OBSERVATIONS POINTING TO THE CONJUNCTIVA AS THE PORTAL OF ENTRY IN SALMONELLA INFECTION OF GUINEA-PIGS

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(With Plate 5)

The established view that salmonellosis is a food-borne infection has scarcely been questioned in recent years. Where man is concerned, the evidence supporting the alimentary route leaves no room for doubt, although experimental work (McCullough & Eisele, 1951) shows that a large dose of salmonellae is in general required to produce food poisoning, and the possibility remains that some sporadic human salmonella infections are not food-borne. In animals such as mice or guinea-pigs, on the other hand, rapidly spreading epizootics of salmonella infection have been described, and a very much smaller minimal infecting dose of salmonellae than that required to cause food poisoning in man must be postulated to explain the spread of the disease in such outbreaks.

The literature on salmonella infections of guinea-pigs offers no convincing evidence that in this species natural infection can be ascribed to contaminated food. Petrie & O'Brien (1910) reported two very extensive outbreaks of *Salmonella typhi-murium* infection in young stock guinea-pigs at the Lister Institute during the winter of 1909. In one, all the animals in a large pen, numbering nearly 200, died within 3 weeks; in the second outbreak, some 90 % of a herd of 300 guinea-pigs died within 5 weeks. Despite the unusual virulence of the causative strain, experimental infection of healthy guinea-pigs by feeding broth cultures of the organism proved extraordinarily difficult. A subcutaneous dose of 0·001 ml. of broth culture, for instance, was invariably lethal in about 5 days, but even with a massive oral dose of 60 ml., eleven out of twenty test guinea-pigs survived, the remainder dying after 5–15 days. From this difficulty in initiating infection by feeding and on the results of some experiments with spleen filtrates from infected animals, Petrie & O'Brien suggested that a virus was primarily responsible for the two epizootics they described and that *Salm. typhi-murium* was merely a secondary invader; they made the point, however, that a virus aetiology could not reasonably be invoked unless it could be shown that salmonellae alone were incapable of penetrating the outer body defences of healthy guinea-pigs.

Bainbridge & O'Brien (1911), who described two outbreaks of *Salm. enteritidis* infection with high mortality in guinea-pigs, also showed that very large doses of the infecting strain were required to infect healthy guinea-pigs by mouth. A similar difficulty in producing infection by feeding was reported by Howell & Schultz (1922), Raebiger (1923), Okamoto (1926) and Maternowska (1930). The last-named author suggested that infection of guinea-pigs with salmonellae might

occur by the respiratory route as well as by mouth. Smith & Nelson (1927) fed two adult and four recently weaned guinea-pigs through a pipette with doses of 800,000,000 *Salm. typhi-murium*; no disease followed. The older animals excreted the organism in the faeces for about 3 weeks, but no lesions were found in them at autopsy; the salmonella was isolated only from a Peyer's patch of one and from the spleen of the other. In the young guinea-pigs a delayed and intermittent excretion of the salmonella was observed, one animal giving only a single positive faecal specimen; the spleen was culture-positive in three out of four and the intestine in one out of four. Uchida (1926) concluded that infection of guinea-pigs by the alimentary route was almost impossible, and that the deaths claimed by other workers after feeding experiments in guinea-pigs were probably a toxic effect of the large doses given. In his opinion, greatly lowered resistance must be the main factor in the pathogenesis of guinea-pig salmonellosis.

The work reported in this paper would appear to show that natural infection of healthy guinea-pigs with organisms of the *Salmonella* group occurs by the conjunctival route. This conclusion is based on (i) observation of natural epizootics caused by *Salm. enteritidis* and *Salm. typhi-murium* in laboratory guinea-pigs; (ii) the finding that a generalized infection could be produced in guinea-pigs by conjunctival instillation of tiny doses of salmonellae although control animals were very refractory to oral infection with organisms of the same strains, and (iii) the demonstration that healthy guinea-pigs adequately protected from conjunctival infection by goggles were resistant to salmonella infection when exposed to heavily contaminated environments in which a high proportion of ungoggled guinea-pigs became infected.

MATERIALS AND METHODS
Guinea-pig maintenance

Observations on guinea-pig epizootics were made in our own laboratory animal houses. When salmonella infections were first diagnosed, all guinea-pigs were being supplied by one breeder whose herd, including sows, boars and young stock averaged 400–500 animals. On arrival at the laboratory the guinea-pigs were placed in stock cages in a large shed until required. Inoculated animals, used mainly for the examination of school milks and of specimens from tuberculous patients, were housed two to a cage in an infected house measuring 16 by 10 ft., which was always seriously overcrowded. Later, guinea-pigs from other breeders were used, and animals from different breeders were always kept segregated in the stock house until inoculated in due course.

Experiments on the initiation of epizootics were carried out in a separate wooden shed, which was disinfected with formalin at the termination of each experiment. The guinea-pigs in this latter shed were always fed last, and careful precautions were taken to prevent the cross-infection of animals in the routine infected house by the wearing of special gowns and careful disinfection of the hands.

Autopsy and culture techniques

Guinea-pigs were killed by a sharp blow on the back of the neck. A median skin incision was made from the submental region to the brim of the pelvis and the skin reflected. Blood was collected from the heart with a Pasteur pipette and the lungs were removed for examination. After searing the anterior abdominal peritoneum the peritoneal cavity was opened. The spleen was removed with sterile instruments, the liver and mesenteric lymph nodes inspected and taken for culture if macroscopically abnormal, and samples of gut contents obtained by taking swabs through an incision in the caecum.

Tissues for culture were ground in Griffith tubes with three to five times their volume of nutrient broth. Direct cultures of heart blood, spleen and lymph nodes were made on horse blood agar, direct cultures of gut swabs on deoxycholate citrate agar. Enrichment cultures were made in nutrient broth and in selenite-F medium.

Experimental inoculation and feeding

The *Salm. enteritidis* strain used in the greater part of this work (strain no. 2014) was isolated from spleen cultures of the first guinea-pig found dead in our infected house on 8 December 1952. The strain was originally maintained on Dorset egg slopes, but was later freeze-dried through the kindness of Dr S. T. Cowan, Director of the National Collection of Type Cultures, and catalogued as N.C.T.C. no. 8515. Subcultures from the Dorset egg stock cultures, or broth washings of freeze-dried cultures, were put up on the afternoon of the day before they were required. At 9 a.m. on the following morning the primary slopes were subcultured into fresh nutrient broth and the fresh cultures incubated for 6 hr. at 37° C., after which suitable ten-fold dilutions were made in one-quarter strength Ringer's solution to provide the approximate inoculum required for a given experiment. Viable counts of the culture dilutions were done by Miles and Misra's method and the inoculum used was then calculated in retrospect.

For the instillation of known volumes of diluted culture into the eye, Pasteur pipettes calibrated to deliver 0·025 ml. drops were used, this being about the largest volume accommodated in the guinea-pig's eye without a spill-over on to the face. In feeding experiments, the desired inoculum was added from a pipette to crushed diet eighteen pellets and immediately fed to individual guinea-pigs on small pieces of cabbage leaf. Control experiments showed no evidence of an inhibitory effect of the crushed pellet on the salmonellae used.

Initiation of guinea-pig epizootics

The general plan used was to expose equal numbers of goggled and ungoggled guinea-pigs to an environment contaminated with *Salm. enteritidis* and observe the infection rates and mortality in both groups after exposure for 10–14 days. In the earlier experiments the test environment was contaminated by 3–12 guinea-pigs infected conjunctivally with *Salm. enteritidis* 5 days before the main experiment was due to start. The test animals were then introduced into the same cage;
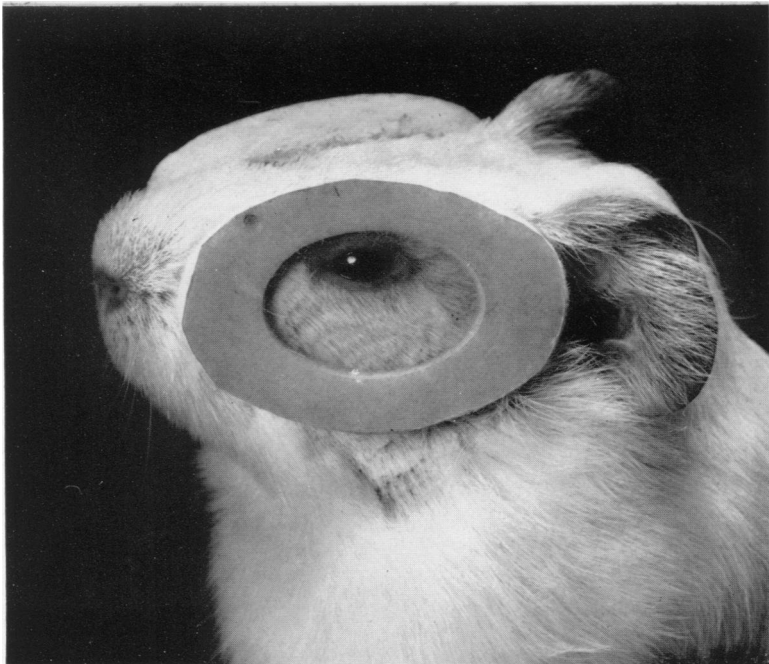
Fig. 1

Fig. 2

the main contamination of the cage at the start of the experiment was thus provided by conjunctival discharge from the infected animals, although after a number of days the excreta of these guinea-pigs were also infective.

In a number of experiments in cages contaminated by this method all exposed guinea-pigs, whether goggled or ungoggled, showed a low proportion of 'takes'. In the final experiment, therefore, a very much heavier degree of contamination was arranged for by exposing guinea-pigs to the excreta of mice infected with *Salm. enteritidis* and placed in a wire cage about a foot above the floor of the guinea-pig pen. The details of this procedure, which gave very satisfactory results, are described in the protocol of the experiment in question.

Techniques used for goggling guinea-pigs

Prolonged attempts were made to devise goggles which would completely protect the guinea-pig's eye from direct or dust-borne infection while not interfering with its normal activities. Operative suture of the eyelids was considered an unsatisfactory procedure, not only on humanitarian grounds but because guinea-pigs so treated could scarcely be considered normal healthy animals. The first goggles to give fairly satisfactory results, which are referred to below as the A type, were made from plastic watch-glasses, embedded in a double layer of adhesive plaster cut to fit approximately the contours of the guinea-pig's head and applied with a latex adhesive (Pl. 5, fig. 1). More cumbersome harnesses were not tolerated and led to prolonged struggles which dislodged the goggles. The great advantage of direct application as above described was its speed, due to the rapid drying of the adhesive used. Guinea-pigs so goggled and returned to their cages behaved quite normally. This type of goggles had, however, two serious disadvantages. One was the rather close approximation of the 'lenses' to the guinea-pig's eye, which caused a low-grade infection of the eye in a number of animals and possibly a number of non-specific deaths as described below. Another disadvantage was that after a number of days the goggles became unstuck and were no longer airtight in a certain proportion of goggled guinea-pigs. They were inspected and repaired daily, but the uncertainty as to whether the conjunctivae of a given animal had been adequately protected during the exposure period sometimes made the interpretation of post-mortem findings difficult.

A very much better type of goggles, the B type, later overcame the drawbacks of the first model. In the later procedure each eye was goggled separately, thus easing greatly the strain on the goggles' framework, and the 'lenses' were raised some distance from the conjuctival surface. Each eye was first ringed by a proprietary 'bunion pad' attached with latex adhesive to the shaven skin of the face. The pad consisted of an O-shaped ring of semi-compressed felt $1\frac{1}{2}$ in. in its long diameter and 3/16 in. thick. To the free surface of the pad was attached, with adhesive, an accurately fitting elliptical patch of cleared X-ray film (Pl. 5, fig. 2). To strengthen further the attachment of the goggles to the guinea-pig's skin, the margins of the goggles were liberally coated with a paste containing latex adhesive and clippings of guinea-pig fur.

RESULTS

I. *Observations on natural infections in guinea-pigs*

Two series of observations are described in this section. In December 1952, a number of *Salm. enteritidis* deaths in our animal house led to investigations which showed that salmonella infection of the conjunctiva was widespread in our own guinea-pig herd and in that of the breeder supplying the laboratory, and that guinea-pigs with systemic salmonella infection, assessed by the presence of spleen lesions, almost invariably gave positive cultures from conjunctival swabs.

Later experiments were to show that the instillation of a small inoculum of salmonellae into the conjunctiva of the healthy guinea-pig set up a conjunctivitis followed by a blood-borne infection *via* the cervical lymph nodes. During the first epizootic, however, it was still thought that generalization of salmonella infection from the conjunctiva occurred by way of the lachrymal duct, and no attention was paid at autopsy to the cervical lymph nodes of infected guinea-pigs. Later the opportunity occurred to examine a number of guinea-pigs from another breeder's herd which were naturally infected with *Salm. typhi-murium*. The findings in this second group of animals, which are also summarized below, confirmed that lymphatic spread of infection from the conjunctiva occurred under natural conditions as well as experimentally.

A. *An outbreak of* Salmonella enteritidis *infection*

On 8 December 1952, four guinea-pigs in our infected house, inoculated a few weeks previously with routine non-salmonella material, were found dead. *Salm. enteritidis* was isolated at post-mortem from three out of the four. It was noted at autopsy that two of the animals had sticky eyes, and eye swabs from both yielded a heavy growth of *Salm. enteritidis*.

On 10 December 1952, five more guinea-pigs in the house showed a partial closure of one or both eyes, and these animals were killed and examined for evidence of salmonella infection. Eye swabs from all five yielded *Salm. enteritidis* on direct plating. As rectal swabs from the same animals were all negative on culture, it seemed possible that eye swabbing would give a better indication of the extent of infection in the herd than would the examination of rectal swabs, and that the segregation of eye-positive animals might limit the spread of the outbreak. Accordingly, on 12 December 1952, eye swabs were taken from the remaining 226 guinea-pigs in our infected house, one swab moistened with nutrient broth serving for the swabbing of both eyes of individual animals. The swabs were immersed in selenite-F medium and tubes showing evidence of growth plated out after 24, 48 and 72 hr. In all, twenty-one guinea-pigs yielded *Salm. enteritidis* from eye-swab cultures; of these, four had shown no macroscopic evidence of conjunctival infection at the time of swabbing, and the remainder a variable degree of closure of the eyes with crusting at the inner canthus or along the lid margins and watery discharge. The twenty-one guinea-pigs with positive eye cultures were killed; seven yielded *Salm. enteritidis* from cultures of gut contents. Cage-mates of eye-positive guinea-pigs were also slaughtered.

On 19 December 1952, the herd was again swabbed and five further guinea-pigs with positive eye-swabs were removed. The remaining animals were now kept under observation and no further eye infections were detected. As all the guinea-pigs concerned had been inoculated with pathological material or with school milks for tubercle bacilli, they were left until due for killing in the normal course 6–8 weeks after inoculation. A third of the remaining animals to the number of sixty-one in all, were then carefully examined at post-mortem for evidence of salmonellosis and cultures taken from eyes, spleen and gut; all were negative for *Salm. enteritidis*.

The positions of infected cages in the animal house during this epizootic suggested a gradual spread of infection, first to cage-mates and then to contiguous cages, possibly by the dissemination of fairly large infected dust particles. As the guinea-pigs had come from one breeder, however, the possibility that guinea-pigs with *Salm. enteritidis* infection had been infected while in the breeder's herd could not be excluded. A visit to the breeder's herd now showed a number of guinea-pigs with eye discharges, and one batch of seven animals from a single pen in which eye lesions were evident was brought back to the laboratory and killed. The post-mortem culture findings on this series of naturally infected guinea-pigs are given in Table 1.

Table 1. *Isolation of* Salm. enteritidis *from breeder's guinea-pigs*

		Result of culture from					
Guinea-pig no.	Condition of eyes	Eyes	Naso-pharynx	Spleen	Heart blood	Lungs	Gut
1	Normal	+	−	−	−	−	−
2	Normal	−	−	−	−	−	−
3	Normal	−	−	−	−	−	−
4	Slight discharge	+	+	−	−	−	−
5	Purulent	+	−	+	−	+	+
6	Purulent	+	−	−	−	−	−
7	Purulent	+	−	+	+	+	+

It will be noted that five of the seven guinea-pigs yielded positive eye swab cultures, and that systemic invasion of the salmonella had occurred only in guinea-pigs showing a purulent conjunctival discharge, two of the three animals with such discharges giving positive cultures from spleen and gut.

An unexpected opportunity occurred a few months later to investigate the natural outcome of salmonella infection of the conjunctiva in guinea-pigs not slaughtered when the infection was diagnosed. The breeder whose guinea-pigs had been infected with *Salm. enteritidis* suffered considerable economic hardship through the temporary cancellation of his accreditation certificate. His entire herd of some 400 animals was therefore eye-swabbed at the end of February 1953, as he reported that no eye discharges had been noted in the meantime. All swabs were negative for *Salm. enteritidis* and his stock was therefore accepted again for laboratory use. On 7 April 1953, however, five guinea-pigs in different

cages in our infected house were found to have conjunctival infection due to *Salm. enteritidis*, and a surprise visit to the breeder disclosed that although his main stock was apparently clear of infection, he had segregated in a smaller hut some distance away a whole collection of guinea-pigs with eye lesions, including a number with the eyeball completely enucleated. Our own infected house was now again swabbed, and eye swabs from 35 out of 280 guinea-pigs yielded *Salm. enteritidis*. These animals were segregated and kept under observation. Only one died, of typical acute salmonella infection; the remaining 34 remained alive and well, and all were negative for *Salm. enteritidis* when they came to autopsy in due course. This confirmed the general experience that salmonella infection in normal guinea-pigs is not a serious disease.

B. *An outbreak of* Salmonella typhi-murium *infection*

A death from acute *Salm. typhi-murium* infection in a stock cage containing fifteen guinea-pigs obtained from a breeder in Scotland led to slaughter of the remaining fourteen. As mentioned above, these were examined particularly for evidence of lymphatic spread of infection from the eye. The cultural findings are given in Table 2.

Table 2. *Isolation of* Salm. typhi-murium *from breeder's stock guinea-pigs*

Guinea-pig no.	Results of culture from				
	Left eye	Right eye	Cervical lymph nodes	Spleen	Gut
1	+	+	+	+	+
2	−	−	−	−	−
3	+	+	−	+	−
4	−	+	−	+	+
5	+	−	+	+	+
6	+	+	+	+	+
7	+	+	−	+	+
8	+	−	+	+	+
9	+	+	+	+	+
10	+	+	+	+	+
11	+	+	+	+	+
12	−	−	−	−	−
13	+	−	+	+	+
14	+	−	−	−	−

It will be noted that once again salmonella infection of the conjunctiva was widespread in this herd; *Salm. typhi-murium* was isolated from one or both eyes of twelve out of fourteen guinea-pigs. The cervical lymph nodes were enlarged and culture-positive for *Salm. typhi-murium* in eight out of fourteen animals; the spleen was positive in eleven out of fourteen.

II. *Experimental inoculation of guinea-pigs with salmonellae*

Apart from the preliminary experiment described below, in which guinea-pigs from the original infected herd were used after preliminary eye swabs had proved negative for *Salm. enteritidis*, all the experiments described in this section were

done on guinea-pigs from herds with no recent history or laboratory evidence of salmonella infection. The greater part of the work was concerned with the *Salm. enteritidis* 2014 strain. The virulence of this strain on parenteral inoculation into guinea-pigs was certainly at least of the same order as that of strains described by previous workers, e.g. an intraperitoneal dose of 0·001 ml. of an overnight broth culture was lethal in 24–48 hr. and 0·0001 ml. intraperitoneally killed guinea-pigs after about a week.

(a) *Infection of guinea-pigs with* Salmonella enteritidis *by the conjunctival route*

In a preliminary orientating experiment, six healthy guinea-pigs were inoculated, each into the left eye, with falling ten-fold dilutions of a broth culture of *Salm. enteritidis* 2014, the viable inoculum per animal ranging from 3,500,000 down to 35 organisms. They were placed in widely separated individual cages in the laboratory attics to prevent cross-infection. After 3 days the two guinea-pigs receiving the highest inocula showed a purulent conjunctival discharge in the inoculated eye, with partial closure and oedema of the lid margins. A day later, the next three animals in the series showed a watery discharge from the inoculated eyes. The guinea-pig receiving the smallest inoculum showed no ocular abnormalities. All six animals were killed after a week, and the cultural findings at autopsy are summarized in Table 3.

Table 3. *Conjunctival inoculation of guinea-pigs with graded doses of* Salm. enteritidis

Guinea-pig no.	Inoculum left eye	Results of culture from				
		Left eye	Right eye	Spleen	Lungs	Gut
1	3,500,000	+	−	+	+	−
2	350,000	+	−	+	−	−
3	35,000	+	−	+	+	−
4	3,500	−	−	+	−	−
5	350	+	+	+	−	−
6	35	−	−	−	−	−

Apart from the guinea-pig inoculated with 35 organisms, which showed no evidence of conjunctivitis and was culture-negative at autopsy, all the test animals showed spleen infection after the conjunctival instillation of doses ranging from 3,500,000 organisms down to 350 organisms. This is in remarkable contrast with the great difficulty reported in the literature of infecting guinea-pigs with salmonellae by mouth. Table 3 also shows that two guinea-pigs gave positive lung cultures, and that all six gut swabs were negative for *Salm. enteritidis*.

(b) *The relative effectiveness of the oral and conjunctival routes of infection*

Preliminary experiments showed that, with doses of the order of 1000 organisms of *Salm. enteritidis* 2014, infection of guinea-pigs by the oral route was impracticable. In the first comparative experiment with larger doses of the same strain, twelve guinea-pigs were given a dose of 160,000 organisms, six by the conjunctival and six by the oral route. When killed a week later, all animals inoculated by the

conjunctival route showed enlarged nodular spleens positive on direct culture for *Salm. enteritidis*, whereas none of the guinea-pigs receiving the oral dose showed any evidence of salmonella infection. In later experiments, all attempts to infect guinea-pigs by mouth were uniformly unsuccessful with doses up to 100 million viable organisms. Experiments with still higher doses, to which guinea-pigs were very unlikely to be exposed under natural conditions, were not considered necessary. In contrast with this failure to infect guinea-pigs by mouth, the 50 % spleen infecting dose of strain 2014 by the conjunctival route was of the order of 100 organisms (see §II*d* below).

(c) *The further course of infection in guinea-pigs inoculated by the conjunctival route*

From the results so far described it seemed highly probable that the initial lodgement of salmonellae in the guinea-pig occurred on the surface of the conjunctiva. Various possible routes by which the organism reached the blood stream from the conjunctiva could be envisaged, of which the most likely were: (i) the lymphatic route, through the extensive lymphatic drainage of the eye, (ii) the passage of infected lachrymal secretions through the lachrymal duct into the nose and thence either into the lungs by aspiration, or (iii) into the gastro-intestinal tract. The relevant evidence for deciding between these possibilities is given below.

Alimentary infection was discounted by our complete failure to infect guinea-pigs with large doses of salmonellae by mouth, by findings, such as those given in Table 3, that the spleen was frequently culture-positive without any evidence of gut infection, and finally by the evidence quoted below that in guinea-pigs gut infection was secondary to systemic infection.

Observations made from June 1953 onwards, during the autopsy of experimentally infected guinea-pigs, pointed to lymphatic spread as the most likely mode of penetration of salmonellae from the conjunctiva. The finding at autopsy of a spleen showing naked-eye lesions and yielding *Salm. enteritidis* in large numbers on culture could almost invariably be predicted from examination of the lymph nodes of the neck. If in such animals the deep cervical lymph node on the side of the inoculated eye was enlarged, e.g. to $12 \times 8 \times 4$ mm. to quote a particular instance, and showed some degree of suppuration, usually of pinhead size but sometimes involving the entire node, the spleen was virtually always pathological. In acutely infected animals with no complicating condition, a spleen with naked-eye lesions has never been seen in the absence of lymphadenopathy of the neck as above described. That this enlargement of cervical lymph nodes occurred under natural conditions as well as experimentally was first shown by examination of the guinea-pigs naturally infected with *Salm. typhi-murium* as described in §I B above.

It was still possible, however, that the picture of salmonella infection of the cervical lymph nodes was merely a coincidental finding. As the eye is in direct connexion with the respiratory tract through the lachrymal duct, various possibilities of natural infection with salmonellae by the respiratory route required consideration. First, infection might occur primarily by inhalation, the conjunctival infection being then under natural conditions secondary to nasopharyngeal

infection by *upward* spread of infection along the lachrymal duct, and systemic infection a result of further penetration of the infecting organism into the lung and thence into the blood-stream. Cervical lymph node enlargement and suppuration would then be a natural sequel to conjunctival infection but of no further moment. The only satisfactory method of testing this possibility appeared to be to expose goggled and ungoggled guinea-pigs to an infected environment. If infection occurred by inhalation, the application of goggles should have no material effect on infection rates. As shown below, however, adequate goggling virtually prevented the infection of healthy guinea-pigs with the salmonella strain used in this work.

A second possibility also required consideration, viz. that salmonella infection in the guinea-pig occurred primarily in the eye, where bacteriological enrichment of the primary inoculum took place, and that this was followed by a heavy nasopharyngeal infection, the infected discharges from the nasopharynx then draining back into the lungs. No evidence in support of this mode of infection was found in the present study. Nasal discharge due to *Salm. enteritidis* was sometimes seen, but was by no means common, e.g. only one out of twenty-one guinea-pigs with conjunctival infection in the outbreak described in §I A above showed such a discharge. In the experiment recorded in Table 3, only the guinea-pig inoculated conjunctivally with 3,500,000 organisms developed a nasal discharge; this appeared 7 days after the instillation of salmonellae into the eye, but by then the guinea-pig inoculated with 350 organisms already had splenic lesions. Again, reference to Table 1 shows that even nasopharyngeal swabs were culture-negative for salmonellae in four out of five guinea-pigs with infected conjunctivae.

Autopsy of the lungs of experimentally infected guinea-pigs also failed to show evidence of a massive infection of the lungs from the nasopharynx. In the few animals dying spontaneously of salmonella infection, the lungs were congested, but the general picture was more suggestive of a septicaemia secondary to suppuration of the cervical lymph nodes than of a primary pulmonary infection with secondary septicaemia. One such guinea-pig, for instance, died on the tenth day after its first exposure to infection in a cage containing experimentally infected guinea-pigs. Its left eye was discharging and yielded a heavy growth of *Salm. enteritidis* on culture. A deep cervical lymph node on the left side was considerably enlarged, to $13 \times 6 \times 6$ mm., and showed four discrete points of suppuration. The lungs showed a patchy congestion but no evidence of consolidation. The spleen was enlarged and slightly nodular. Plate counts for *Salm. enteritidis* were done on the lungs, cervical lymph nodes and spleen, giving the following approximate figures: lymph node, 500,000,000; spleen, 10,000,000; lungs, 2,000,000. It seems probable that the lung count could be attributed largely to organisms present in the blood flowing through the pulmonary circulation at the time of death.

From these results, it would seem probable that the generalization of salmonella infection in the guinea-pig occurs by a blood-borne invasion from the cervical lymph nodes, and that the lungs play no essential part in the process.

How the gut becomes infected after the primary blood-borne infection has

been investigated less fully. Some evidence was obtained, however, to suggest that the mode of infection may be broadly similar to the picture seen in mice as described by Ørskov, Jensen & Kobayashi (1928). Briefly, these authors found that when mice were fed with relatively large doses of *Salm. typhi-murium* (10 drops of a 20-hr. broth culture), most of the organisms were destroyed in the alimentary tract, but a few were absorbed and reached the spleen. A secondary bacteraemia followed, and the organism was excreted in the bile, thus infecting the gut. The following experiment suggests that in the guinea-pig the same sequence of infection from spleen to gut may operate. In a group of twelve guinea-pigs, six were inoculated in the left eye with 160,000 *Salm. enteritidis* 2014, and the remaining six were fed with the same dose. All the animals were killed after a week and cultures made from spleen, gall-bladder and gut. In agreement with previous findings, no evidence of salmonella infection was found in the guinea-pigs receiving the oral dose. Of those infected conjunctivally, all six had pathological spleens positive on culture for *Salm. enteritidis*; four of the six were gall-bladder-positive and two of the guinea-pigs with positive gall-bladder cultures were also gut-positive. The descending sequence of positive findings from spleen, gall-bladder and gut, respectively, together with the failure to isolate the salmonella from guinea-pigs fed with *Salm. enteritidis*, all suggest that in the guinea-pig, as in the mouse, gut infection is secondary to bacteraemia.

(d) Conjunctival infection with other salmonella strains.

It was necessary to determine whether the results of experimental infection with *Salm. enteritidis* 2014 were applicable to other salmonella strains infecting guinea-pigs, and not to be explained by some peculiarity of this strain alone. As above mentioned, investigation of a naturally arising *Salm. typhi-murium* epizootic in stock guinea-pigs showed the same widespread conjunctivitis, with cervical lymphadenopathy and splenic infection, as in guinea-pigs infected with *Salm. enteritidis* 2014. In addition, a strain of *Salm. typhi-murium* and one of *Salm. enteritidis*, both isolated from animal infections, were kindly supplied by Dr Joan Taylor, director of the Salmonella Reference Laboratory. Both strains behaved very similarly to *Salm. enteritidis* 2014. A more careful comparison between the Salmonella Reference Laboratory (S.R.L.) strain of *Salm. enteritidis* and strain 2014 showed that both strains were about equally invasive when judged by the minimal dose producing generalized infection by the conjunctival route. Once established in the spleen, however, the S.R.L. strain multiplied more rapidly and produced a different naked-eye picture.

(i) *The relative invasiveness* of the two strains was assessed by inoculating conjunctivally groups of four guinea-pigs at four dosage levels with each strain, using suspensions of equal density as starting cultures for the preparation of falling inocula of each organism. The viable inoculum of each strain was calculated as usual by Miles and Misra counts. The thirty-two guinea-pigs were killed after a week and examined for evidence of spleen infection. The results are given in Table 4.

It would appear from this table that the 50% spleen infecting dose for each

Table 4. *Relative invasiveness by the conjunctival route*
of Salm. enteritidis *strains*

Approximate conjunctival dose*	Number of spleen-positive guinea-pigs	
	Salm. enteritidis 2014	*Salm. enteritidis* S.R.L.
100	2/4	2/4
1,000	4/4	3/4
10,000	4/4	4/4
100,000	4/4	4/4

* Miles and Misra counts showed that the actual viable inocula corresponding to an estimated inoculum of 100 organisms were as follows for the two strains used: *Salm. enteritidis* 2014 130, *Salm. enteritidis* S.R.L. 106.

strain by the conjunctival route was approximately the same and was of the order of 100 organisms.

(ii) *The spleens of guinea-pigs* inoculated with *Salm. enteritidis* S.R.L. in the experiment above described all showed a characteristic picture which differed from that seen with *Salm. enteritidis* 2014. Edington (1929) described two types of splenic lesion in salmonella-infected guinea-pigs, one with focal necroses and the other with abscess formation. The spleens of pigs inoculated with *Salm. enteritidis* S.R.L. consistently showed abscesses up to 5 mm. in diameter, while animals infected with the strain 2014 had spleens with considerable numbers of smaller greyish nodules of pinhead size, slightly raised above the surface when inspected by reflected light. Viable counts on spleens with abscess formation were approximately a thousand times greater than on spleens showing focal necroses without abscess formation.

(e) *Attempts to increase the invasiveness of* Salmonella enteritidis 2014.

The work described in the previous section suggested the existence of at least two components of virulence in salmonella strains, one reflected in invasive power and the other in the capacity to multiply in the spleen once established there. A number of attempts were made to increase the invasive power of strain 2014. First, a series of passage transfers was done, using material from cervical lymph nodes for inoculation into the conjunctiva of the succeeding guinea-pig in the series. At the twenty-first passage the invasiveness of the strain was apparently no greater, but the spleens of guinea-pigs inoculated by the conjunctiva with the passaged strain, and also the cervical lymph nodes, showed a picture of suppuration more like that produced by *Salm. enteritidis* S.R.L. than that found with the original 2014 strain.

Because of statements in the literature that guinea-pigs suffering from tuberculosis or on deficient diets were more susceptible to salmonella infection than normal guinea-pigs, attempts were also made to determine whether the invasiveness of *Salm. enteritidis* 2014 was greater in such animals than in healthy guinea-pigs. No clearcut evidence of increased invasiveness was found in experiments on conjunctival instillation of salmonellae. On the results of goggling experiments

the suggestion is made below that the increased susceptibility to salmonella infection of guinea-pigs with complicating conditions may be associated with penetration of the organisms into the body by a route which normally resists penetration.

III. *The protective effect of goggles on guinea-pigs exposed to salmonella infection*

If salmonella infection of guinea-pigs occurs naturally by the conjunctival route, it should be possible to prevent infection by hindering the access of salmonellae to the surface of the conjunctiva, or by establishing an antibiotic barrier on the conjunctiva to cope with the organisms at the time of lodgement. The latter method of preventing infection was used first, in an experiment in which twelve guinea-pigs were placed in a cage containing three guinea-pigs with heavy conjunctival discharges due to *Salm. enteritidis* 2014. Both eyes of six of the exposed guinea-pigs were treated 2-hourly during the day with chloramphenicol ointment, and all the test animals were removed from the infected environment during the night. The guinea-pigs were killed after 10 days, but the results were inconclusive as only a single untreated guinea-pig showed evidence of salmonella infection. The experimental procedure was, however, open to criticism, since, apart from the difficulties of regular inunction of guinea-pigs' eyes with chloramphenicol, the objection that guinea-pigs so treated were being protected not by the local effect of the antibiotic in the conjunctiva, but by systemically absorbed antibiotic was difficult to meet. The only technique that promised reliable results was the application of closely fitting goggles protecting the conjunctiva from dust-borne or direct contact infection. The construction of effective goggles has already been described above. The crucial experiments were as follows:

Experiment I. To provide an infected environment, twelve healthy guinea-pigs were inoculated into the left eye with a loopful of a nutrient agar slope culture of *Salm. enteritidis* 2014. Five days later, all the inoculated animals were showing a profuse conjunctival discharge, and it may be mentioned here that nine of the twelve guinea-pigs died of salmonella infection from 9 to 19 days after inoculation. A group of twelve ungoggled guinea-pigs and twelve fitted with the A type goggles described above were now placed in the same wire cage as the experimentally infected animals and exposed to infection for a fortnight, after which all surviving guinea-pigs were killed.

Results. The first deaths in the uninoculated guinea-pigs occurred in goggled animals, apparently as a complication of the goggling. Three goggled guinea-pigs died 5, 6 and 7 days, respectively, after their first entry into the infected cage. At autopsy they showed an acute lobar pneumonia with bilateral pleural effusions. Cultures from the lungs yielded a micrococcus, which was also isolated in large numbers from conjunctival swabs of the same animals. One of the three guinea-pigs also yielded *Salm. enteritidis* in large numbers from the lungs, heart blood and gut, but, as the other two gave negative cultures for salmonellae, and as, moreover, an acute lobar pneumonia has not yet been seen to follow experimental infection with *Salm. enteritidis* 2014, the salmonella infection in this guinea-pig was considered to have been secondary to the micrococcal pneumonia.

One of the ungoggled guinea-pigs died after 10 days and showed a picture typical of acute salmonella infection as described above, with direct-plate positive cultures for *Salm. enteritidis* from eyes, cervical lymph nodes and spleen, and positive cultures by enrichment from the heart blood and gut.

The remaining twenty guinea-pigs were killed after a fortnight's exposure to infection. The results of culture of eye swabs, cervical lymph nodes, spleen, gut swabs and heart blood of these animals are shown in Table 5.

Table 5. *Goggle experiment I. Results of cultures on surviving guinea-pigs*

| | Guinea-pig no. | Results of culture from | | | | |
		Eyes	Lymph nodes	Spleen	Gut	Heart blood
Ungoggled	1	+	+	+	−	−
	3	+	+	−	−	−
	4	+	+	+	−	−
	7	−	−	+	−	−
	8	+	+	+	−	−
	9	+	+	+	−	−
	11	+	+	+	−	−
	2, 5, 6, 10	−	−	−	−	−
Goggled	4	−	−	+	−	−
	5	−	+	+	−	−
	6	−	+	+	−	−
	1, 2, 3, 7, 8, 9	−	−	−	−	−

The preponderance of positive results from ungoggled guinea-pigs will be evident from the table. Only three of the goggled guinea-pigs yielded positive cultures for *Salm. enteritidis*. In two out of the three the cervical lymph nodes yielded *Salm. enteritidis* on culture. In both animals these nodes were enlarged and suppurating, and as the goggles used in this experiment had been patched in a number of animals during the course of the experiment, the possibility that the cervical lymph nodes in question had been infected *via* the conjunctiva could not be excluded. This was particularly the case with goggled guinea-pig no. 5, as a note had been made to the effect that its goggles had become detached during the first week-end of the experiment, so that penetration of salmonellae through the eye could have occurred.

Two further experiments with the A type goggles were attempted. In one, however, the goggles from most animals came off within a few days, and in the second experiment the infection rate even in ungoggled guinea-pigs was too low to yield significant results. Three out of twelve ungoggled animals became infected as against one out of twelve goggled ones, the single infected goggled animal again showing an enlarged cervical lymph node which yielded *Salm. enteritidis* on culture. Further attention was now given to devising more effective goggles and also to increasing the degree of environmental contamination to which the test guinea-pigs were exposed. This work led to the use of the B type goggling procedure above described, and to the contamination of the environment to which guinea-pigs were exposed by a subsidiary epizootic in mice.

Experiment II. In previous experiments the guinea-pigs were kept in large wire cages, made by joining two rabbit cages end-to-end. In this experiment they were placed in a 250-gal. galvanized iron tank. Suspended across the middle of this was a wire cage the bottom of which was approximately 1 ft. above the floor of the tank. Into the wire cage were placed twelve mice infected with *Salm. enteritidis* 2014. The excreta of these mice produced the environmental contamination to which the guinea-pigs were to be exposed. The mice were infected by feeding with biscuits heavily contaminated with *Salm. enteritidis*. Excreta from each mouse were collected by placing individual mice on a sheet of paper and pulling their tails, this serving as a stimulus to defaecation. As soon as a mouse was known to be excreting the salmonella it was placed in the wire cage. By the time twelve infected mice had been accumulated in this way, the environmental contamination resulting from their excreta, which fell through the mesh of the wire cage, was considered adequate. At this stage, twenty-four ungoggled guinea-pigs and twenty-four guinea-pigs with the B type goggles were placed in the galvanized iron tank. The original intention was to kill surviving guinea-pigs after 14 days' exposure to infection, but on the 12th day a few of the X-ray film 'lenses' were found to have been torn off and eaten. The surviving animals were therefore killed between the twelfth and sixteenth days of exposure to infection, an equal number of guinea-pigs from each group being taken for killing each day, but including in each day's kill the animals whose goggles were becoming ineffective.

Results. The final autopsy and culture findings on the forty-eight guinea-pigs used in this experiment showed salmonella infection in nineteen out of twenty-four ungoggled animals, and in only two out of twenty-four with goggles. Moreover, one of the two infected goggled guinea-pigs was found to be advanced in pregnancy, a condition known to increase susceptibility to salmonellosis in guinea-pigs (Friedlander & Hertert, 1929), and the second one showed appearances at autopsy very similar to those described in the exudative hepatitis of guinea-pigs reported by Paget (1954). No other animals in the series showed evidence of these complicating conditions. The preponderance of infection in the ungoggled guinea-pigs was therefore overwhelming. Findings in this experiment are summarized in Table 6.

In all, four ungoggled guinea-pigs died between the tenth and twelfth days of exposure, three of them with typical acute salmonella infection. One goggled animal died rather suddenly on the tenth day, but showed no splenic lesions. Its lungs were heavily congested and a large adherent mass of caseating mesenteric lymph nodes was found at autopsy; cultures were negative for salmonellae (and also negative for *Pasteurella pseudotuberculosis*).

Details of the findings in guinea-pigs surviving at the end of the period of exposure to infection are as follows:

(1) *Goggled guinea-pigs.* As mentioned above, only two guinea-pigs in this group showed evidence of salmonella infection, viz.

(*a*) *Guinea-pig* 14. This animal showed the curious gelatinous oedema of 'dry' consistence described by Paget (1954) as a feature of exudative hepatitis of unexplained aetiology. In addition, the spleen was nodular, and *Salm. enteritidis*

was isolated in small numbers from direct plating of spleen suspensions and also by selenite enrichment from the gut. The goggles of this guinea-pig were intact, and cultures from eyes, cervical lymph nodes and blood were negative for salmonellae.

Table 6. *Goggle experiment II. Summary of results*

| | Guinea-pig no. | Results of culture from | | | | |
		Eyes	Lymph nodes	Spleen	Heart blood	Gut
Ungoggled	1 (d.)*	+	+	+	+	+
	2 (d.)	+	+	+	−	+
	3 (d.)	−	+	+	+	−
	4 (d.)	−	−	−	−	+
	5	+	−	+	−	+
	6	−	−	−	−	+
	7	+	+	+	−	−
	8	+	+	+	−	−
	9	+	+	+	−	−
	10	+	+	+	−	−
	11	+	+	+	−	−
	12	+	+	+	−	−
	13	+	+	+	−	−
	14	−	+	+	−	−
	15	−	+	+	−	−
	17	−	−	−	−	+
	19	−	+	+	−	−
	22	−	+	+	−	−
	24	−	+	−	−	−
	16, 18, 20, 21, 23	−	−	−	−	−
Goggled	14	−	−	+	−	+
	24	−	−	+	−	−
	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 21, 22, 23	−	−	−	−	−

* (d.) = died during the experiment.

(b) *Guinea-pig 24.* This pig, which was advanced in pregnancy, had an enlarged and nodular spleen. *Salm. enteritidis* was isolated in large numbers from spleen suspensions. Cultures from eyes, cervical lymph nodes, heart blood and gut were negative for salmonellae. This animal's goggles were also intact.

(2) *Ungoggled guinea-pigs.* In this group nineteen out of twenty-four animals showed in greater or lesser degree evidence of salmonella infection. The findings were as follows:

(a) In three animals, including one that died on the twelfth day of exposure, *Salm. enteritidis* was only isolated from selenite enrichment cultures of gut swabs. Two of these guinea-pigs showed a caseating mesenteric lymphadenopathy similar to that seen in the salmonella-negative goggled animal the death of which was described above. It is possible, therefore, that in these three guinea-pigs the

salmonella was a secondary invader in animals already diseased; the considerable degree of fibrosis surrounding the caseating abdominal masses also suggested that these lesions must have been present before the beginning of the experiment. Cultures from the caseating masses of the two guinea-pigs in this group were negative for *Salm. enteritidis*.

(b) In eight guinea-pigs the now familiar combination of *Salm. enteritidis* infection of eyes, cervical lymph nodes and spleen was found. One of these yielded in addition a positive culture by selenite enrichment from the gut.

(c) In four guinea-pigs, conjunctival swabs were negative for *Salm. enteritidis*, but the cervical lymph nodes were enlarged and yielded the organism on direct plating. The spleens of these animals also yielded *Salm. enteritidis*, two on direct plating and two by selenite enrichment only.

(d) One pig yielded *Salm. enteritidis*, on direct plating, from the cervical lymph nodes only.

(e) Apart from the death mentioned under (a) above, three further ungoggled guinea-pigs died during the experiment. All three yielded a heavy growth of *Salm. enteritidis* from the cervical lymph nodes and spleen. One yielded in addition positive cultures from eyes, gut and heart blood, one from eyes and one from heart blood.

DISCUSSION

The findings reported in this paper would seem to support strongly the thesis that in normal guinea-pigs salmonella infection is transmitted by the conjunctival route. No previous suggestion to this effect has been found in the literature studied. Pandalai (1934) gives an excellent account of salmonella conjunctivitis in a short paper describing an epizootic in guinea-pigs due to an organism which was not examined serologically, but was probably *Salm. enteritidis*. The emphasis of the paper is, however, on careful description of the clinical picture seen, and the mode of transmission of the infection is not discussed. Duthie & Mitchell (1931), in a series of twenty-three experiments on the infection of guinea-pigs with salmonellae by different routes, inoculated two guinea-pigs on the conjunctiva with a drop of salmonella culture and found that one died of septicaemia in four days and the second became acutely ill but recovered. These workers were, however, unable to determine the mode of transmission of salmonella infections of guinea-pigs under natural conditions.

The present observations were concerned only with guinea-pigs, and no evidence has so far been obtained that other animal species are also infected by the conjunctival route. A few experiments have shown that mice are very readily infected by this route, and the presence of conjunctivitis in salmonella-infected mice is well established (e.g. Ratcliffe 1946). No attempt has been made, however, to measure the relative effectiveness of ocular and oral routes in mouse salmonella infection, but the wide literature on salmonella infections in mice at least suggests that they are more susceptible to alimentary infection than are guinea-pigs. Evans, Bruner & Peckham (1955) described blindness in chickens associated with salmonellosis. The causative organism was *Salm. typhi-murium*, and the infection

was clinically manifested as a suppuration in the anterior chamber of the eye and on the retinal surface, followed ultimately by complete enucleation of the eyeball. The lesion did not follow intraconjunctival instillation of salmonellae and was thought to have been blood-borne.

The observations recorded here have an evident bearing on the management of guinea-pig herds. Conjunctival discharge in guinea-pigs is an obvious phenomenon and routine cultures from watery half-closed eyes would probably detect the introduction of salmonella infection into a herd at an early stage. Some evidence has also been obtained that other bacterial infections of guinea-pigs may be transmitted through the conjunctiva. Thus, one animal in our herd which showed a unilateral conjunctival discharge was swabbed routinely, and cultures from the affected eye yielded *Pasteurella pseudotuberculosis* in large numbers. The cervical lymph node on the affected side was enlarged to form an abscess 15 mm. in diameter. The animal also had a typical pseudotuberculous spleen. Again, I am indebted to my colleague Dr G. T. Cook of Guildford, for details of an episode in his laboratory guinea-pig stock, suggesting that a massive cervical adenitis due to coliform organisms was probably transmitted through the conjunctiva from contaminated hay. It is of interest to note in this context that Calmette, Guérin & Grysez (1913) reported that guinea-pigs inoculated conjunctivally with tubercle bacilli developed a cervical adenitis closely resembling scrofula in man. Calmette (1923) speculated indeed that 'Such is the resemblance that, on examining experimental animals, one is immediately struck with the idea that human family contagion probably frequently takes the same path and that it then follows the projection, by a tuberculous cougher, of particles of saliva abounding in bacilli upon the ocular conjunctiva of healthy individuals.'

The findings of this investigation also suggest another approach to the general problem of resistance to infection and its underlying mechanisms. The experimental findings in the second goggling experiment confirm the suggestions in the literature that salmonella infections of guinea-pigs tend to be associated with 'lowered resistance'. In the present instance, this lowering of resistance seems to have taken the form of permitting infection by some route normally resistant to penetration by pathogens. Salmonella infections of guinea-pigs might provide a useful model for the investigation of the factors underlying resistance of healthy animals to infection, and further work on these lines is in progress.

No evidence has been found in the literature of an association of salmonella infections in man with the conjunctiva. The ocular route has long been known to play a part in certain diseases such as leptospiral, erysipelothrix and brucella infections. More recently, evidence has accumulated that the adenoviruses may be transmitted naturally via the conjunctiva, and Papp (1956) claims to have shown that measles is transmitted by the conjunctival route and that susceptible infants who are effectively goggled escape infection when placed in contact with infective patients. It may well be that the conjunctival route is worth considering in the epidemiology of other bacterial or virus infections of man.

SUMMARY

In the course of salmonella epizootics in guinea-pigs due to *Salm. enteritidis* and *Salm. typhi-murium*, respectively, salmonella conjunctivitis was widespread, and conjunctival swabbing was found to be a more effective procedure for detecting the spread of infection than the examination of rectal swabs. Eye-swabbing with segregation of eye-positive guinea-pigs and their cage-mates was apparently successful in controlling two *Salm. enteritidis* epizootics.

Later experimental observations showed that the conjunctival route was a far more effective one for producing systemic salmonella infection in guinea-pigs than the oral route. The 50 % spleen infecting dose by the conjunctival route was of the order of 100 organisms, while a dose of 100,000,000 organisms of the same strain of *Salm. enteritidis* was ineffective by mouth. Blood-borne invasion from the primary conjunctival lesion occurred by the lymphatic route. Gut infection of guinea-pigs infected experimentally throughout the eye was secondary to spleen infection and probably occurred by excretion of salmonellae through the gall-bladder.

The importance of the conjunctival route in natural salmonella infections of guinea-pigs was confirmed by goggling experiments, which showed that, except in animals with complicating conditions such as pregnancy, effectively goggled guinea-pigs were highly resistant to infection in environments heavily contaminated with *Salm. enteritidis* which produced high infection rates in ungoggled control animals.

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EXPLANATION OF PLATE

Fig. 1. Guinea-pig with the 'A' type goggles, made from plastic watch-glasses embedded in a double layer of adhesive plaster and attached with a latex adhesive.

Fig. 2. Guinea-pig with the 'B' type goggles, consisting of two independent elliptical pads of semi-compressed felt covered with patches of cleared X-ray film and attached with latex adhesive. The goggles are shown before the application to the margins of a paste made of latex adhesive and guinea-pig fur clippings, which strengthens their attachment to the guinea-pig's skin.