed. Before assay for interferon, media from SFV-infected cultures were treated at pH 2 overnight at 4° C., neutralized to pH 7, and heated at 56° C. for 30 min., in order to destroy all infective virus present (R. F. Sellers, personal communication).

RESULTS

Plaque sizes with MB and CK viruses on L cells

On L cell monolayers under agar, MB virus formed plaques which ranged from pin-point in size to 3 mm. diameter at 48 hr. after overlaying, and increased to between 0·5 and 5 mm at the 4th day. On other monolayers tested in parallel, CK virus produced plaques which were only pin-point in size at 48 hr.; additional plaques often became visible on the 3rd day, and on the 4th day the plaque sizes ranged from pin-point to 2·5 mm. With both strains the plaque sizes varied considerably at all times of reading, but MB virus formed plaques which were definitely the larger.

Serological identification of CK virus

It was necessary to demonstrate that CK virus was a variant of SFV, and not a bovine virus picked up from one of the serial passages in calf cells. Cross-neutralization tests were therefore carried out. In two experiments, an antiserum from a rabbit immunized with MB virus had a geometric mean titre of 1/224 against MB virus, and 1/110 against CK virus. An antiserum to CK virus had a geometric mean titre of 1/105 against MB virus, and 1/107 against CK virus. These results may be analysed for evidence of antigenic similarity by the method of Archetti & Horsfall (1950). The ratios (r_1 and r_2) of the titres with heterologous virus and with homologous virus are calculated with each serum, and also the geometric mean (r) of these ratios. The value r gives in a single figure the extent of the antigenic difference between the two viruses, a value for $1/r$ of between 1/1 and 1/5 implying that the strains concerned are antigenically indistinguishable. Applying this analysis to the data above, values are obtained for r_1 of 105/107, for r_2 of 110/224, and for $1/r$ of 1·4. It is concluded that CK virus is in fact a SFV variant.

Growth curve studies with MB and CK viruses in calf kidney cells

(i) *Formation of extracellular virus.* Feeding bottle cultures of calf kidney cells were inoculated with 100 pfu (approximately 2×10^{-5} pfu added per cell) of either MB or CK virus. With each virus, medium was sampled from one group of six bottles at intervals during the next 72 hr. Some of the medium harvested at 42 hr. was incubated for a further 24 hr. *in vitro* at 37° C. to give the rate of thermal inactivation of the progeny viruses. It will be seen from Fig. 1 that, compared with MB virus, CK virus accumulated more rapidly in the medium and reached a higher final titre at an earlier time. Under the microscope, cell damage was seen earlier and ultimately became more extensive in the cultures infected with CK virus than in those infected with MB virus.

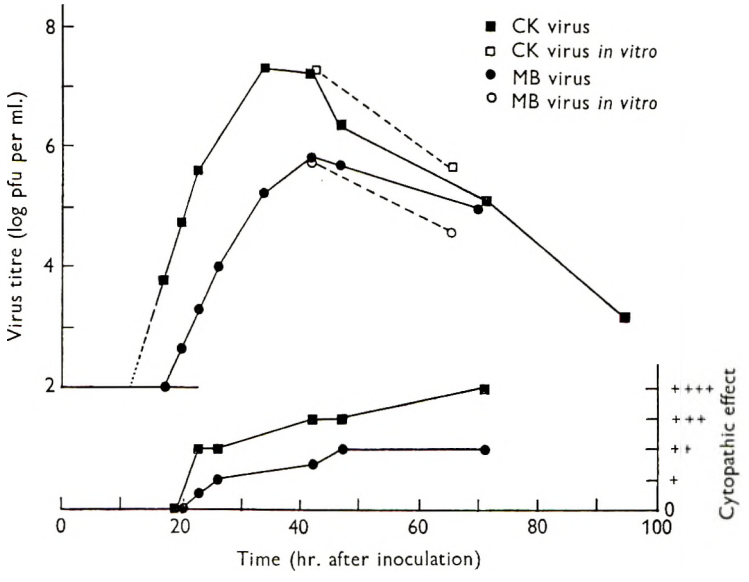

Fig. 1. Growth curves for MB and CK viruses in extracellular fluid of calf kidney cells. Inocula were 2×10^{-5} pfu per cell. Harvests at 42 hr. were incubated *in vitro* at 37° C. for 24 hr.

The rates of thermal inactivation *in vitro* of the medium harvested at 42 hr. were similar, at 0·053 and 0·057 log pfu per hr. for MB and CK viruses, respectively (Fig. 1). The level of infective virus in the medium of the cultures infected with CK virus declined from its peak at a comparable rate. In contrast, the rate of decline in the MB-infected cultures was definitely slower (0·029 log pfu per hr.) between 42 and 72 hr., suggesting that significant amounts of additional virus were being liberated from the cells.

In the same experiment, another group of six bottles infected with each virus was harvested at 24 hr., and again at 48 hr. and 72 hr. At each time, the medium was completely harvested, the cell sheets were washed once, and 20 ml. of fresh medium were added. These harvests contained the total amounts of infective virus liberated into the medium during the successive 24 hr. periods. These were found to be $10^{7\cdot4}$, $10^{6\cdot9}$ and $10^{5\cdot3}$ total pfu in the cultures infected with CK virus, and

$10^{4\cdot7}$, $10^{6\cdot8}$ and $10^{5\cdot9}$ total pfu in those infected with MB virus. It was thus confirmed that after an inoculum of MB virus, virus was released from the cells more slowly and for a longer time than after a comparable inoculum of CK virus.

A final group of cultures received a 10,000 times larger inoculum of MB virus (0·2 pfu per cell). The virus in the medium rose to a peak level ten times higher than after the smaller inoculum of the same virus. This probably resulted from the operation of interferon mechanisms, as discussed below.

In a series of other growth curve experiments with relatively small inocula, it was again found that CK virus grew faster than MB virus, and reached a higher final titre (Table 1).

Table 1. *Rates of increase in and maximum titres of virus after equivalent inocula of MB and CK viruses*

Inoculum (pfu/cell)	Rates of increase in infective virus (log pfu/hr.) Virus		Maximum titre of infective virus (log pfu/ml.) Virus	
	MB	CK	MB	CK
2×10^{-5}	0·23	0·29	5·8	7·3
5×10^{-5}	0·30	0·40	n.d.*	n.d.*
2×10^{-3}	0·31	0·47	6·8	8·2
7×10^{-2}	0·47	0·55	6·7	7·4
$3{\cdot}8 \times 10^{-1}$	0·31	0·38	7·2	7·9
$7{\cdot}5 \times 10^{-1}$	0·30	0·48	7·2	8·2

These data from six growth curve experiments with calf kidney cells relate to extracellular infective virus.

* n.d. = not determined.

(ii) *Formation of extracellular virus in cells pre-treated with interferon.* A batch of calf kidney tubes was divided randomly into four groups, of which two were treated with a 1/10 dilution of interferon prepared from calf cells with Kunz virus. The remaining tubes were changed to medium without interferon and served as controls. On the following day, $10^{3\cdot1}$ pfu of CK virus were added to all tubes of one interferon-treated and one control group. The remaining interferon-treated and control tubes similarly received $10^{3\cdot1}$ pfu of MB virus. The tubes were incubated on a roller apparatus throughout this experiment. At various times between 1 and 96 hr. after inoculation of the seeds, medium was harvested and pooled from six tubes in each group. Results of assays are shown in Fig. 2.

The growth curves for infective virus in the medium of control tubes show the features described in the previous section. In this particular experiment, the differences between the two viruses were rather small. About 4 hr. after inoculation of MB virus, the amounts of infective virus in the medium of the control tubes increased rapidly. In the interferon-treated tubes, there was no increase until 15 hr. Thereafter infective virus increased slowly until about 60 hr., and reached a maximum level about 1000-fold lower than in the controls. The level then remained unchanged for at least a further 30–40 hr., during which virus must have been liberated from the cells at a rate sufficient to balance the amounts thermally inactivated.

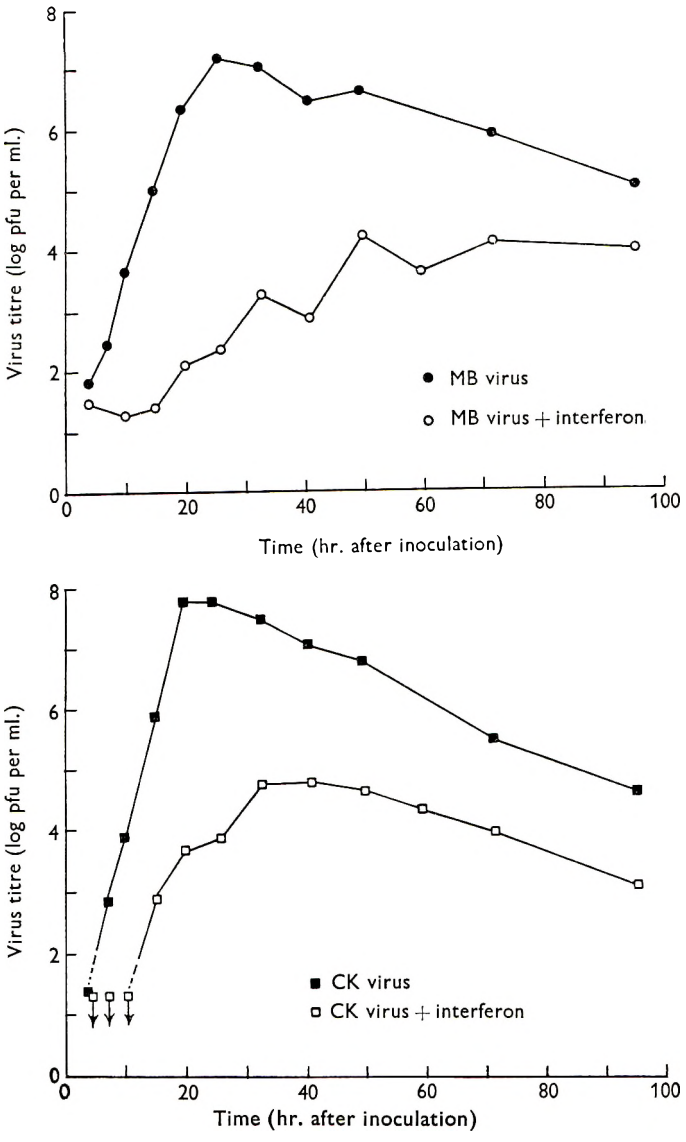

Fig. 2. Growth curves for MB and CK viruses in calf kidney cells which had been pre-treated with calf interferon, and in control cultures. Inocula of each virus were $10^{3 \cdot 1}$ pfu per culture.

The effects of added interferon on the growth of CK virus were similar but much less marked. Infective virus increased in the medium somewhat more slowly than in the controls, but the peak level was reached only 12 hr. later than in the controls. This level was again approximately 1000 times lower than in the corresponding controls, but it was five times greater than in the interferon-treated tubes infected with MB virus. From the shape of the growth curve, there was probably also some prolongation of the period of liberation of CK virus from the interferon-treated cells.

Similar results were obtained in a replicate experiment.

(iii) *Formation of interferon by MB and CK viruses.* CK virus was added to groups of twenty medical flat bottle cultures of primary calf cells at a multiplicity, in terms of added virus, of 1·15 pfu per cell, and at 1/1000th of this level. Similarly MB virus was added to further bottles at 1·61 pfu per cell and at 1/1000th of this level. After incubation at 37° C. for 2 hr., the bottles were completely drained and washed, and fresh medium was added. From assays of the amounts of unadsorbed virus, it was calculated that 72 % of the larger inoculum of CK virus was taken up by the cells, and 38 % of that of MB virus. At various times up to 96 hr. after inoculation of virus, samples were taken from four or more bottles in each group, pooled and titrated for infective virus and interferon. Results from this experiment are shown in Fig. 3.

Considering first interferon production with the concentrated inocula, it will be seen that small amounts of interferon were first detected 10 hr. after inoculation of MB virus. After CK virus, interferon was not detected at this time, but thereafter the rates of formation were similar with the two viruses, and levels increased rapidly to reach the same maximum. After CK virus, the level of interferon declined after 48 hr. and became undetectable by 96 hr. In contrast there was little change over this period after MB virus. In other experiments, interferon formed after infection with CK virus consistently declined more rapidly than that formed after MB virus. Possibly the earlier and more extensive cell damage caused by CK virus leads to earlier release of enzymes which break down interferon.

After the dilute inoculum of CK virus, interferon was not detected in the tissue culture fluid until 25 hr. Thereafter it increased rather slowly to reach a peak which was the same as after the more concentrated inocula of either virus. In contrast, the dilute inoculum of MB virus led to a more rapid increase in interferon, and harvests at 36, 48, and 60 hr. after infection contained significantly more interferon than after any other inoculum in this experiment.

These differences between the two viruses in their relative production of and sensitivity to interferon may account for certain differences in the growth curves obtained after small inocula of the two viruses (Fig. 3). They may also account for differences in the curves after large and small inocula of MB virus. After the dilute inocula, the rates of increase in extracellular virus appear identical with the two viruses during the first 7 hr. Thereafter that of MB virus slowed relatively, and this virus grew to a lower maximum than CK virus. With the 1000 times larger inocula of the two viruses, the rates of growth and the maxima differed little. However, with MB virus the peak titre was more than twice that after the smaller inoculum. A tenfold increase in the peak virus titre after a 10,000 times greater inoculum of MB virus was described earlier. It seems likely that with dilute inocula, the cells infected initially produce not only infectious virus but also interferon. This will act on the cells which have not yet been infected and diminish their response to virus infection. In calf kidney cell cultures, such an effect will be more apparent with MB virus, which forms more interferon and is also more sensitive to its effects, than with CK virus. If on the other hand most or all of the cells are infected initially, interferon will be formed too late to influence virus growth. Under these conditions MB virus will grow at the same rate and to the same peak titre as CK virus.

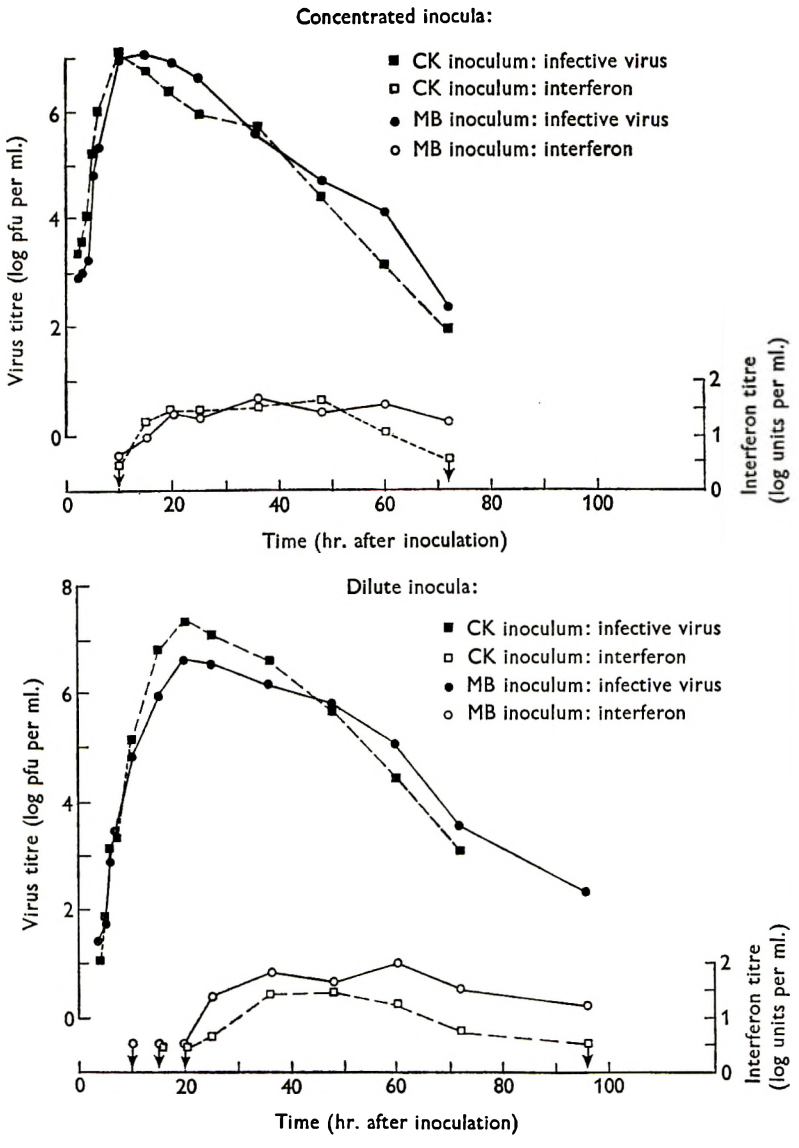

Fig. 3. MB virus was added to groups of calf cell cultures at 1·61 pfu per cell, and at $1{\cdot}61 \times 10^{-3}$ pfu per cell. CK virus was similarly added at 1·15 and $1{\cdot}15 \times 10^{-3}$ pfu per cell. After 2 hr., cultures were drained and washed and fresh medium was added. Samples of medium were taken from four or more bottles in each group at the times indicated.

In two comparable experiments, results were in general very like those shown in Fig. 3. The relative and absolute amounts of interferon formed after the different amounts of the two viruses varied somewhat from experiment to experiment. However, CK virus usually formed less and never more interferon than a comparable inoculum of MB virus. The differences between the experiments probably resulted from the use of particular batches of calf cells; when cells from several different kidneys were infected in parallel with the same inoculum of MB virus, the amounts of interferon formed after 48 hr. differed more than five-fold.

Attempts to detect the presence of particles of CK type in MB virus

During the serial passages in calf cells, CK virus replaced MB virus as the predominant type, presumably because of selection under the conditions of passage. Attempts were made to isolate clones of large and small plaque-forming particles by picking plaques from L cell monolayers which had been inoculated with highly diluted MB virus under an agar overlay. The precautions suggested by Mosley & Enders (1961) were carefully observed to minimize the chances of contamination of the selected clones with other virus particles. In two experiments, no significant differences were found in the mean diameters of the plaques formed by progeny from the different clones. Thus the particular plaques selected may have differed in size at the time of picking due to physiological rather than genetic reasons. Alternatively, a high rate of mutation and back-mutation between particles forming plaques of the two sizes may occur on L cells.

These results suggest that particles of CK virus are present in MB virus in a proportion too low to detect without difficulty. Alternatively, they may have appeared as the result of chance mutation during the calf cell passages.

Growth of MB and CK viruses in other systems

Results of repeated assays of MB and of CK viruses in three different host cell systems are shown in Table 2. With both seeds, approximately the same figures were obtained for the amounts of infective virus per ml. using mice inoculated intracerebrally, and in plaque assays with L cells and in chick embryo fibroblasts. Thus particles infectious in the mouse brain were also infectious in L cells and in chick cells.

Table 2. *Titres of MB and CK viruses in three assay systems*

Assay		Virus			
		MB		CK	
Assay system	Units	No. of assays	Mean log titre	No. of assays	Mean log titre
L cells	log pfu/ml.	6	6·88	3	7·23
Chick embryo cells	log pfu/ml.	6	7·05	1	7·0
Mice infected intracerebrally*	log MLD50/ml.	2	7·0	2	6·93

* 17–18 g. mice inoculated with 0·03 ml. Titres are the 50% mouse lethal dose end-points, calculated by the method of Thompson (1947).

In a growth curve study, 18–20 g. mice were infected intracerebrally with 100 pfu of MB or CK virus. At intervals, mice were killed and the amounts of virus in 10% (w/v) brain extracts were measured. The mice infected with MB virus were moribund at 72 hr., at which time the virus content of the brains was $10^{8\cdot2}$ pfu per ml. (Fig. 4). In contrast, CK virus grew to a maximum titre of only $10^{6\cdot6}$ pfu per ml., and less than half of the mice inoculated were dead 8 days later. Thus CK virus grew less readily in the mouse brain than MB virus and was less virulent.

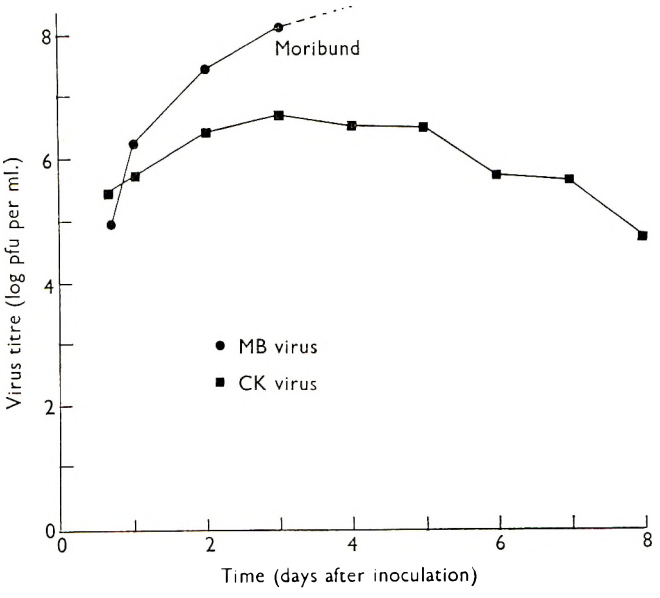

Fig. 4. Growth curves MB and CK viruses in the brains of mice. The inoculum of each virus was 100 pfu.

DISCUSSION

The two variants of SFV described in this report, MB, derived from mouse brain passages, and CK, from calf kidney cell passages, were first distinguished by the sizes of the plaques which they form on L cells under agar. In growth curve experiments with calf kidney cells, it was found that the MB variant was the more sensitive to the effects of added interferon. Also this variant usually stimulated production of more interferon, and never less interferon, than CK virus. It has already been pointed out that these differences probably account for the observed differences in the rates of growth of the two variants after small inocula, and for the differences in the rates of growth of the MB variant after large and small inocula.

Differences in interferon production and interferon sensitivity between strains of the same virus have been previously reported. De Maeyer (quoted by Enders, 1960) found that a monkey-virulent strain of measles virus produced 2–4 times less interferon in human cell cultures than an avirulent strain derived from it by egg passage. Sellers (1963) reported that attenuated strains of foot-and-mouth disease virus appear to produce more interferon, and to be more sensitive to its action, than cattle-virulent strains. Wagner *et al.* (1963) studied two variants of vesicular stomatitis virus which resembled the SFV variants described in this report in that they differed in plaque morphology as well as in rates of growth and in interferon sensitivity. No definite conclusions could be reached about their relative production of interferon.

The differences in interferon production and in interferon sensitivity between the two SFV variants may also be relevant to the phenomena of virus adaptation

and virus virulence. Compared with MB virus from which it was derived, CK virus grew poorly in the mouse brain and was less lethal. In calf cells, however, it grew faster, and it caused cell destruction sooner. Thus CK virus was less virulent than MB virus in the mouse brain, but more virulent in calf cells to which it had been adapted. The mechanisms behind these changes are of interest. It has been suggested that adaptation of a virus results from selection of variants capable of more rapid growth in the new host system (Burnet & Bull, 1943; Wang, 1948). Such variants may be present in the original inoculum as a small minority, or may arise during the serial passages as the result of chance mutations. Wang (1948) showed that mouse-adapted influenza virus grew considerably more rapidly, and to a 10 times higher titre, in the lungs of mice than an egg-passaged line of the same virus. These results are comparable to those found here with CK and MB viruses in calf cells.

A number of factors have been described which favour the growth of one variant of a virus rather than another. Such factors include the pH and bicarbonate content of the medium (Vogt, Dulbecco & Wenner, 1957; Mosley & Enders, 1962), and the temperature of incubation (Lwoff, 1959; Carp & Koprowski, 1962). The relative sensitivity of two virus variants to an inhibitor, such as a sulphated polysaccharide, may encourage the growth of one of them (Takemoto & Liebhaber, 1961). It is here suggested that another factor may be the relative production of, and sensitivity to, interferon of two variants.

In studies on viral interference in the allantois, Fazekas de St Groth, Isaacs & Edney (1952) inoculated eggs with heated influenza B virus, and challenged them after 24 hr. with influenza A virus. The total yield and the rate of growth of the challenge virus was markedly less than in controls. Similar results have been found with cells treated in tissue culture with interferon by Ho & Enders (1959), by Wagner (1961) and in the present study. The slower rate of growth and reduced final yields of challenge virus from cells previously treated with either interfering virus or interferon closely resemble the features described above for the growth of a virus in a new host system. This suggests that a common mechanism may be operating.

In support of this idea, it was found that when growing in calf cells, the unadapted MB variant usually stimulated production of more interferon than the adapted CK variant, and was also the more sensitive to the effects of added interferon. Such differences in interferon production and sensitivity could lead to overgrowth of MB virus during serial passages in calf cells by any particles of virus of the CK type present in the MB seed, or arising as the result of mutations. Thus while the evidence can only be regarded as circumstantial, these interferon mechanisms could play a part in, or even be mainly responsible for, the process whereby a virus adapts to a new host. In general, adapted viruses are more virulent in the host concerned. A correlation between increased virulence and decreased interferon production among virus variants has already been noted (Enders, 1960; Sellers, 1963).

Adaptation of SFV to calf cells was accompanied by loss of adaptation to growth in the mouse brain. The CK variant grew more slowly in the mouse brain, reached

a lower final titre and was less virulent than MB virus. It would be interesting to know whether the relative sensitivity to, and production of, interferon by the two strains in the mouse brain are, as expected, the converse of what obtains in calf cells.

SUMMARY

Semliki Forest virus was passaged 10 times in series in calf kidney cells, starting with virus passaged in the mouse brain (MB virus). A variant was obtained (termed CK virus). The two viruses were antigenically indistinguishable. When grown on L cells, CK virus formed smaller plaques than MB virus. In parallel growth curve studies in calf kidney cells, a small inoculum of CK virus grew more rapidly and to a higher final titre than a comparable inoculum of MB virus, and usually stimulated production of less interferon. Pre-treatment of cells with calf interferon reduced the growth of both viruses, but especially that of MB virus. The results are considered in relation to the phenomenon of virus adaptation. It is suggested that such differences between the two variants in their relative sensitivity to and production of interferon could have played a part in the emergence of CK virus as the predominant type during serial passages in calf cells.

I wish to thank Mr H. Moores and Mr R. Sims for skilful technical assistance.

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J. Hyg., Camb. (1964), **62**, 351
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The relationship of a type of *Mycoplasma* isolated from tissue cultures to a new human oral *Mycoplasma*

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INTRODUCTION

In a previous communication (Lemcke, 1964), four strains of *Mycoplasma* isolated from tissue cultures and one from a line of Eaton agent (*M. pneumoniae*) were found to be serologically related to one another but distinct from sixteen other species or serotypes. It was suggested that this group constituted a new serological type or species. As these strains did not belong to any of the species of known origin with which they were compared, there was no indication of the source from which the tissue cultures or the *M. pneumoniae* culture were contaminated.

More recently a comparison of these strains with four others has thrown some light on their origin. Of these new strains, three were described by Herderscheê, Ruys & van Rhijn (1963) as a new human oral *Mycoplasma*. The fourth, although isolated from a culture of *M. pneumoniae*, was known to be identical with strains occurring in the human oropharynx (Chanock, personal communication).

METHODS

The five strains previously described (Lemcke, 1964) were 823, 826, 837 and 844 from tissue cultures and BM from a culture of *M. pneumoniae* (PI 898) propagated in primary monkey kidney cells. Three of the new strains were isolated by Herderscheê *et al.* (1963); H and S from the throats of two patients with scarlatina and E from a tissue culture. The fourth, 898/2, was isolated by Dr R. M. Chanock (Bethesda) from the same source as BM.

Complement fixation and gel diffusion tests were carried out as described by Lemcke (1964). Antigens for gel diffusion tests were prepared by the ultrasonic treatment of washed saline suspensions, using a Branson S-75 Sonifier at 20 kc./sec. for 10 min.

RESULTS

In complement-fixation tests, antigens of strains H, S, E and 898/2 reacted to titre or to $\frac{1}{2}$ of the titre with antisera against the 'tissue culture' strains 823 and 837. In contrast, these antigens reacted only to 1/32 or less of the homologous titre with antisera against strains of *M. hominis* type 1, *M. pneumoniae*, *M. salivarium*, *M. fermentans*, *M. pulmonis*, *M. arthritidis*, *M. neurolyticum*, *M. mycoides* var. *mycoides*, *M. bovigenitalium*, *M. agalactiae*, *M. laidlawii*, *M. gallisepticum*, *M. gallinarum* and three other serotypes represented by the strains Navel (human), A 36 (non-pathogenic avian) and pp. goat (contagious caprine pleuropneumonia).

In gel diffusion tests, reactions of identity were given by antigens of 837, BM, H, S, E and 898/2 with antiserum against 837.

The new strains, like the five previously examined, were distinctly filamentous when grown in liquid medium.

DISCUSSION

The four 'tissue culture' strains, the two contaminants from *M. pneumoniae* PI 898 and the three strains belonging to the new oral type described by Herderscheê *et al.* (1963) are not only indistinguishable serologically but constitute a group distinct from sixteen other serological types or species already described.

As the evidence suggests that this type is primarily an inhabitant of the human mouth and throat, it is possible that the tissue cultures were contaminated by droplet infection during maintenance and transfer. If one tissue culture can be contaminated from another maintained in the same laboratory, the infection of only one cell line could serve as a reservoir for the contamination of others. In view of the failure to find mycoplasma in sera used in tissue culture media (Rothblat & Morton, 1959; Coriell, Fabrizio & Wilson, 1960; Klieneberger-Nobel, unpublished observations) it is unlikely that, as suggested by Herderscheê *et al.* (1963), the source of the contaminant was human serum.

It was suggested (Lemcke, 1964) that BM, the first contaminant of *M. pneumoniae* examined, was present in one of the tissue cultures used for passage. Now that the relationship of BM and 898/2 to a human oral mycoplasma has been demonstrated, it seems more likely that BM was present together with *M. pneumoniae* in the sample taken from the patient's throat.

SUMMARY

A previously unidentified type of *Mycoplasma* found as a contaminant in tissue cultures and in a culture of Eaton agent, is identical with a newly recognized human oral type.

I am indebted to Drs Herdersheê and Chanock for sending me their cultures and relevant information.

Note. Since this paper was submitted, the name *Mycoplasma orale* has been suggested for the new oral type (Herderscheê & Taylor-Robinson, personal communication).

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The specificity of cellular immunity*

By DAVID F. GRAY† and ROSALIND POOLE‡

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INTRODUCTION

In diseases such as tuberculosis, pasteurellosis, pertussis, brucellosis, salmonellosis, listerosis and many viral infections, where the infecting organism occupies a predominantly intracellular position, protective antibody, even if it is produced, seems to be denied effective immunological contact with the pathogen (Lurie, 1936, 1950; Osebold & Sawyer, 1957; Kadull, Reames, Coriell & Foshay, 1950). However, as Mackaness (1962) has pointed out, unless circulating antibody can be excluded as a factor in acquired immunity, difficulties arise in assessing the actual significance of non-humoral factors, even though these may be playing the major role. One indirect piece of evidence that a significant degree of immunity can be achieved in the virtual or complete absence of antibody is provided by the response of agammaglobulinaemic patients to diseases of the type under discussion (Bruton, 1952). For the purpose of the present discussion it is accepted that a degree of cellular immunity is probably a feature of all immune responses and that it is independent in origin of the plasma cell system responsible for antibody production that may or may not function concurrently with it (Gray, 1964).

In the case of tuberculosis, cellular mechanisms certainly appear to play a major role (Mackaness, 1954) and while there have been several reports of humoral tuberculostatic factors (Dubos, 1954; Seibert *et al.* 1956; Zitrin & Wasz-Hockert, 1957) there is little convincing evidence (Raffel, 1961) that these factors take the form of specific protective antibodies, or even that they are necessary for opsonization, since phagocytosis occurs quickly and completely in the non-immune animal (Gray, 1959).

The degree of specificity underlying cellular immunity has recently been under discussion (Rowley, 1962; Mackaness, 1962). While it is evident that cellular immunity normally follows a specific antigenic stimulus, a phenomenon of current interest to immunologists is the discovery that an increase in resistance to the type of infection under consideration may also arise following several kinds of non-specific stimulus involving unrelated microbial antigens as well as substances presumably unrelated to microbial antigens in any way. These include:

(*a*) Unrelated intracellular infections (Lurie, 1939; Dubos, 1954; Edsall, 1955; Parry, 1956; Rowley, 1956; Smith, 1956; Mackaness, 1962).

(*b*) Bacterial lipopolysaccharides (endotoxins), particularly those derived from Gram-negative bacteria (Westphal, 1957; Boehme & Dubos, 1958; Rosen, 1961).

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(*c*) Certain simple lipids, such as the triglycerides, tri-olein or tri-caprin (Stuart *et al.* 1960; Cooper & Stuart, 1962; Cooper & West, 1962; Stuart & Cooper, 1962).

(*d*) Certain surface-active detergents represented by polyoxyethylene ethers (Cornforth, Hart, Rees & Stock, 1951; Hart, Long & Rees, 1952; Solotorovsky & Gregory, 1952; Mackaness, 1954; Niffeneger & Youmans, 1960).

It is pertinent to question whether these stimuli were all acting on the same cellular mechanism to induce the same phenomenon, or whether increased cellular resistance may be the product of a number of different factors and systems. Taking tuberculosis as a model, since it is the subject of the present paper, the important change occurring under both specific and non-specific stimulation appears to be in the ability of macrophages to kill ingested bacilli, since phagocytosis in itself is never a problem in this disease. This implies that macrophages must undergo a period of training, differentiation, induction or selection, culminating in the emergence of a large population of actively bacteriolytic cells. Incidentally, and this is of importance in assessing reports on the duration of non-specific immunity discussed below, it must be remembered that the period of active bacteriolysis is confined to the first week or two of the immune phase and thereafter is replaced by a steady state in which multiplication and bacteriolysis maintain an equilibrium (Affleck & Gray, 1957; Cheers & Gray 1964).

As has just been implied, the literature suggests the existence of certain apparent points of difference between specific and non-specific cellular immunity requiring further study. Boehme & Dubos (1958), for example, pointed out that the protective effect of endotoxin against *Mycobacterium fortuitum* lasts longer than the increased reticulo-endothelial activity promoted by the endotoxin, and Howard, Rowley & Wardlaw (1958) reported that the non-specific activity was of shorter duration than specific immunity, usually lasting only 7–10 days. Comparing our own observations on specifically induced antituberculous immunity with those of Cornforth and others, it also seemed that the non-specific stimulation may induce increased resistance a good deal more rapidly.

Of immediate practical importance is that, if an effective and durable cellular immunity could be induced non-specifically, it would have a profound effect on current immunoprophylaxis against those diseases for which living vaccines, with their attendant risks, are commonly employed.

The experiments reported here were proposed by Hart in 1960 (personal communication). It had been shown previously that the detergent Triton* enhanced the resistance of guinea-pigs to tuberculosis and apparently suppressed tuberculin allergy in these animals (Hart *et al.* 1952). At the time, Hart & Rees (1960) had also just completed experiments showing that Macrocyclon, a surface-active agent similar in many ways to Triton, suppressed the acute, but not the chronic stage of the disease in intravenously infected mice. Hart suggested that these results might profitably be compared with those obtained from intranasally infected mice, in which changes in the disease pattern with the onset of the immune phase are customarily followed by means of culturable lung counts and footpad tuberculin

* Triton, WR 1339 (Rohm and Hass Co., Philadelphia).

tests (Gray & Jennings, 1955). In this way it was likely that some evidence might be obtained of the possible interference of Triton with the process of antituberculous immunity.

MATERIALS AND METHODS

Experimental mice. Two strains of mice were used: (*a*) C57 Black mice originally obtained from the Roscoe Jackson Memorial Laboratories in 1947, and since randomly mated; and (*b*) MUA (Melbourne University albino) mice, randomly mated for 15 years. The results were similar and apparently independent of strain differences in immunity (Gray, Graham Smith & Noble, 1960). They were fed on Barastoc pellets* supplemented twice weekly with green lucerne. Water, to which 250 mg./l. of chloramphenicol was added to control intercurrent infection with *Corynebacterium murium*, was available *ad lib.*

Infection of mice and assessment of disease. Mycobacterium tuberculosis, strain H37Rv, freeze-dried for storage after animal passage, was grown in dispersed cultures of Tween-albumin medium. Serial dilutions of coarse-filtered, 10-day cultures consisting predominantly of single cells were prepared as previously described (Gray & Mattinson, 1952). This 1952 paper also described in detail the culturable counting technique used and the precautions associated with the intranasal infection of anaesthetized mice. The footpad tuberculin test, using 1/25 old tuberculin in saline, was described by Gray & Jennings (1955).

Triton WR 1339. Distilled water was added to 12·5 g. of Triton in a beaker to make up 50 g. and the mixture stirred during gentle heating. This produced a cloudy mixture which cleared on cooling to give a 25 % solution. An equivalent volume of normal saline was added to provide a stable solution of 12·5 % concentration. This was autoclaved at 10 lb. for 10 min in flasks which were shaken during cooling to maintain solution of the detergent when cold.

Triton was administered by intraperitoneal injection as either a 10 or 12·5 % solution, using a tuberculin syringe fitted with a 26-gauge needle. In a series of tests to confirm the findings of earlier workers, which need not be recorded here, the antibacterial activity of Triton *in vivo* became evident about 5–7 days after a dose of 25 mg. and was adequately maintained by repeating this dose every 2 or 3 weeks. Toxic effects characterized by muscular spasms and some deaths first occurred after the use of 40 mg. twice weekly, by the time the fourth dose was given.

RESULTS

In vitro *antimycobacterial activity of Triton*

The reported absence of any *in vitro* antituberculous activity by Triton was confirmed for the H37 Rv strain used in these laboratories, by exposing heavily inoculated Loewenstein slope cultures to assay disks impregnated with the detergent solution in concentrations ranging from 1 to 20 %. After 4 weeks' incubation no inhibition had occurred in the vicinity of the highest Triton levels to which the culture was exposed.

* Barastoc Grower's Pellets—a growing ration for chickens—W. S. Kimpton and Sons, Melbourne.

The influence of Triton on footpad conversion rate

During the course of these experiments, Triton was administered at several different dose levels (25–80 mg./week) to mice infected intranasally with small, medium or large doses of tubercle bacilli, commencing 5 weeks before, during, or shortly after infection and often continuing through the course of the disease. In every experiment records were made of the allergic state of the mice, using bi-weekly footpad tests immediately before selecting those to be killed. Whereas these mouse experiments failed to show any reduction in the degree of tuberculin allergy similar to that reported by Hart and his colleagues (1952) in treated guinea-pigs, in certain combinations of infecting dose and Triton administration there was a significant delay observed in the appearance of measurable allergy.

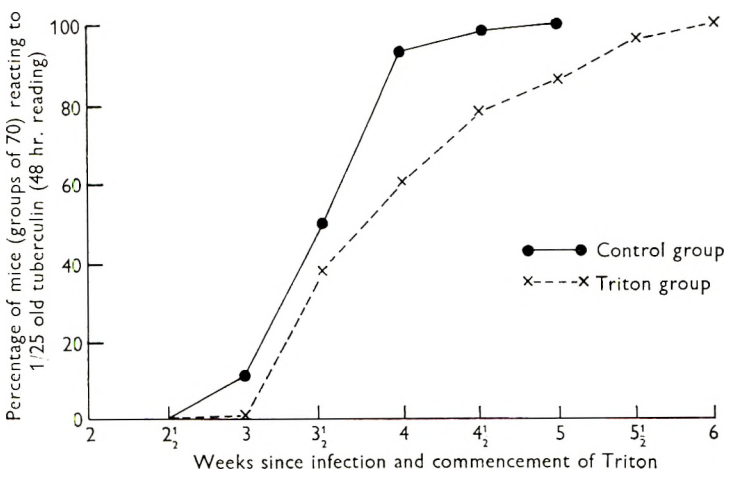

Fig. 1. The influence of Triton WR 1339 on footpad conversion rates in tuberculous mice receiving a small infecting dose. Infecting dose: 150 culturable units of *M. tuberculosis* (H 37 Rv). Triton dose: 25 mg. every 3 weeks, commencing at infection.

Where the infecting dose was small (150 culturable units) and commenced concurrently with the Triton treatment (25 mg. at 3-week intervals) a significant reduction occurred in the conversion rate. A typical result is presented in Fig. 1, where the conversion of groups of seventy mice with and without Triton are compared. However, when the infecting dose was increased above 1000 units the apparent difference in conversion rate became unpredictable, while a very large infecting dose ($2{\cdot}25 \times 10^6$ units) gave a uniform and more rapid conversion in both treated and control mice.

These findings, assessed in relation to earlier work on the influence of infecting dose size on conversion (Gray & Jennings, 1955) strongly suggest that the observed delay in this instance was due to the retarded progress of the disease in Triton-treated mice when the initial dose was small. There was no real evidence that even large and prolonged Triton dosing suppressed the severity of the tuberculin reaction in individual mice, as judged by measuring the thickness of the footpad swelling. It seems possible in the light of these findings that the reduction in

guinea-pig reactions comparable with that produced by cortisone which was reported by Hart and others (1952) might well be masked in rats and mice by their higher allergic threshold (Gray & Jennings, 1955; Gray, Noble & O'Hara, 1961).

The effect of Triton on the progress of pulmonary tuberculosis

We now have a very clear picture of the normal course of murine pulmonary tuberculosis induced by small intranasal infecting doses (Gray & Affleck, 1958; Gray, 1959; Gray *et al.* 1960). An initial lag of about 5 days gives way to rapid multiplication within macrophages lasting from $3\frac{1}{2}$ to 6 weeks, when it is followed by a dramatic reduction of the lung population by well over 90 %. This bacteriolytic

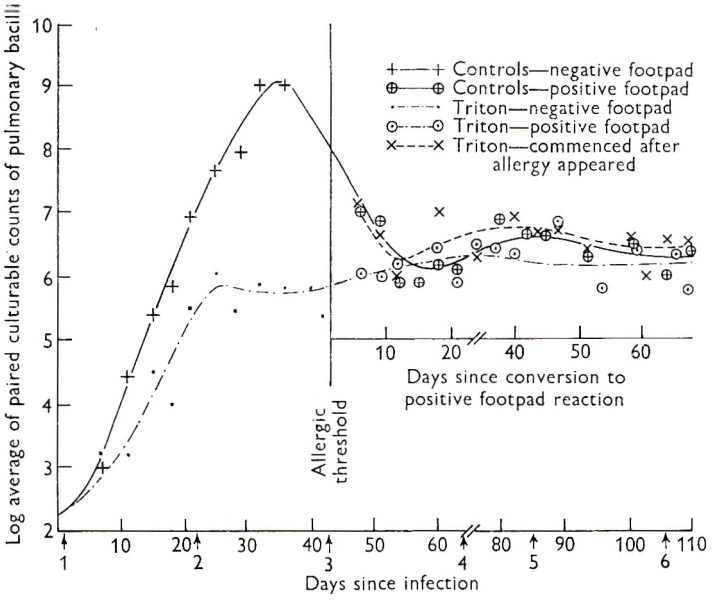

Fig. 2. Effect of Triton on pre-allergic and immune phases of murine pulmonary tuberculosis induced with a small infecting dose. Infecting dose: 150 culturable units. Triton dose: 25 mg. every 3 weeks ($\frac{1}{1}$, $\frac{1}{2}$, etc.).

phase, in turn, is quickly replaced by a steady state of usually long, but unpredictable duration (the immune phase), which is normally paralleled by tuberculin allergy. This typical picture is illustrated by the control group, Fig. 2, in which the pre-allergic peak of log 9 was followed by a steady state between logs 6 and 7.

(*a*) *Small infecting dose, with Triton treatment started in pre-allergic or allergic phase*

This trial is typical of several tests on both C 57 and MUA mice, which will not be recorded because of the essential agreement obtained in each case. The conditions included infection with 150 units, and while one treated group of seventy mice received 25 mg. of Triton the following day and again after 3 and 6 weeks, the other group, of similar size, received no Triton until they had converted to the allergic state. The control group received no Triton at any stage.

Two mice from the appropriate groups were killed every 3–4 days immediately

the results of the concurrent tuberculin tests were known, and the bacilli in the lungs of these were counted, together with those of any mice which died. For convenience of presentation and interpretation, only tuberculin-negative mice were killed in each group as long as they lasted. Meanwhile mice showing positive footpad tests were identified and held. When they were killed it was in order of conversion, so that the times on the graph to the right of the line marked 'Allergic threshold' represent the number of days that had elapsed between conversion and sacrifice in each case.

(1) It will be observed from the results recorded in Fig. 2 that the control mice followed the anticipated pattern, reaching the steady state of the immune phase with a lung population between 10^6 and 10^7 bacilli and remaining there with minor fluctuations throughout the experiment.

(2) The group receiving Triton concurrently with infection not only failed to show a pre-allergic peak, but also entered the steady state on the average 3 weeks after infection, i.e. at least 2 weeks before the controls did so. Although their culturable counts during these 2 weeks were at least as low as those reached later by the immune controls it is noteworthy that these mice were still negative reactors to tuberculin.

(3) The effect of Triton if any, equalled but did not exceed that of the normal immune phase, except in appearing earlier.

(4) Triton administered to mice already in the immune phase did not enhance the existing immunity.

(5) These findings are compatible with an hypothesis that the action of Triton is to accelerate the onset of cellular immunity.

(*b*) *Large infecting dose, preceded or accompanied by Triton*

The large infecting dose was used to determine whether the delay in conversion reported above was due merely to the small number of bacilli lysed at the beginning of the immune phase. It was also of interest to determine the degree of protection afforded against death when a combination of high infecting and low Triton dose was given and whether it would influence the relative number of organisms surviving to the steady state in the treated animals. In this experiment, the infecting dose of H 37 Rv selected was $2{\cdot}25 \times 10^6$ units accompanied by a single, 25 mg. dose of Triton. The results which are set out in Fig. 3 have been interpreted as follows:

(1) The single dose of Triton administered protected a high proportion of the heavily infected mice against the chances of pre-allergic death (4/40 deaths compared with 13/40 in the control group). It must therefore be concluded that the protective effects of the single dose persisted in most mice through the pre-allergic phase. No observations have yet been made to ascertain whether higher or repeated dosing with Triton would have increased the resistance at this infecting dose level.

(2) The Triton-protected mice, although they tended to survive the pre-allergic stage, were less well able to restrain pre-allergic multiplication than mice receiving a smaller infecting dose (see Triton group, Fig. 2).

(3) Allowing for a tendency for heavy-handed procedures to dampen minor differences, this experiment requires no significant modification of the interpretations based on the smaller infecting dose (Fig. 2). It will be noted that, although more treated than control mice entered the immune phase, there was again no significant difference between the steady state levels reached by the two groups.

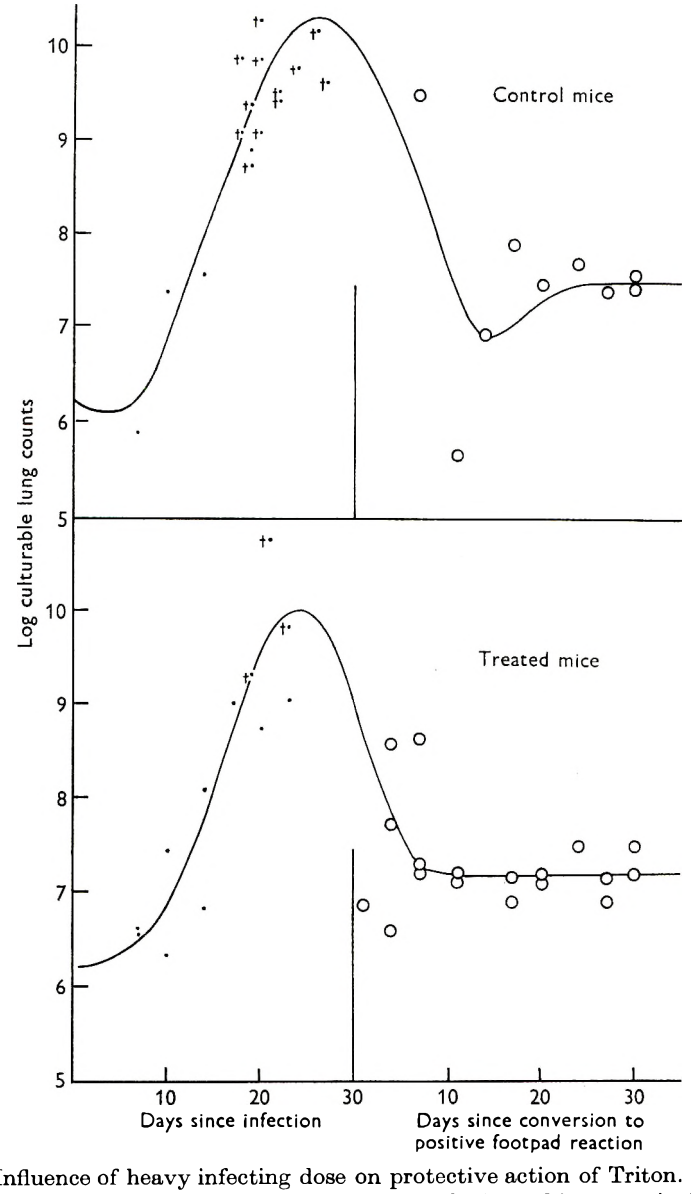

Fig. 3. Influence of heavy infecting dose on protective action of Triton. Infecting dose: $2{\cdot}25 \times 10^6$ culturable units. Triton dose: Single dose of 25 mg. coinciding with infection. † = Died. Pre-allergic deaths = 13/40 controls; 4/40 treated mice.

DISCUSSION

The most important obstacle to a clearer understanding of the nature of cellular immunity does not appear to be concerned with opsonization and ingestion of bacteria, but rather with the changes occurring in the intracellular activities of macrophage cells which convert tolerance into bacteriolysis. This is perhaps best illustrated by chronic infections such as tuberculosis, in which normal macrophages rapidly ingest tubercle bacilli and allow them to multiply freely for several weeks before any detectable bactericidal properties are acquired. It is not clear whether this represents a period of training for existing cells, or one of replacement by selected or educated immunologically competent cells. Moreover, how widely and how completely this acquired competence embraces enhanced resistance against other intracellular pathogens is still under discussion (Crowle, 1963).

A puzzling feature is the variation in time needed to reach a state of competence under different conditions. As we have seen, in mice infected with tubercle bacilli or immunized with BCG, it may take from 3 to 6 weeks to develop bactericidal properties, but lipopolysaccharide or Triton can apparently produce the same antituberculous efficiency within a few days. Moreover, specifically acquired competence against *Listeria monocytogenes* and *Salmonella typhi-murium*, which are stated to afford cross-protection for tuberculosis, can be developed in a few days. The question of whether training or replacement of macrophages occurs is perhaps of less immediate importance than the probability that these competent cells can be induced to appear in the body at varying rates and be maintained for varying periods by a group of unrelated, specific and non-specific stimuli. These appear to have little or nothing in common and some of them must be reasonably supposed to be antigenically inert. The present study leaves the more provocative aspects of cellular immunity still to be answered and, indeed, probably raises more new questions than it answers existing ones. It does permit certain tentative conclusions to be drawn, however, and suggests further profitable experiments.

Pulmonary tuberculosis produced in mice under conditions simulating a natural infection (viz. with a tiny intranasal infecting dose) was profoundly modified by Triton used prophylactically, despite the failure of Triton to exhibit either bacteriostatic or bactericidal activity *in vitro*, thus confirming earlier reports to this effect. Reference to Fig. 2 shows that this effect was well established by about the 7th day and continued up to the onset of the immune phase. At this stage the activity of Triton merged smoothly into the altered immunological state and added nothing to it.

Furthermore, Triton given for the first time to mice as they converted to the allergic state appeared to add nothing to the existing, specifically acquired resistance. This observation confirms the findings of Kátó & Gözsy (1957) that Triton failed to increase the existing level of immunity in mice specifically immunized with BCG, and suggests the possible identity of the two states. Such a concept would imply that Triton might in some way be capable of inducing the same degree of enhancement of antituberculous activity as follows specific immunization, but that it does so very much more quickly. However, there is no indication at present of the existence of a mechanism which might allow this to take place. The absence

of allergy from the early part of the Triton-induced steady state may be unimportant, since by the time this work was undertaken the evidence against allergy *per se* playing any important role in immunity was already quite strong (Raffel, 1948; Gray, 1958, 1964). That the two phenomena are normally concurrent may mean no more than that they are both dependent on the same sort of stimuli, i.e. those arising from the products of lysed tubercle bacilli. If the action of Triton does actually stimulate an accelerated immune response, it is not only notably independent of allergy but also of the need for specific antigenic stimulation.

Such a supposition requires that the mechanisms governing cellular immunity be less precise than those of antibody production, allowing for possible induction by several apparently unrelated stimuli, some of which bear no apparent resemblance to known antigens, and there is certainly no proof at the present time that this is so. Another possible explanation is that specific cellular immunity does not exist at all, but that macrophages possess a latent, non-specific, bactericidal ability which is inducible by a variety of stimuli. Thus, there are many questions still to be answered.

Experiments are now under way to compare the relative speed of onset, efficiency and duration of cellular immunity produced by specific and non-specific means and to study the effects of Triton on the immune and steady states in an acute disease, viz. listerosis.

It is yet to be decided whether an animal in the steady state with respect to one disease is automatically in the same state with respect to all other intracellular infections as some of the recorded observations suggest. If this were so it would presuppose involvement of the entire macrophage system whenever cellular resistance is increased, and this, again, is inconsistent with what we know of specific immunological behaviour.

Whatever the nature of the steady state may be, it is clearly a dynamic compromise between host and parasite rather than a quiescent state in tuberculosis. Recently completed experiments (Cheers & Gray, to be published) have shown that alterations produced in the bacterial population of the steady state by chemotherapy and/or by superinfection are rapidly readjusted to the previous steady state at the earliest opportunity. The duration of this readjustment is now being examined.

SUMMARY

1. Non-specific stimulation of mice by a detergent lacking *in vitro* antibacterial activity, Triton WR 1339, produces a steady state in the course of experimental tuberculosis at about the same level as specific immunization, and does so independently of delayed allergy.

2. This effect is achieved in a much shorter time than is required by the normal immunological processes and the relative duration of enhanced resistance in each case is yet to be worked out.

3. Triton fails to enhance an existing, specifically acquired immunity.

4. There is no evidence so far that the mechanism involved is the same in both instances. If it were found to be the same, then the present concept of a specific cellular immunity would need to be revised.

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J. Hyg., Camb. (1964), **62**, 365
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Experiments on the spread of colds

1. Laboratory studies on the dispersal of nasal secretion

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It is now known that colds are due to infections with a number of different viruses and that it is therefore unwise to generalize about the epidemiology of the disease. We have, nevertheless, attempted to measure some of the processes which may operate when infected nasal secretion leaves the upper respiratory tract of a model 'patient' and reaches that of another subject.

This paper describes work using biological tracers; in this we have measured the clearance of foreign material from the nose, mouth and conjunctiva and the dispersal of material from the mucous surfaces of normal subjects on sneezing, coughing and so on.

METHODS

We wished to use a tracer which could be detected after great dilution, could be measured accurately, was completely unaffected by the activity of biological fluids, and could be detected in the presence of these fluids or of common house dust. It had, of course, to be completely non-irritating and harmless to man even when administered repeatedly. We considered the use of fluorescent or radioactive powders and tried suspensions of uniform plastic beads stained with fluorescent dyes, and detected by ultra-violet microscopy. We finally chose to use a suspension of spores of the non-pathogenic organism *Bacillus mycoides*, which was recommended and prepared for us by the Microbiological Research Establishment, Porton. This organism is a saprophyte which forms large colonies in 18 hr. at 30° C. on a medium (1 % peptone, 2 % agar) on which the normal human flora will not grow at all, and the saprophytes of the laboratory and home will grow very little. The colonies are so characteristic that they are immediately distinguished from the occasional contaminant (see Plate 1*a*). The suspension used contained 10^9 spores per ml.; it was stored at 4° C. and the viable count was unchanged at the end of 1 year. It was shown that the viable counts were quantitatively satisfactory provided that not more than fifty colonies on a 4 in. plate were counted; also the counts were unaffected by inoculating spore suspensions mixed with human saliva and nasal secretion. If spores were spread on glass, enamel and other surfaces and allowed to dry they could be completely recovered by washing the surfaces with 1 or 2 % of calf serum in isotonic saline buffered at pH 7·1 with 0·01 M sodium phosphate buffer (sampling fluid).

We had also to choose methods of collecting material for assay. We found that the absorbent cotton bacterial swab used in this laboratory took up about

0·04–0·1 ml. when rubbed on to a moist mucous surface. This method was the best available for taking repeated samples from the respiratory tract. However, we also modified previous methods for studying the expulsion of droplets from the nose and mouth. In order to measure the total number of spores expelled by sneezing or coughing, the subject was seated on a chair with his head over the top of a large air-filled polythene bag (120 cm. × 60 cm.) of the type used for storing clothes, containing 190 ml. of sampling fluid. The air sampler, which was held in a retort stand, was lowered to the bottom. The plastic was drawn round the subject's neck while he coughed, spoke or stimulated his nose with a wooden applicator in order to induce a sneeze. The applicator was inside the bag, but grasped by the subject's fingers through a fold in the bag. Immediately after the experimental procedure the subject's head was withdrawn, and the neck of the bag was closed. The air pump was then switched on and in about 2 min. it removed through the sampler practically all of the air in the bag. The sampler was then extracted, the fluid or slides removed for assay (see below); the spores on the sides of the bag were removed by closing the neck again and swirling the sampling fluid all over the inside of the bag; an aliquot of the sampling fluid was removed for assay.

To sample air we used one of two methods. The first was a pre-impinger (type A) designed by May & Druett (1953) added to an impinger through which air was drawn in series at 11 l./min. (Plate 1*b*); the pre-impinger collects in 4 ml. of sampling fluid particles of about the size trapped in the nose, i.e. those which behave like spheres of unit density, and a diameter of 4 μ or greater (Landahl & Black, 1947); the impinger collects smaller particles down to about 1 μ in diameter. It was necessary to wash the dome and neck of the pre-impinger to collect those spores which were not trapped in the fluid. Spores in the fluid were assayed as indicated above. In order to obtain fuller information about the size distribution of airborne droplets we used as a second method a cascade impactor as designed by May (1945). In this apparatus air is drawn at 17 l./min. through four slits of decreasing size and particles are trapped on glycerine-coated slides (Plate 1*c*). The sizes of airborne particles trapped on each slide were determined by May (1945) and we used his data. The deposit was washed off each slide and assayed for spores. Only in a few preliminary experiments did we use settling plates. These give valuable information on how many infectious particles may drop on to a vulnerable surface, such as a wound, but we thought that the most important material epidemiologically would be what remained airborne and was therefore likely to be inhaled. We also wished to extrapolate from our results to the probable behaviour of virus-containing material of much lower infectivity; it was therefore less important to know the number of airborne particles which were carrying infectivity than the amount of infectivity which was being carried in particles of a certain size range. We therefore used impingers and impactors rather than apparatus such as the Bourdillon slit sampler, on which particles are impacted directly on to an agar surface.

Except where noted the experiments were reproducible although the results of only one of each type are presented. Several others, sometimes performed with minor modifications, have given similar results.

RESULTS

Transport of spores in the nose and mouth

We first studied the fate of spores placed in a small volume of fluid on various mucous surfaces of a normal subject. About 0·05 ml. of spore suspension was placed on both sides of the nasal septum just inside the nasal vestibule. The subject then

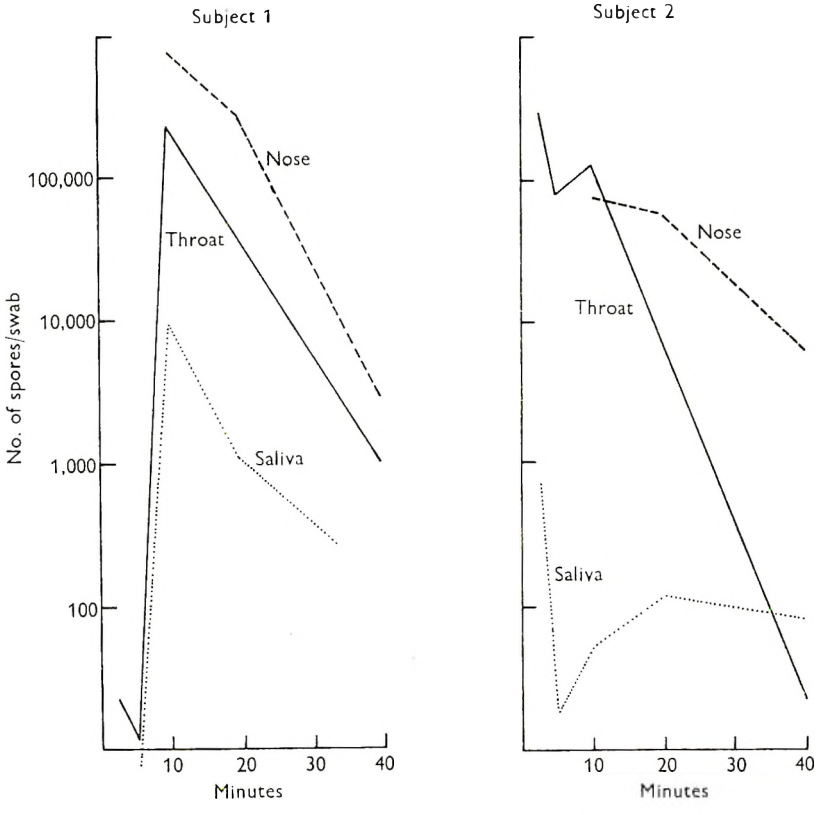


Fig. 1. The recovery of spores from the nose, throat and saliva following the inoculation of 10^8 spores in 0·1 ml. of fluid on to the anterior nasal septum of two normal subjects.

continued his laboratory work, speaking if necessary but avoiding sneezing or blowing the nose. Swabs were collected at intervals from the throat and mouth and after 5 or 10 min. from the anterior part of the nose too. The 'decay' curve of spore concentration was apparently exponential as can be seen from Fig. 1. The spores took 5–10 min. to reach the throat and at about the same time appeared in the saliva. The concentration in the saliva was lower than and followed that of the throat. This suggested that not only did ciliary activity move nasal mucus rapidly into the throat, but that some unknown mechanism moved throat secretions forwards into the mouth. Experiments on subject 1 always gave smooth curves, but in subject 2 the process of clearance seemed to be much less regular. Spores were placed under the tongue in the same way and swabs were collected

from the top and side of the tongue; they were steadily removed as can be seen from Fig. 2; this was thought to be due to the fact that they were being diluted with saliva and swallowed.

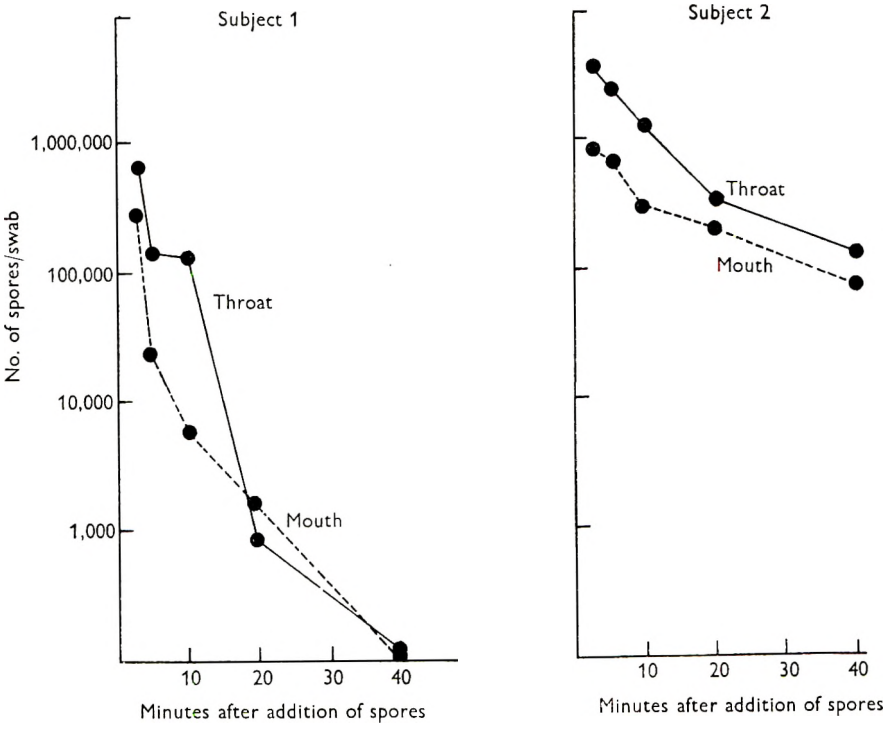


Fig. 2. Recovery of spores from the throat and mouth following inoculation of 10^8 spores into the mouth of the same two subjects as in Fig. 1.

Rate of clearance of spores

Assuming that spores were rapidly and uniformly diluted in saliva we calculated from the intercept and slope of the curve the apparent volume and rate of secretion of saliva. The subjects also dribbled saliva into a Petri dish and the rate of secretion was calculated directly. By direct measurement subject 1 produced 1·8 ml./min. of saliva and 2 produced 0·22 ml./min. By calculation from the spore counts 1 produced 1·8 ml./min. and 2 formed 0·27 ml./min. From the ratio of the concentrations of spores in the throat and saliva we calculated that throat secretion was being carried forward into the mouth at the rate of 0·09 ml./min. in subject 1 (Fig. 1) and 0·0012 ml./min. in subject 2 (data of Fig. 3). We tried to make similar calculations from the curves of nasal clearance, but found unreasonable results, presumably because most of the spores passed through the nose in a little 'packet' (as was suggested by experiments with dye powders, see below) and therefore the mathematical model did not apply. The 'half life' of spores in the nose was 3 and 7 min. and of spores in the mouth was 2 and 6 min. in subjects 1 and 2, respectively. These experiments were all repeated with bacteriophage T3 and almost identical results were obtained, indicating that a

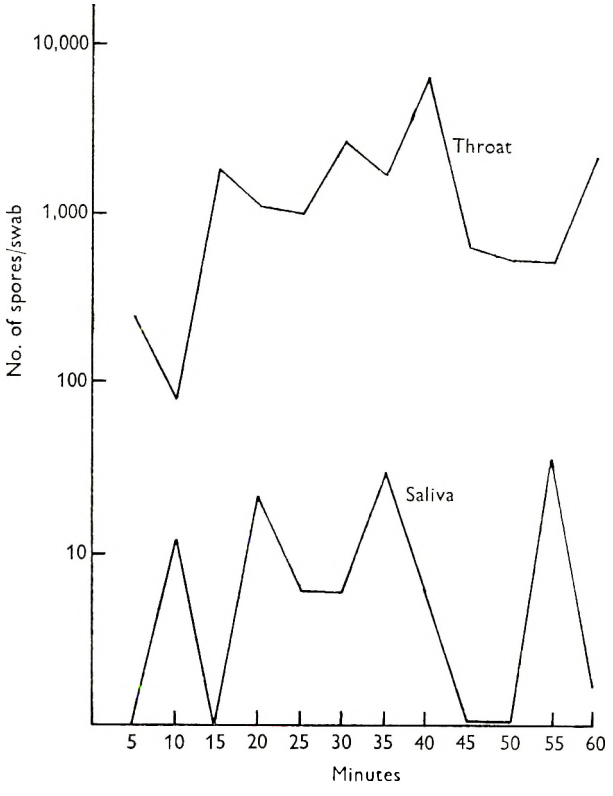


Fig. 3. Recovery of spores from throat and saliva during continuous infusion by nasal catheters of a suspension containing $10^{7\cdot1}$ spores/ml. The concentration in saliva was at all times much lower than in the throat.

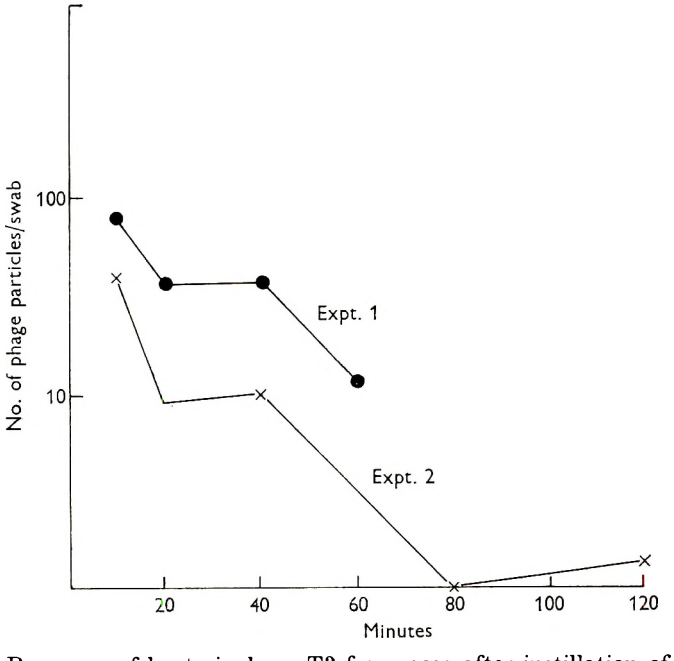


Fig. 4. Recovery of bacteriophage T3 from nose after instillation of $3{\cdot}3 \times 10^5$ pfu into conjunctival sac.

particle with the dimensions of a medium-sized virus might be expected to be transported in the same way as the spores. Bacteriophage particles were also added to the conjunctiva. They were removed rapidly (Fig. 4), but could not be detected regularly in the anterior nares.

It was desirable also to mimic the way in which infectious secretions are constantly being formed in the nose during a cold. By means of a sinusoidal pump 3×10^6 spores per min. were delivered in 0·25 ml. of phosphate buffered saline through two fine nasal catheters (Jacques E.G. 3 with one side hole) inserted 4 cm. into the nostrils. The fluid emerged as a slow trickle from each catheter which lay on the floor of the nose; the subject sat erect with his head tilted backwards just enough to prevent the suspension running out of the nose. The number of spores recovered from the throat and mouth swabs are shown graphically in Fig. 3. This shows that even when nasal material containing about 10^6 spores per 0·1 ml. was being constantly carried down into the throat, spores were detected only intermittently in the mouth of this subject although they were constantly present in the throat. We thought that spores were forced into the mouth by an occasional imperfection in the swallowing mechanism; this was supported by another experiment in which it was found that the concentration of spores in the mouth increased greatly when the head was tilted forwards. Negus says (personal communication) 'I see no reason why peristaltic contraction of pharyngeal constricters should not force material into the mouth after the pharynx has been elevated'.

We also followed nasal clearance non-quantitatively by placing a powder of edicol orange and calcium phosphate inside the anterior nares. This was usually observed on the pharyngeal mucosa between 5 and 15 min. later, but the rate of clearance could not be evaluated quantitatively.

The dispersal of tracers from the nose and throat

Spores were placed in the nose of a subject as described in the section on transport of spores, and those expelled were then recovered after three coughs, each of which was as deep and vigorous as the subject could make it; in another experiment he vigorously declaimed ten lines of Shakespeare, sneezed hard, or blew his nose into a cotton handkerchief 40×40 cm. The results shown in Table 1 indicate that over 1000-fold more spores were recovered after sneezing or blowing the nose than after the other manoeuvres. Hare & Thomas (1956) found that the snorting occurring in blowing the nose released more *Staphylococcus aureus* from the nose than did sneezing. It has been observed in high-speed photography (Jennison, 1942; Bourdillon & Lidwell, 1941) that the droplets expelled by sneezing come mainly from the mouth. This was confirmed in our experiments by weighing the secretion expelled into two separate small plastic bags held over the nose and mouth during an experimental sneeze. We recovered 0·15 g. from the mouth and 0·025 g. from the nose. However, there were more spores in the nasal secretion than in the saliva which apparently forms most of the droplets coming from the mouth; it was therefore thought likely that more spores would be shed from the

nose than from the mouth during a sneeze. This was confirmed in other experiments also shown in Table 1, in which the nose or mouth were closed during the sneeze and in which the secretions of the nose and mouth were collected separately with plastic bags. Immediately after sneezing into the two bags swabs were collected from the nose and mouth—190,000 spores were recovered from the former and 1500 from the latter. In five experiments carried out between 1 and 2 min. after putting spores on to the anterior nasal septum we recovered from the large plastic bag between 6 and 38 % of the spores added, the average recovery being 25 %.

Table 1. *Activities which disperse spores placed on the anterior nasal septum*

	Time between adding 10^8 spores and making experimental manoeuvre	Spores recovered from plastic bag after indicated activity*	
		Surface	Air
	Experiment 1		
Talking	5 min.	380	40
Coughing	5 min.	380	0
Sneezing	1½ min.	5,700,000	1,910
Blowing the nose	2 min.	1,400,000†	4,100‡
	Experiment 2		
Sneeze with mouth covered	10 min.	11,400,000	2,020
Sneeze with nose covered	10 min.	14,200	470
	Experiment 3		
Sneeze with: 2 small bags	10 min.	—	—
Mouth bag	—	1,500	—
Nose bag	—	1,040,000	—

* Subject 1.
† 32,800,000 spores were recovered from the handkerchief.
‡ In experiments with subject 2 counts in this category were much lower.

It was thought that during talking or sneezing rapid airflows might move spores from the nose into the mouth or from the mouth into the nose. This was tested by swabbing one site after adding spores to the other and carrying out the manoeuvre. There was no evidence of any movement of spores at all in this way.

It was concluded that under the conditions of a common cold, in which infectivity was present mainly in the nasal secretion, the infectious particles would be expelled mainly during sneezing and mainly from the nose. A substantial proportion of fluid on the anterior part of the nasal septum would be expelled. Hamburger, Green & Hamburger (1945) found that patients who had throat and nose swabs which were positive for haemolytic streptococcus shed eighty times more of these organisms than patients in whom only the throat swab was positive.

The size of droplets in which spores are sneezed out

In many experiments the number of spores which could be recovered from the air in the 1½–2 min. after sneezing into a bag, and the number of spores which were recovered from the wall of the bag were measured separately as indicated in

Table 1. This suggested that most of the spores were found in droplets which remained airborne for a very short time. A subject sat in front of a bench on which a large number of glass slides were spread. He sneezed in a horizontal direction over them and the droplets on the slides were allowed to dry and were then stained and measured with a microscope with a micrometer eyepiece. The size of droplets before drying was assumed to be 1/2·5 of the diameter measured (Liddell & Wootten, 1957), and it was calculated that the diameter of the dried droplets ranged from 50 to 860 μ—76 % were between 80 and 180 μ. The number of spores found in individual drops ranged from one or two to uncountable masses.

Table 2. *Number of spores carried in droplets of various sizes expelled into plastic bag by sneezing*

	Total number of spores recovered	
Sample collection from:	Expt. 1	Expt. 2
Bag surface	36,000,000	37,050,000
Impinger sampler	48,000	—
Impactor: (1) > 10 μ*	—	2,130
(2) 16–3 μ	—	24,000
(3) 6–1·5 μ	—	10,600
(4) 2½–0·8 μ	—	95

* Approximate size range of particles of unit density on numbered slides—there is some overlap in the sizes collected (see May, 1945). Stages 1 and 2 approximate to upper respiratory collection. Stages 3 and 4 approximate to lower respiratory collection.

We then studied the distribution of spores in the smaller sized particles. Some typical results are shown in Table 2. This indicated that about two-thirds of the spores were carried in particles collected on slides 1 and 2: and such particles were therefore in the larger size range which might be trapped in the nose. This was confirmed by other experiments with preimpinger and impinger samplers. It is, of course, impossible for many spores to be found on slide 4 of the cascade impactor because there is not room for a spore inside a droplet of less than about 1 μ diameter yet such droplets might well carry virus particles. The experiment was therefore repeated using T3 bacteriophage, but the results were very similar; from 2 to 6 % of plaque-forming units were recovered from slide 4.

Model experiment on possible routes of transmission to other subjects

We next repeated the experiment under conditions which approached more closely those in which sneezing normally occurs. The experiment was done in a quiet laboratory in which the windows were closed. The subject sneezed with his head just above the level of a bench on which was placed a rectangular glass plate 56 × 30 cm. This was intended to collect droplets which would normally drop to the floor. Beyond the plate a microscope slide was placed vertically, to represent the conjunctival surface of a person in the vicinity of a sneezer; next to this stood an impinger with attached preimpinger and a cascade impactor. The glass surfaces were all coated with a thin film of a mixture of glycerine and gelatine and the spores

Table 3. *Spores recovered after sneezing in an open room. Number of spores recovered by sampling method*

Method of dispersal	Horizontal glass plate	Vertical microscope slide	Pre-impinger	Impinger	Impactor 1	Impactor 2	Impactor 3	Impactor 4
Sneeze	2,000,000	10	970 65 %*	500 35 %	500 24 %†	1,000 48 %	510 25 %	30 3 %
Artificial coarse spray	6,400,000	150	82,400 59 %	60,000 41 %	26,000 21 %	95,000 55 %	41,000 23 %	1,270 1 %

* Percentage of all airborne spores recovered in preimpinger–impinger sample.
† Percentage of all airborne spores recovered in impactor sample.

were recovered by washing the glass plate with 100 ml. sampling fluid and the slide with 5 ml. The arrangement of the apparatus is shown schematically in Fig. 5 and some typical results in Tables 3 and 4. The vast majority of spores (99·9 %) dropped rapidly towards the ground and only a small proportion remained airborne in the vicinity of the sneezer for as long as a minute. Rather over half the spores were carried on droplets which might be trapped in the nose. Very few spores were picked up by the vertical slide. It was possible that some of these results were due to difficulty in 'lining up' the sneeze when powerful reflex movements began, so the experiment was repeated with a hand spray specially made for us by Dr B. M. Wright. This device produced a rather coarse spray of droplets which resembled in size distribution that formed during a sneeze. As can be seen from Table 3 the results were the same as those of a sneeze.

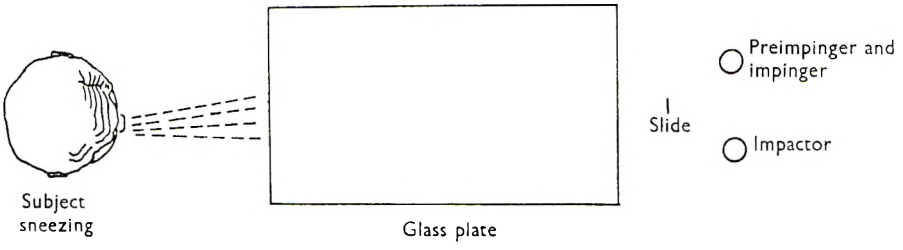


Fig. 5. Scale drawing of a subject sneezing in an open room, showing the position of the horizontal glass plate, the vertical slide and the impactor and the pre-impinger and impinger samplers. (Scale 1:12.)

Table 4. *Rate of dispersal of sneezed material in a quiet room*

Time of collection in minutes from time of sneeze	Spores recovered at indicated times		
	Horizontal plate	Vertical slide	Impinger
0–¼	1,800,000	30	1,350*
¼–½	2,800	0	90
½–¾	600	0	518
¾–1	5,000	0	330

* The neck of the impinger was not washed out.

We concluded that just after a sneeze only a tiny fraction of the expelled material would remain airborne in droplets near the patient, but that over half of these might be of a size which could be trapped in the upper respiratory tract.

We attempted to detect spores by swabbing the nose and conjunctiva after opening the eyes in or inhaling a droplet suspension produced by the spraying device. Small numbers of spores were recovered and we attempted to extrapolate from these to the number picked up, using the known clearance rates to account for the number which had disappeared. The results were unfortunately too erratic to be significant. The results obtained in the model system seemed to be a much more satisfactory way of estimating the uptake.

DISCUSSION

It has long been known that the mucociliary blanket clears particulate material from the nose (Proetz, 1953). Much early work on the disappearance of bacteria from the nose is difficult to interpret because some organisms might have been inactivated by lysozyme, but it is generally believed that bacteria and viruses would be treated like inert particles. Our results form, therefore, only a small addition to the many facts about the nasal mucous membranes which have been summarized by writers such as Rivera (1962) and Negus (1958). We measured the rate at which the normal nasal mucociliary blanket removes spores and bacteriophage deposited in the nose and transfers them to the pharynx; in a patient with a cold the rate might be greatly modified by increases in the amount or viscosity of nasal secretion and damage to the cilia, but virus formed primarily in the nose would probably be found in high concentrations in the pharynx. Because of the flow of saliva and the efficiency of the mechanism of swallowing it seemed that virus being formed in or passing through the pharynx would usually appear in the saliva in low concentrations.

The dispersal of bacteria and secretions from the respiratory tract has been studied since the end of the last century, and it has been shown that although coughing and talking may disperse some droplets from the respiratory tract, sneezing produces many more, and that most of these droplets originate from the mouth. They range in size from coarse droplets which fall rapidly to the floor to fine ones which dry off while airborne and form droplet nuclei of Wells (1956). It is widely believed that bacteria which are present in the mouth become airborne directly, but that others such as streptococci and staphylococci contaminate the skin, or fabrics and then dry off and become airborne later. The number of coarse droplets bearing bacteria which are produced and the direction in which they travel varies with the activity which produces them (Hare & MacKenzie, 1946). It is also known that bacteria found in air are usually attached to particles 4–20 μ in diameter (Noble, Lidwell & Kingston, 1963) whether they are believed to have been formed directly from expelled secretions or resuspended after drying. All this and much other excellent research is summarized by Duguid (1945) and in reviews such as those by Williams (1960), and Hare (1964), but we felt that tracer experiments specifically designed to study the fate of materials arriving in or being produced by the nose would be helpful in interpreting the epidemiology of colds, the agents of which are readily destroyed by drying and can only be efficiently transmitted experimentally if infectious material is put up the nose (Lovelock *et al.* 1952); in addition virus is usually found in the nose and throat of patients with colds and rarely in their saliva.

The present experiments suggest to us that since virus particles, such as a bacteriophage, which have no affinity for human cells, are rapidly cleared from the nose infectious viruses must rapidly attach themselves to cells or perhaps to cilia because only a few tissue culture infectious doses can infect when given as a small intranasal drop or aerosol.

The artificial sneezes were probably comparable with those occurring naturally

during a cold, but direct experiments on subjects with colds will be needed to show whether the amount of infectious secretion shed, and the proportion of virus carried in small particles and coarse droplets are the same as those found in our model experiments. If our analysis is correct, saliva is likely to contain very little of the virus produced in the nose and throat: the number of droplets of saliva in the air and the salivary bacteria therein will therefore have only an indirect relationship to the transmission of colds.

Organisms which have been resuspended after drying apparently play a large part in the transmission of bacterial infections of the respiratory tract. It remains to be seen whether infectious virus particles can be dispersed in the same way. Volunteers do not catch colds from living in rooms in which others with colds have spent several hours (Lovelock *et al.* 1952), but colds are transmitted from person to person living in the same house. Therefore the airborne droplets of nasal secretions produced by sneezing or blowing the nose are likely to be the main route of transmission of viruses, although virus will probably not remain infectious in them very long. These droplets are relatively speaking so few in number that it is not surprising that colds are, on the average, transmitted relatively ineffectively, even within a household (Badger *et al.* 1953; Lidwell & Sommerville, 1951).

SUMMARY

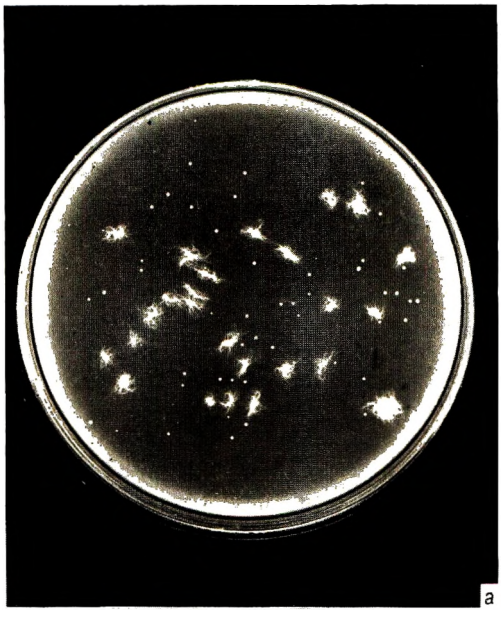
An attempt has been made to study quantitatively the mechanisms by which infectious materials may enter the body by the upper respiratory tract and be shed during a cold.

The rapid clearance of tracers, spores of *B. mycoides* and bacteriophage type T 3, has been measured after adding them in small drops to the nose, conjunctiva and mouth.

Tracers placed in the nose pass rapidly down the throat, but are found in only small amounts in the saliva. They are dispersed by blowing the nose and, more efficiently, by sneezing. Nearly all are shed as coarse droplets. About 0·1 % are shed in droplets small enough to remain airborne and just over half of these are in the size range likely to be trapped in the upper respiratory tract. The droplets are apparently formed mainly in the nose. The larger amounts of droplets formed in the mouth carried relatively few infectious particles.

Testing by experiments in a model system it was concluded that most of the coarse droplets produced by a sneeze or by an experimental spray fall rapidly to the floor. A few of the larger droplets were trapped on a moistened microscope slide intended to mimic the possible trapping of droplets on the conjunctiva. The spores in these represented only a small proportion of those found in droplets which remained airborne and were collected by an air sampler.

We wish to thank Miss P. D. Ball for technical assistance and Dr J. E. Lovelock for helpful discussion in the early stages of this work. We are also grateful to Sir Victor Negus and Dr B. Wyke for discussing the physiology of the nose with us, and to Mr K. R. May for his advice on methods of air sampling. The air samplers were loaned by the Microbiological Research Establishment, Porton, and the photographs taken by the Chemical Defence Experimental Establishment, Porton.



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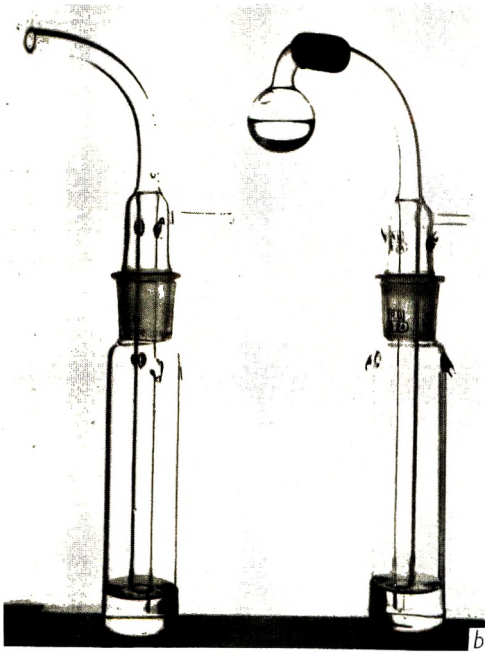

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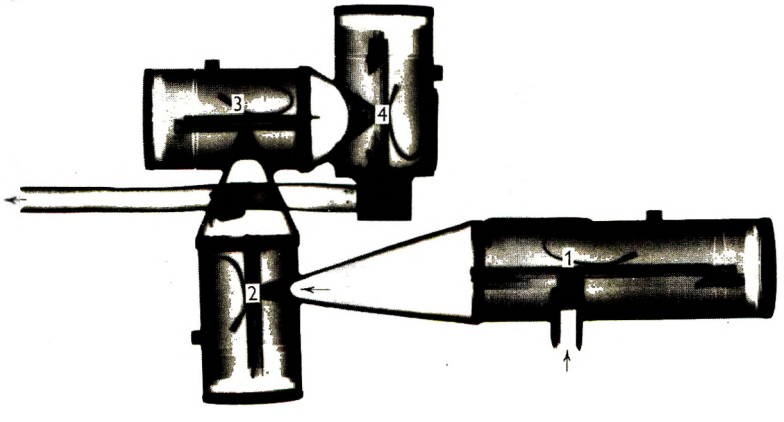

(*c*)

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EXPLANATION OF PLATE 1

(*a*) A plate showing *Bacillus mycoides* colonies growing up from a mixture of spore suspension and room dust.

(*b*) Photograph of a Porton impinger with and without an attached pre-impinger.

(*c*) Hard X-ray photograph of a cascade impactor. The arrows show the route by which air is drawn into and out of the apparatus. The numbers indicate the position of the four slides and the springs which hold them in place.

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Simultaneous active and passive immunization of guinea-pigs against tetanus*

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Antitoxic horse serum when injected into man may induce hypersensitivity reactions to the horse serum proteins. Even though obvious reactions do not occur, the patient may become sensitized so that a reaction develops when antitoxin is given on a subsequent occasion. Furthermore, antibodies against horse serum may arise, which can cause antitoxin injected subsequently to be eliminated from the body too rapidly to be of prophylactic or therapeutic value. Injection of horse antitoxin may therefore leave the patient at a therapeutically induced disadvantage, the dangers of which can largely be overcome by ensuring that patients who receive horse serum are actively immunized against those diseases for which horse serum may be used. Such a procedure is also to be recommended since a person who has been actively immunized is more effectively protected than one who is passively protected at the time of infection.

The main diseases of man in which antitoxin is used are diphtheria and tetanus and, as a result of diphtheria immunization campaigns, tetanus prophylaxis is now the common reason for injecting horse serum. It is therefore considered that all who receive tetanus antitoxin in the form of horse serum should also be actively immunized against tetanus. This is most conveniently achieved by beginning the course of tetanus toxoid at the same time as the antitoxin is given.

Simultaneous active and passive immunization against tetanus has not been regularly practised because the antitoxin may interfere with the active response (Cooke & Jones, 1943; Barr & Sachs, 1955; Prudovsky & Turner, 1958; Philipson, 1959). On the other hand, evidence is available that interference is less evident when toxoid containing an adjuvant is used (Wolters & Dehmel, 1937; Gold & Bachers, 1943; Ericsson, 1948). The studies of Uhr & Baumann (1961), on diphtheria immunization in guinea-pigs, suggest that although interference with the immediate antitoxin response may occur when antitoxin is given with toxoid, nevertheless, a basal immunity develops which enables a booster response to be induced by a later dose of toxoid. Indeed simultaneous active and passive immunization using adsorbed toxoid is successfully practised for the protection of children exposed to diphtheria (Fulton, Wells, Taylor & Wilson, 1941).

It was therefore considered that simultaneous immunization against tetanus would probably be effective if toxoid containing adjuvant was used, and that, even though some interference might occur, the active response would probably

* Some of the results in this paper were first presented at an International Conference on Tetanus held in Bombay in November 1963.

be sufficient to provide acceptable levels of circulating antitoxin. Consequently a trial of simultaneous active and passive immunization against tetanus was made in man and the results, combined with those of another group, have already been reported (Smith, Evans, Jones, Gear, Cunliffe & Barr, 1963). In conjunction with these field studies, laboratory experiments were made using guinea-pigs in order to examine the response to simultaneous immunization carried out in different ways. The experimental results are reported here and the findings related to those obtained in man.

MATERIALS AND METHODS

Antitoxin and toxoid

Tetanus antitoxin (Wellcome) containing 1500 units per ml. was used. A subcutaneous injection of 2·0 ml. of a 1/10 dilution in normal saline was given into the shaved lateral aspect of the left thigh of guinea-pigs. The dose was therefore 300 units, i.e. 1/5th of the usual human dose.

Plain tetanus toxoid (Wellcome) containing 40 Lf/ml.was used. The dose given was 1 ml. of a 1/10 dilution in normal saline, i.e. 1/5th of the human dose, and was injected subcutaneously into a shaved area of the right anterior quadrant of the abdomen.

The aluminium hydroxide adsorbed tetanus toxoid (Wellcome) used also contained 40 Lf/ml. and 1·8 mg. of aluminium per ml. It was given in the same dilution and by the same route as the plain toxoid.

Tetanus antitoxin titrations were performed by the method of Glenny & Stevens (1938) and the results recorded in international units per ml. of serum. The smallest amount of antitoxin which was tested for was 0·01 units per ml.

Experimental animals

Female albino guinea-pigs were used and at the time of active immunization were approximately 6 weeks old, weighing between 300 and 400 g. They were housed five to a cage and fed on guinea-pig pellet food supplemented by cabbage daily.

Blood samples of 2·0 ml. were obtained from the guinea-pigs by cardiac puncture under ether anaesthesia, and the serum was separated after overnight storage at 4° C.

Immunization procedure

The guinea-pigs were randomly divided into six groups (A to F) each of fifteen animals. Serum samples were obtained from three animals of each group before immunization and none was found to contain detectable tetanus antitoxin. Toxoid was given in three doses spaced as in the schedule used in Britain for the immunization of children, the second dose being given 6 weeks after the first and the third dose 6 months after the second. Serum samples were taken 1 day before and 2 weeks after both the second and third doses of toxoid.

Group A was immunized with plain toxoid and group B with adsorbed toxoid. Group C was immunized with plain toxoid and group D with adsorbed toxoid, but in these two groups the first injection was accompanied by a dose of tetanus antitoxin given into a different site as described above, i.e. simultaneous active and passive immunization was employed. In all groups, however, the third dose of toxoid, given 6 months after the second, was plain toxoid.

A fifth group, E, was immunized both actively and passively using plain toxoid, but in this group the injection of antitoxin was given 24 hr. after the toxoid. The sixth group, F, was given simultaneous active and passive immunization using adsorbed toxoid but, in addition, the guinea-pigs received an injection of tetanus antitoxin 3 weeks before the simultaneous immunization, i.e. when they were 3 weeks old.

The immunization régimes employed are summarized in Table 1.

Table 1. *Immunization régimes employed in six groups each of fifteen guinea-pigs*

Group	Preliminary procedure	1st injection	2nd injection (6 weeks after 1st)	3rd injection (6 months after 2nd)
A	None	Plain toxoid	Plain toxoid	Plain toxoid
B	None	Adsorbed toxoid	Adsorbed toxoid	Plain toxoid
C	None	Plain toxoid and simultaneous antitoxin	Plain toxoid	Plain toxoid
D	None	Adsorbed toxoid and simultaneous antitoxin	Adsorbed toxoid	Plain toxoid
E	None	Plain toxoid and antitoxin 24 hr. later	Plain toxoid	Plain toxoid
F	Antitoxin 3 weeks before 1st toxoid injection	Adsorbed toxoid and simultaneous antitoxin	Adsorbed toxoid	Plain toxoid

RESULTS

Response to the first and second doses of tetanus toxoid

Table 2 shows the response of the guinea-pigs of groups A to D to the first and second doses of toxoid. These responses are plotted in Fig. 1 which also shows the responses obtained to the third dose of toxoid.

The response to immunization with adsorbed toxoid alone (group B) was considerably better than with plain toxoid (group A), the difference in response being approximately 60-fold after the first dose of antigen and 7-fold after the second. Furthermore, four of the guinea-pigs failed to show a response to the first dose of plain toxoid, whereas all the guinea-pigs immunized with adsorbed toxoid produced more than 0·2 units of antitoxin.

The antitoxin response to simultaneous active and passive immunization using the plain toxoid (group C) was very poor, thirteen of fourteen animals having less than 0·01 units of antitoxin 6 weeks after the first injection. On the other hand, when adsorbed toxoid was used (Group D) only three of twelve animals failed to

Table 2. *Tetanus antitoxin titres in guinea-pigs after receiving the different immunization régimes shown in Table* 1

Group	Serum sample taken after	No. of sera with antitoxin titres of (units per ml.)														No. of sera tested	Geometric mean titre
		< 0·01	0·01–	0·02–	0·05–	0·1–	0·2–	0·5–	1·0–	2·0–	5·0–	10–	20–	50–	100–200		
A	1st injection	4	1	4	2	1	1	—	—	—	—	—	—	—	—	13	0·03
	2nd injection	—	—	—	—	—	—	—	2	—	1	4	3	—	—	10	11·0
B	1st injection	—	—	—	—	—	1	3	—	10	—	—	—	—	—	14	2·0
	2nd injection	—	—	—	—	—	—	—	—	—	—	1	1	2	5	9	79·0
C	1st injection	13	1	—	—	—	—	—	—	—	—	—	—	—	—	14	< 0·01
	2nd injection	3	—	1	—	5	3	—	1	—	—	—	—	—	—	13	0·06
D	1st injection	3	1	1	—	1	1	4	1	—	—	—	—	—	—	12	0·12
	2nd injection	—	—	—	—	—	1	1	—	—	3	—	6	1	2	14	17·0

Serum samples taken 6 weeks after the 1st injection and 2 weeks after the 2nd injection of toxoid.

respond to the first dose. Two weeks after the second dose of toxoid three of the animals given plain toxoid still showed no response, whereas all the animals given adsorbed toxoid had more than 0·2 units of antitoxin. Furthermore, it may be seen that the response of guinea-pigs to simultaneous active and passive immunization using adsorbed toxoid (group D) was greater than that to immunization with plain toxoid given without any antitoxin (group A).

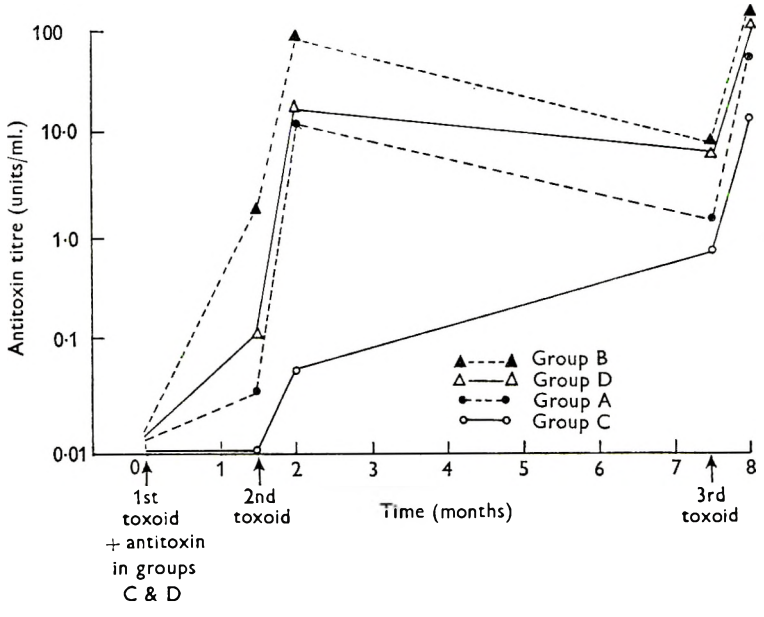

Fig. 1. Geometric mean tetanus antitoxin titres in guinea-pigs. Group A, plain toxoid; group B, adsorbed toxoid; group C, simultaneously, plain toxoid and antitoxin; group D, simultaneously, adsorbed toxoid and antitoxin.

Antitoxin titres 6 months after primary immunization and the response to the third dose of toxoid

The geometric mean antitoxin titres found when the guinea-pigs were bled 6 months after the second dose of antigen and the titres produced in response to a third dose of toxoid, are shown in Fig. 1.

It can be seen that the effect of interference by antitoxin was less evident at this later stage. The two groups in which adsorbed toxoid was used (groups B and D) had similar antitoxin titres 6 months after the second injection, and they showed a similar response to the booster of plain toxoid, even though in the early stages of immunization the group which also had antitoxin (group D) showed definite evidence of interference.

The animals of group C, which had simultaneous active and passive immunization using plain toxoid, responded well to the booster injection, but the effect of interference was still evident as the mean antitoxin titre was lower than in group A which had no passive antitoxin.

The guinea-pigs given simultaneous active and passive immunization with plain toxoid (group C and also group E shown in Fig. 2) had a higher antitoxin titre

at 6 months than at 2 weeks after the second injection of toxoid. Furthermore, the groups receiving simultaneous active and passive immunization with adsorbed toxoid (group D and also group F shown in Fig. 3) showed a slower decline in antitoxin titres after the second dose of antigen than guinea-pigs not given antitoxin (group B). It appears that the passive antitoxin not only reduced the antibody response to toxoid, but also caused the response to be delayed. A similar delaying effect has also been observed in diphtheria immunization both in guinea-pigs (Mason, Robinson & Christensen, 1955) and in man (Downie, Glenny, Parish, Smith & Wilson, 1941) and also in tetanus immunization in man (Tasman & Huygen, 1962).

The effect of delaying the injection of antitoxin for 24 *hr.*

Group E was included to test whether interference with plain toxoid could be avoided by delaying the antitoxin injection, so that the toxoid could have access to the antibody-forming cells for 24 hr. unhampered by antitoxin. Such a delay

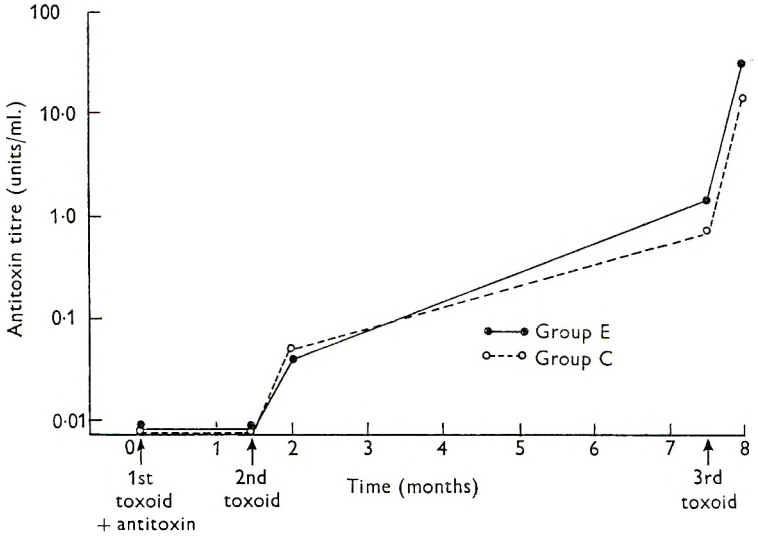

Fig. 2. Geometric mean tetanus antitoxin titres in guinea-pigs. Group C, simultaneously, plain toxoid and antitoxin; group E, plain toxoid and antitoxin 24 hr. later.

might be effective if interference is due to an afferent inhibition of the immunological response (Billingham, Brent & Medawar, 1956). The results obtained in group E are shown, together with those of group C, in Fig. 2.

It can be seen that the antitoxin response of group E was not appreciably different from that of group C, in which the antitoxin injection was not delayed.

The effect of an earlier injection of passive antitoxin

Group F was included to examine the possibility that simultaneous active and passive immunization might fail in animals already sensitized to horse serum, owing to the 'crowding out' effect of a secondary response to the horse serum (Glenny, Hopkins & Waddington, 1925).

The results obtained in group F are shown in Fig. 3 together with those of group D, which had no previous experience with horse serum. When adsorbed toxoid was used, instead of finding evidence of 'crowding-out', the responses, 6 weeks after the simultaneous immunization, were 10 times higher than in guinea-pigs with no previous experience of horse serum. This difference is highly significant ($P = < 0{\cdot}005$).

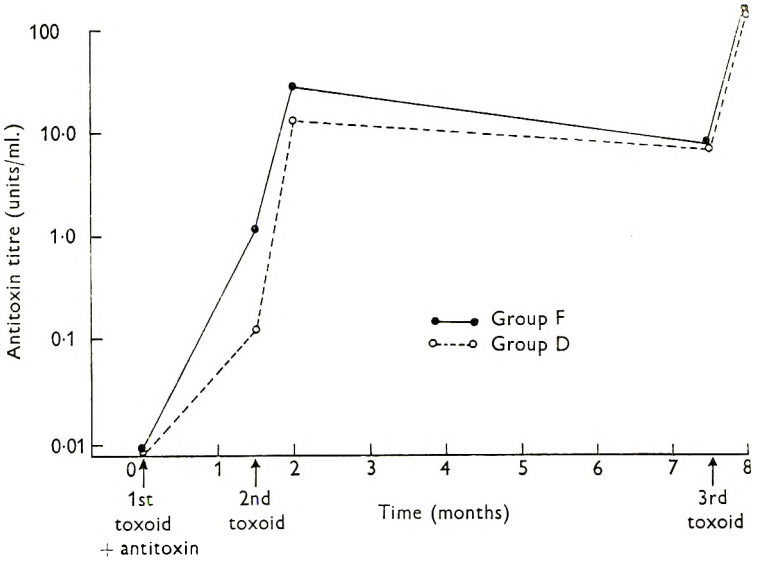

Fig. 3. Geometric mean tetanus antitoxin titres in guinea-pigs given, simultaneously, adsorbed toxoid and antitoxin. Group D, not sensitized to horse serum; group F, sensitized to horse serum by an injection 3 weeks earlier.

DISCUSSION

Whilst a direct comparison of results obtained in man and in experimental animals cannot be made, it is probably justifiable to use the results obtained in animals as a guide to what might be expected of an immunization régime in man. It is in an attempt to relate the laboratory to the field work that the results obtained in simultaneously immunized guinea-pigs are presented in Fig. 4, together with those obtained from simultaneous immunization with adsorbed toxoid in eighty-two patients (Smith *et al.* 1963). The titres shown are those found 2 weeks after the second dose of toxoid in both guinea-pigs and man.

Before making any comparison two points should be considered. First, the doses of toxoid and of antitoxin used in guinea-pigs, although given in the same relative proportions as in man, must be considered large in relation to the body weight of the animals. The doses were chosen to provide circulating passive antitoxin for a time similar to that in human patients given 1500 units (i.e. approximately 20 units per kg. body weight), as it was thought that the duration of passive immunity might be an important factor in determining the degree of interference. In preliminary experiments it was found necessary to give guinea-pigs 1000 units per kg. body weight to provide antitoxin levels just detectable two weeks later.

It appears that in guinea-pigs, and in rabbits, horse antitoxin is eliminated from the circulation more rapidly than is usually the case in man (Madsen, 1936; Suri & Rubbo, 1961).

The second point is the fact that in the clinical study, patients in whom antitoxin was detectable before the second dose of toxoid was given were excluded from

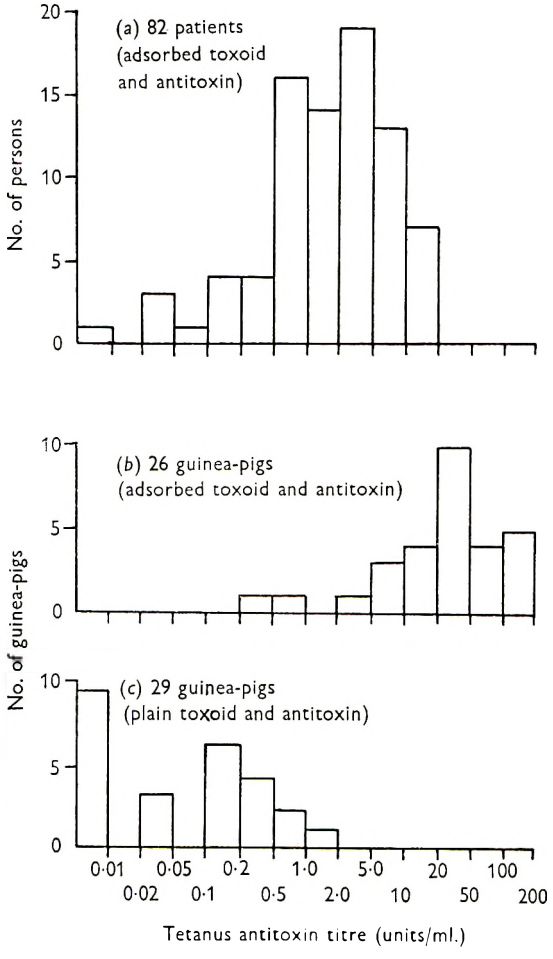

Fig. 4. Antitoxin titres 2 weeks after the second dose of toxoid in: (*a*) eighty-two patients immunized with adsorbed toxoid and antitoxin; (*b*) twenty-six guinea-pigs immunized with adsorbed toxoid and antitoxin, groups D and F; (*c*) twenty-nine guinea-pigs immunized with plain toxoid and antitoxin, groups C and E.

analysis. This was done to ensure that all previously immunized persons were excluded, but it is likely that some non-immunized persons were also excluded. Furthermore, the proportion of non-immunized patients excluded may have been high owing to the possibility, suggested by the results obtained in guinea-pigs (see Fig. 3), that patients previously injected with horse serum can respond particularly well to simultaneous immunization.

With these points in mind, the results obtained in guinea-pigs suggest that the response of man to simultaneous immunization using plain toxoid is likely to be

markedly inferior to the response using adsorbed toxoid. Thus if the same relative response is found in man as occurs in guinea-pigs, the use of the plain toxoid for simultaneous immunization in man is likely to yield titres of only 0·005–0·006 units per ml., 2 weeks after the second injection of antigen.

The deficiencies of plain toxoid for simultaneous immunization are most evident early in the course of immunization. After a third dose of toxoid, which should be considered an integral part of the tetanus immunization régime, the difference in responses obtained with plain and with adsorbed toxoid became less marked. In man therefore plain toxoid might produce satisfactory antitoxin titres after injection of the third dose. Nevertheless, it is considered that adsorbed toxoid is preferable for simultaneous immunization, since it should lead to the earlier development of immunity and to higher antitoxin titres after the third injection. Furthermore, patients who fail to return for either the third or second injection are more likely to be effectively immunized if they have received adsorbed toxoid.

A number of workers have examined the possibility that interference may be overcome by delaying the injection of antitoxin. Glenny, Buttle & Stevens (1931) reported that diphtheria antitoxin would cause interference when given to guinea-pigs 3 days after they had received diphtheria plain toxoid, and Uhr & Baumann (1961) found that interference occurred even when a 5-day interval was employed. On the other hand, Moloney & Fraser (1929) obtained no evidence of interference when antitoxin was given 2 hr. after toxoid, although interference occurred when the interval was 10 min. In the experiments reported here, tetanus antitoxin given to guinea-pigs 24 hr. after plain tetanus toxoid had no advantage over giving the antitoxin and toxoid at the same time.

The work of Glenny *et al.* (1925) and of Barr & Llewellyn-Jones (1953) suggests that, when two protein antigens are injected together, the antibody response to one antigen may be 'crowded-out' if the animal is in a state of secondary responsiveness to the other antigen. In the experiments reported here it was found that in guinea-pigs sensitized to horse serum, simultaneous active and passive immunization produced better responses than in guinea-pigs encountering horse serum for the first time. It therefore appears that, when one of the competing antigens (tetanus antitoxin) is an antibody to the second (tetanus toxoid), then a booster response to the first antigen can prevent it, to a large extent, from interfering with the second antigen. Whilst the mechanism responsible deserves investigation, the finding suggests that simultaneous active and passive immunization in man is unlikely to fail in patients who have previously received horse serum.

SUMMARY

1. Simultaneous active and passive immunization of guinea-pigs using tetanus toxoid adsorbed on to aluminium hydroxide produced a higher antitoxin response than simultaneous immunization using plain toxoid. The response was also better than that produced by plain toxoid alone.

2. A 24 hr. interval between the injection of plain toxoid and the subsequent injection of antitoxin gave no better results than when toxoid and antitoxin were given at the same time.

3. The response to simultaneous immunization, using adsorbed toxoid, of guinea-pigs sensitized to horse serum, was superior in the first 6 weeks to that of guinea-pigs which had not been sensitized.

4. The suggestion that simultaneous active and passive immunization of man should be performed with adsorbed toxoid rather than plain toxoid is supported by these results.

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The toxicity of 0·025% warfarin to wild house-mice (*Mus musculus* L.)*

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INTRODUCTION

The most extensively used rodenticide in Britain over the last 10 years has been warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin]. During this time a considerable amount of information has been accumulated, both in the laboratory and in the field, on its toxicity to rats, and in particular to *Rattus norvegicus* Berk. In contrast, the toxicity of warfarin to the wild house-mouse (*Mus musculus* L.) has been less well investigated. However, recent improvements in rat control methods have resulted in the house-mouse supplanting the rat as the major pest in some habitats. Furthermore, in the last 2 years there have been reports of some house-mouse populations proving extremely difficult to kill with warfarin. For these reasons a laboratory investigation of the toxicity of warfarin to mice was undertaken and the results of this are described below.

MATERIALS AND METHODS

The mice used in the tests were drawn from thirteen different localities. The majority were hand-caught in corn-ricks at threshing time; only ricks in which no warfarin treatment had been carried out were used. After capture, the mice were kept in metal pens, 6 ft. square, erected in an unheated building. Animals from the same source were penned together in colonies of fifty or less. They were given excess whole wheat and water, and hay for nesting material, and were provided with wooden nest-covers.

At least 2 weeks before a test, each mouse was put individually in a metal cage (14 in. × 11 in. × 6 in.) containing a metal food-pot, a water bottle and a wooden nesting box (3 in. × 1 in. × 1 in.). During this period it was supplied with a diet of mixed whole wheat and pinhead oatmeal and with water *ad lib.*

The method employed in all tests was to offer each mouse excess amounts of a bait containing 0·025% warfarin for a fixed number of days. This warfarin concentration is normally used against *Mus musculus* in Britain and is based on early work carried out in the United States of America. The poison was dispersed thoroughly in fine oatmeal to give a master-mix containing 0·5% warfarin. One part of the master-mix was then added to nineteen parts of a bait-base consisting of pinhead oatmeal, castor sugar and a technical grade 'white oil' in the proportions

by weight of 17:1:1, respectively. Before poison was administered the mice were fed on the plain bait-base for 4 days. The total amount of bait-base eaten in this period was recorded, paper placed in the tray underneath each cage facilitating the collection of spilled food. Animals that failed to eat adequately during this period were discarded. Wherever possible an equal number of each sex and animals of comparable weight were chosen for the toxicity tests. None of the females was pregnant. In a majority of the tests the amount of warfarin bait eaten was recorded daily and the food pots were replenished each day with fresh poison bait. Occasionally in tests exceeding 14 days, the poison bait was left unchanged for a single 2- or 3-day period. The time of death and weight of mice that died was recorded and the dead animals were autopsied. Mice still alive at the conclusion of the test period were given a wheat/oatmeal diet until 30 days had elapsed from the beginning of the test. Animals still surviving at the end of this period were killed, weighed and autopsied.

RESULTS

The results of the toxicity tests are summarized in Table 1. In all, 345 mice were tested over feeding periods ranging from 4 to 28 days. The data show that there was considerable variation in the susceptibility of individual mice to warfarin at 0·025 %. The lowest fatal dosage was 26·1 mg./kg. of body weight. The animal concerned, a male, which weighed 13·4 g., ate only 1·4 g. of 0·025 % warfarin bait and died with normal warfarin symptoms on the fourth day of a specified 6-day test period. At the other extreme, a female weighing 11·9 g. survived the equivalent of 1067·2 mg./kg. of body weight after eating 50·8 g. of 0·025 % warfarin bait over 21 days. It is noteworthy that a complete kill was not obtained in any feeding test of less than 18 days' duration and five mice fed for 21 days survived total dosages of 666·7, 671·9, 689·0, 790·2 and 1067·2 mg./kg., respectively. These five animals were taken from three different localities, as also were the four that survived 14 days. The time required to kill the first mouse in each test was similar—between 2 and 4 days. Not unexpectedly, because of the mode of action of warfarin,

Table 1. *Mortality of* Mus musculus *after unrestricted feeding on bait containing* 0·025 % *warfarin for a limited number of days*

No. of days feeding	Mortality	Total dosage range that killed (mg./kg.)	Average total lethal dose (mg./kg.)	Highest total dose survived (mg./kg.)	Range of days until death
4	6/30	90·4–152·9	120·4	189·8	4–23
5	16/35	113·3–251·8	167·7	220·0	3–30
6	23/33	26·1–303·6	157·0	322·0	3–10
7	36/46	41·0–439·9	190·6	319·6	3–13
8	35/41	67·3–253·6	164·0	329·0	4–14
10	31/37	68·3–471·6	192·2	557·5	4–12
14	41/45	106·2–571·4	248·2	642·2	4–30
18	12/12	68·2–307·2	174·7	—	2–17
21	48/53	104·7–805·7	268·6	1067·2	3–20
28	13/13	82·4–238·1	164·4	—	4–10

there was considerable variation in the time to death of mice in the same test; for example, in a 5-day test the first death occurred on day 3 and the last on day 30. The lethal feeding period corresponding to a 95% kill (LFP 95) with 0·025% warfarin, calculated from a regression line obtained by transforming the data into probit mortality and log(days − 3·85), was about 22 days.

Table 2. *Mortality of* Mus musculus *taken from one rick site after unrestricted feeding on bait containing* 0·025% *warfarin*

No. of days feeding	Mortality	Total dosage range that killed (mg./kg.)	Average total lethal dose (mg./kg.)	Highest total dose survived (mg./kg.)	Range of days until death
4	3/11	115·2–152·9	128·0	158·7	5–23
5	7/14	118·0–184·7	151·5	220·0	3–7
6	8/14	26·1–181·8	120·7	322·0	3–8
7	10/14	92·8–199·2	146·3	218·2	3–8
8	12/14	67·3–212·8	147·8	287·6	4–11
10	10/12	110·4–206·5	152·2	250·0	4–9
14	12/14	106·2–494·2	256·2	642·2	5–30
18	12/12	68·2–307·2	174·7	—	2–17
21	18/20	104·7–545·1	242·1	671·9	4–18
28	13/13	82·4–238·1	164·4	—	4–10

Table 3. *The mortality of male and female house-mice in each of four weight classes after unrestricted feeding on* 0·025% *warfarin for periods of from* 4 *to* 28 *days*

Weight class (g.)							
10·0		10·1–15·0		15·1–20·0		20·1	
M	F	M	F	M	F	M	F
3/3	10/11	59/70	52/72	55/71	60/80	14/18	8/20

Examination of the toxicity data indicated no obvious difference in the susceptibility of mice from different localities, but some of the samples were small. By far the largest sample (138 mice) came from three wheat ricks standing on one site. The data for these animals have been abstracted from Table 1 and are set out in Table 2. The lowest lethal dose for the sample was 26·1 mg./kg. of body weight spread over 4 days (see above), and the highest dose survived by any animal was 671·9 mg./kg. spread over 21 days. This mouse, a female, which weighed 11·2 g., ate 30·1 g. of 0·025% warfarin bait. One other female survived a feeding period of 21 days.

The data are indicative of a slight difference in susceptibility between the sexes. Of 211 animals (92 males, 109 females) tested over 8 days or longer (Table 1) five males survived (5·4%) compared with sixteen females (14·7%) [$\chi^2 = 3{\cdot}62$; $P = 0{\cdot}05$–$0{\cdot}1$]. In all but two of the test periods (18 and 28 days, in which complete kills were obtained), the average time to die of animals killed by warfarin was longer for females than for males. The greatest difference occurred in the two shortest feeding periods (4 and 5 days), the figures for males being 4·5 and 5·8 days,

and for females 10·8 and 8·9 days, respectively. There was little obvious difference in susceptibility based on weight. Females weighing 20·0 g. or more appeared to be the most difficult to kill (Table 3) but the sample was small and the range in weight of mice surviving 8 days feeding or longer was considerable (11·6–19·1 g. for males and 10·0–21·9 g. for females).

Most of the survivors showed signs of illness at some time during the test; some produced bloody droppings and ate little or no poison bait for a day or two, but then subsequently recovered and ate normally. The bait consumption of each of the five females that survived 21 days feeding was in fact as great at the end of the test period as it had been at the beginning.

The examination of animals killed by warfarin poisoning showed that haemorrhages occurred at several sites in the body and were most common in the thoracic cavity (51·4 %), digestive organs (24·4 %), central nervous system (18·2 %), lungs (15·4 %) and abdominal cavity (8·4 %). The decrease in body weight in some cases amounted to over 40 %.

DISCUSSION

There is little published information available on the toxicity of warfarin to wild mice. Most of the early work in the United States of America involved offering mice either 0·025 % warfarin bait till death or a choice of warfarin bait and a laboratory diet. A straightforward comparison with the toxicity data of these experiments is therefore impossible. Bonnet, Mau & Gross (1951) fed 0·025 % warfarin bait to fifteen mice till death; fourteen died by the 13th day and the last animal died on the 17th day (mean 8·6, S.E. ± 1·0 days). Hayes & Gaines (1959) offered batches of individually caged mice baits containing warfarin of varying concentrations (between 0·0003 and 0·08 %) together with poison-free food for a maximum of 40 days. They concluded that some individuals were extremely susceptible to warfarin but that there was considerable variation in response. Giban (1958) found that the proportion of small rodents (including house-mice) surviving periods of 0·025 % warfarin bait in tests lasting 30 days amounted to between 10 and 20 %. The data from the present study support these findings; some animals succumbed readily to warfarin, whereas others survived substantial amounts of the poison.

From the control standpoint, the most significant aspect of the results of the present tests is the extent to which some mice were able to survive large doses of poison. While it is probably true to say that free-living mice would be more active than caged animals and might as a result be more easily killed by warfarin, it must be remembered that the animals under test had no choice of food. This kind of situation seldom occurs in the field, where the presence of alternative foods may make it difficult to induce mice to feed regularly at baiting points: and tests by Spencer (1950) indicate that the survival of wild mice is increased considerably if gaps of two or more days occur between periods on warfarin bait. The duration of warfarin treatments against mouse populations then will depend mainly on the susceptibility of the populations to warfarin and on their feeding behaviour. Populations fairly susceptible to warfarin poisoning and readily drawn to baiting

points will probably be controlled within one week, whereas a considerably longer time will undoubtedly be required for the eradication of populations more tolerant to warfarin and feeding irregularly at the baiting points.

The results of laboratory tests (unpublished) on small numbers of mice taken from premises treated periodically with 0·025 % warfarin over several months have shown that in some cases a higher proportion of animals tolerant to warfarin has been present than would normally be expected from the data presented in Table 1. The most likely explanation of these difficult cases is that the individuals in the original populations most susceptible to 0·025 % warfarin were killed off, leaving the more tolerant animals to reproduce. Certainly, current laboratory investigations indicate that tolerance to warfarin is a heritable trait in *Mus musculus*. Thus it would seem that, apart from the demand on time and labour involved in a lengthy warfarin treatment (of, say, more than 6 weeks), the prolonged use of 0·025 % warfarin to control such populations is of doubtful value.

SUMMARY

1. Individually caged wild house-mice (*Mus musculus* L.) were fed 0·025 % warfarin in a pinhead oatmeal/oil/sugar base. In all, 345 mice were tested for a fixed period ranging between 4 and 28 days.

2. There was considerable variation in the susceptibility of individual mice. The lowest effective dosage was 26·1 mg./kg. of body weight in 4 days and the highest dosage survived was 1067·2 mg./kg. eaten over 21 days.

3. The time required to obtain a 100 % kill of house-mouse populations with 0·025 % warfarin under field conditions is discussed.

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The incidence of the serological groups of *Candida albicans* in southern England

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INTRODUCTION

It is now known that *Candida albicans* as a distinct species can be classified into groups A and B by means of a direct agglutination test with absorbed rabbit antisera (Hasenclever & Mitchell, 1961*a*). Although these workers attempted to employ double diffusion in agar-gel as an alternative method, the results were reported as being not consistent in showing the differences observed with agglutination tests. Nevertheless, double diffusion has demonstrated that two serological groups do in fact exist and that the so-called group A and group B strains as supplied by Dr Hasenclever can be differentiated by this means (Stallybrass, 1964).

An attempt was made by Hasenclever & Mitchell (1963) to correlate the *C. albicans* groups with the site of isolation in 242 patients, although unfortunately many of the sites were listed together and no figures are available for particular sources such as sputum. In one collection of 55 vaginal cultures from an unspecified number of pregnant Negro women 22 belonged to group A and 33 to group B, whereas in another collection of 653 cultures obtained from 242 patients 68% were A and 32% B. As the particular strains of *C. albicans* isolated from the several sources in individual patients may sometimes have been identical, the figures indicate neither the true incidence of group A and group B strains at the site of isolation, nor the total incidence in the 297 patients.

The present paper reports the results of grouping 234 strains of *C. albicans* obtained from 234 patients in southern England during 1962 and 1963, using the agar-gel double diffusion technique.

MATERIALS AND METHODS

Candida albicans *test strains*

These strains were obtained from primary cultures on Sabouraud dextrose medium or blood agar, from patients attending eight hospital centres situated in London, Berkshire, Surrey, and Hampshire. Of the 234 cultures, 48 were from sputa of patients at one hospital alone, 36 were from vaginal swabs collected at a second hospital, and 150 from various sites including sputum and vagina from patients attending six other well-separated hospital centres.

All the strains, after being checked for purity, were tested for the ability to ferment glucose, maltose, lactose and sucrose (3% in peptone water), and to form

chlamydospores on Taschdjian's medium (Taschdjian, 1957). The fermentation tests were read after 1 week at 37° C., while the Taschdjian plates were left at room temperature and examined daily for the development of chlamydospores. Those strains producing acid and gas from glucose and maltose only, and also producing chlamydospores within 5 days were accepted as *C. albicans*. After identification, these strains were maintained at room temperature by monthly subculture from the main growth on Sabouraud dextrose medium containing 20 and 40 μg./ml. respectively of penicillin and streptomycin.

Candida albicans *control strains*

One strain each of group A (no. 207/5/3) and group B (no. 792/5/3) were supplied through the courtesy of Dr Hasenclever.

Antigen preparations

(*a*) *Formamide extracts.* Between 45 and 60 mg. wet weight of *C. albicans* obtained from two 48 hr. slope cultures was extracted by Fuller's (1938) method with 0·5 ml. formamide at 160° C., treated with acid-alcohol and acetone, and the polysaccharide finally dissolved in 2·0 ml. saline at pH 7·6.

(*b*) *Mickle extracts.* The same amount of growth made as a 50 % (v/v) suspension in peptone water was placed in a polythene tube (capacity 5 ml.) containing 75 stainless-steel balls of $\frac{3}{32}$ in. (2·4 mm.) diameter. After shaking for 15 min. in the Mickle disintegrator the contents of the tube were centrifuged and made up to 1 ml. in peptone water and stored frozen overnight. After thawing, the fluid was centrifuged again, when the supernatant was ready for use as the Mickle extract.

Extracts of both types were prepared from the test and control strains.

Antisera

Two rabbits each weighing 3·5 kg. were inoculated intravenously. Rabbit 'X' received five injections of a formalin-killed vaccine of the group A strain supplied by Dr Hasenclever, in doses ranging from 4 to 20 million cells over a period of 139 days. Rabbit 'T' received four injections of a similar vaccine prepared from another group A strain, in doses ranging from 4 to 40 million cells over a period of 36 days. Blood was taken from the marginal vein of the ear on days 39 and 150 from rabbit 'X' and on day 50 from rabbit 'T'. The samples of serum obtained from these bleedings were stored separately at −79° C. until required for use.

No vaccine of a group B strain was used because a group B antiserum will not differentiate the two groups (Stallybrass, 1964).

Double diffusion method

Tests were performed in flat-bottomed Petri-plates ('Anumbra') of 9 cm. diameter, containing 0·8 % ion-agar (Oxoid) in saline with 1 % sodium azide. Wells of 6 mm. diameter were cut and the bases of these were sealed with melted

medium; they were arranged round a central well so that their margins were separated from this by a distance of 5 mm. After filling the peripheral wells with appropriate extracts and the central one with the group A antiserum the plates were incubated at 37° C. and then inspected for precipitin lines after 24 and 48 hr.

Grouping procedure

The strains were differentiated by three separate precipitin tests. The first test was used simply to select extracts for further examination by the second, and in both of these formamide extracts were employed. The third test was intended as a means of checking the results previously obtained, and in this the Mickle extract was used.

Test 1. The formamide extracts were placed in the wells so that a maximum of four extracts were reacting with the group A antiserum. For the whole batch of tests one group A and one group B extract were included to demonstrate the differential pattern (Plate 1*a*). On the basis of this screening test, those extracts appearing to react as group B were selected for further testing.

Test 2. Each test extract was placed in a well immediately adjacent to the control group A extract (Plate 1*b*) and allowed to diffuse against the group A antiserum; the control group B extract was included in each batch. Those extracts which reacted as group B in this second test were recorded and the appropriate strains subjected to the final investigation.

Test 3. Mickle extracts prepared from fresh 48 hr. subcultures of the original strains were employed, and each extract was placed in a peripheral well adjacent to wells containing similar extracts of group A and group B controls. The group A antiserum was as usual placed in the central well, and the plates read after 24 and 48 hr.

RESULTS

Both in the individual hospital centres making up the series and in the series as a whole, group A strains have been found to predominate. Of the 234 cultures of *C. albicans* 75% were of group A and 25% of group B, figures which agree broadly with those of Hasenclever & Mitchell (1963). The figures here refer to isolations obtained from human sources in eight hospital centres, some of which provided specimens from a single site only (e.g. sputum, vagina, skin) while others provided specimens from various sites.

Table 1. *The incidence of* Candida albicans *groups A and B*

Site of isolation	Nos. of centres and hospitals supplying strains	Total isolations	Sputum isolations	Total group A	Total group B	Sputum group B
All	Nos. 1–8	234 (25)	91 (26)	174	60	24
Various	Nos. 1–4, 6, 8	150 (22)	43 (14)	117	33	6
Vagina only	No. 5	36 (25)	—	27	9	—
Sputum only	No. 7	48 (38)	48 (38)	30	18	18

The figures in parentheses represent percentage values for group B strains.

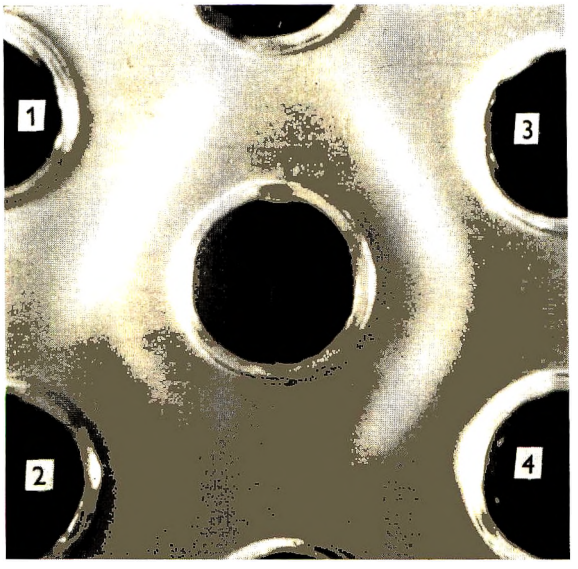
Of 36 isolations from vaginal swabs provided by one hospital, 9 were of group B, figures which contrast with those of Hasenclever & Mitchell (1963) who found group B strains to predominate in Negro women. The significance of race, if any, is as yet unknown. Of 150 isolations obtained from six hospital centres collecting from a variety of specimens 22% were of group B, but of 48 isolations obtained from the sputum of patients under treatment at one hospital 18 (38%) were of group B. These results are summarized in Table 1.

DISCUSSION

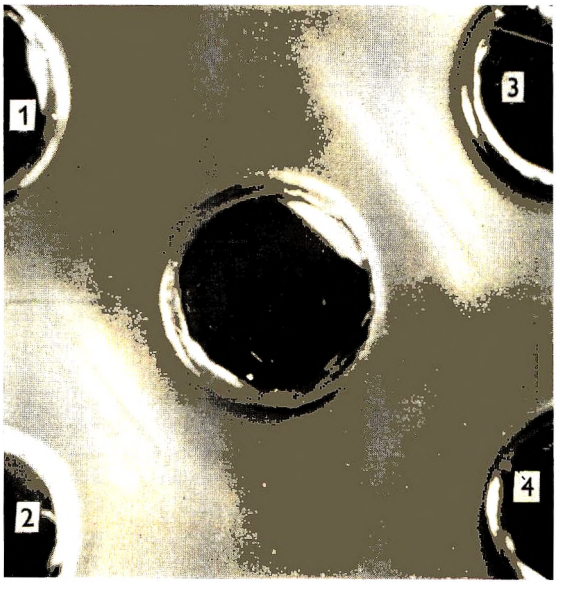
The group characteristics of *C. albicans* appear to be stable within the limits of the experimental conditions outlined in this report. Three group A strains were grown and extracted at monthly intervals for 3 months, and at all times the extracts gave the group A reaction.

The diffusion method itself has proved reliable for differentiating the two groups. One of the group A strains was at first thought to be of group B on the basis of diffusion tests with a formamide extract, but when the Mickle extract was tested the strain reacted as a member of group A, a finding which was confirmed by the preparation and testing of fresh formamide and Mickle extracts. Apart from this single exception, there was complete agreement in the three tests with all strains. It was found that the grouping was more easily achieved by use of the formamide extracts and that the ease with which the strains could be differentiated depended in part on the properties of the group A antiserum employed. In the present series two group A antisera were used and both were satisfactory for the purpose; however, one of them ('T') was found to give sharper differentiation at 24 hr. since the group B precipitin bands were consistently very faint at this time. If grouping is to be performed by one diffusion test only, it is essential to use an antiserum which differentiates clearly.

Little is known of the significance of the two serological groups of *C. albicans*. Apart from the data relating to Negro women all the evidence to date shows that group A strains predominate in man. It seems unreasonable to postulate that strains of the organism can be selected on the basis of their antigenic constitution by the host's tissues at different sites, and it is tentatively suggested that the antigenically deficient group B strains may be attenuated forms produced by environmental factors; these may include prolonged exposure to antibiotics such as tetracycline, or to influences related to survival outside the body in clothing, ward dust, etc. Although it is reasonable to postulate that group B strains may lack a capsular polysaccharide and are avirulent, this has not yet been proved experimentally. No difference between the groups in their virulence for mice was found by Hasenclever & Mitchell (1961*b*), and group B strains certainly proved lethal to rabbits. Unpublished experiments by the present author in which serial blood cultures were taken before and after intradermal inoculation of rabbits with strains of groups A and B have failed to show that group A strains are more invasive than the group B. It would be useful to know if group B strains are ever responsible for systemic candidosis in man.



(*a*)



(*b*)

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Considering all the data available in the present series, no evidence has been found that sputum cultures in particular are associated with a high incidence of group B strains (Table 1). The unusually high incidence of group B in the sputum of long-term hospital patients from No. 7 hospital may be accounted for by a process of cross-infection, but there was in fact no epidemiological evidence to suggest that this cause was operating here. Nevertheless, it is possible that prolonged survival of *C. albicans* in a ward environment may lead to loss of some antigens, so that repeated reinfection with attenuated strains may occur.

SUMMARY

Cultures of *C. albicans* have been classified into two groups by means of a double diffusion precipitin test employing rabbit antisera and polysaccharide extracts. In the present series it has been found that 75 % of cultures belong to group A and 25 % to group B, and it is suggested that the higher incidence of group B strains occurring in the sputum of hospital in-patients with chest disease may be the result of reinfection by attenuated forms which persist in the environment.

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EXPLANATION OF PLATE 1

(*a*) Preliminary screening test of formamide extracts. (1) Group A control, (2) group B control, (3) and (4) tests. The group A antiserum is in the central well.

(*b*) Second test with formamide extracts of suspected group B strains. (1) and (4) test extracts of group B; (2) and (3) control extracts of group A. The group A antiserum is in the central well.