A comparison of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin

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It is now generally accepted that the increased rate of weight gain in chicks receiving dietary antibiotics depends largely, if not entirely, on consequent modification of the microbial population of the chick's alimentary tract. Fundamental investigation of the phenomenon, therefore, calls for the maintenance of chicks free from indigenous organisms so that a defined flora can be introduced as desired. We deal here with experiments on the rearing of chicks in the Gustafsson (1948, 1959) germ-free apparatus and the effects of dietary penicillin on chick growth in germ-free or conventional environments.

EXPERIMENTAL

Chicks. The chicks were produced on our own premises from Light Sussex hens crossed with Rhode Island Red cocks, so that the sexes were distinguishable at hatching by down colour. For much of this work the hens were housed in batteries and artificially inseminated twice weekly, fouling of the egg-shells being thus avoided as far as possible. On some occasions the hens were flock-mated in fold units on grass; some of the eggs from these birds were soiled, and only the cleanest were selected for the production of germ-free chicks.

Germ-free apparatus. Three of the small light-weight tanks (see Pl. 1) designed by Gustafsson (1959) and supplied by Wojidkow and Co. A. B., Malmö, Sweden, were used. The tank consists essentially of a stainless steel box, 34 in. long, 18 in. wide and 20 in. deep, covered by a plate-glass window. Into one of the sides a pair of heavy rubber sleeves is sealed, to which can be fitted rubber gloves of any suitable pattern. A third sleeve is provided on the opposite side. At one corner of the tank is a stainless-steel U-trap to be filled with germicide, through which objects can be passed into or out of the tank. The air supply is sterilized by passing it through a steel cylinder containing granulated carborundum heated electrically to 350°; it is cooled by passing through a tube along the side of the tank. The exhaust air is similarly treated in order to destroy any organisms that may, deliberately or accidentally, have been introduced into the apparatus. In the experiments described here air was passed into the tanks at the rate of 7 l./min during the hatching period, and subsequently the flow was increased to 10 l./min. Positive pressure was always maintained in the tanks.

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The apparatus was originally designed for rats; small modifications had to be made to meet the more exacting environmental requirements of young chicks. During the hatching period a relative humidity of about 65 % is optimal and was achieved by dripping water slowly into the air-intake tube as it entered the air sterilizer. After hatching, ordinary atmospheric air was supplied through the sterilizer until the birds were 2–3 weeks old, by which time humidity in the tanks began to rise because of the chicks' respiration and of evaporation from droppings and water troughs. At this time the air entering the tanks was dehumidified by passing it over a freezing coil at – 5° before it was pumped into the sterilizers. By this means it was usually possible to keep the relative humidity below 75 %, provided care was taken to minimize the water surfaces exposed.

Heat was supplied by three 250 W i.r. lamps clamped about 12 in. below the floor of each tank. Some form of thermostatic control was necessary; because of the difficulties of sterilizing electrical apparatus, the thermostat was not placed directly in the tank. Instead, a stainless-steel tube 5 in. long and ½ in. diam., sealed at the upper end, was placed in the waste outlet provided at the bottom of each tank and held in position by a retaining nut with a rubber gasket to ensure a hermetic closure. Into this tube was inserted a bimetallic thermostat (A.E.I. Ltd, Instruments Division, Harlow, Essex). During hatching the temperature was held at 35–36° and gradually reduced to room temperature (20–22°) during the next 4 weeks. Light was provided by a 20 W fluorescent tube fixed along one of the long sides of the plate-glass window. It was controlled by a time-switch to give 12 h illumination a day.

Management of the chicks. The chicks were housed in galvanized wire cages 18 in. long × 12 in. wide × 10 in. deep, with wire mesh floors. The cages rested about 2 in. above a solid galvanized droppings-tray, which allowed sufficient space for the droppings to accumulate throughout the whole 4-week test period. The cages held up to five chicks. Each tank accommodated two of these cages, which were placed side by side so as to allow a 1 in. space for air circulation between the droppings-trays and the bottom of the tank. The air space was found necessary for maintenance of an even temperature throughout the tank. Food and water containers were of stainless steel. Food was given in troughs 11 in. long × 4 in. wide × 2 in. deep, fitted with wire grids and a 'windmill' device to prevent scattering. A closed reservoir holding about 1 pint of water was provided on each cage. It was fitted with a constant-level device dipping into a small trough in which 2½ in. × 1 in. water surface was exposed for the birds to drink.

Control (hereafter referred to as 'conventional') chicks were housed in the same type of cage fitted with similar equipment for food and water. In an attempt to reproduce as nearly as possible the physical environment of the germ-free tanks, the cages were stacked on racks in a controlled-temperature room (Coates, Hall & Thiel, 1950). The temperature was adjusted to be equal to that in the germ-free tanks, and lighting was provided for 12 h a day. The rate of ventilation was unavoidably higher, and the relative humidity consequently rather lower, than in the tanks. The controlled-temperature room opened directly into one of the main chick-rearing rooms, from which its air supply was drawn, so that the birds were constantly exposed to contamination with air-borne organisms from other conventional chicks.

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Food and water. Sufficient sterilized drinking water, packed in half-pint cans (Smedleys Ltd, Whyteleafe, Surrey), was placed in the tanks at the beginning of each experiment. The reservoirs were filled when necessary and the empty cans taken out through the germicidal trap. The diet was the practical type of chick mash used in our previous work on antibiotics and had the percentage composition: ground wheat 35, ground maize 30, wheat offals 8·5, white-fish meal 10, dried skim milk 7·5, dried grass 3, dried yeast 3, limestone flour 1·5, salt mixture ($MnSO_4.4H_2O$ 6 g, KI 0·06 g, NaCl 93·94 g) 0·5, arachis oil (containing 680 i.u. vitamin A and 64 i.u. vitamin D_3/g) 1. When necessary, procaine penicillin was added at the rate of 45·5 mg/kg diet. In preliminary trials the diet was autoclaved at 121° for 30 min, but this diet failed to support normal growth in chicks even when extra supplements of vitamins were added after autoclaving. For this reason, in all the experiments reported here the diet was treated by γ-irradiation through the kind co-operation of the staff of Wantage Radiation Laboratories. It was packed in Polythene–cellulose laminate bags, each containing 400 g diet, which were evacuated, sealed and then placed in a second bag, which was also sealed. Finally, the sealed packets were put into cylindrical press-lid tins in which they were irradiated at 5 Mrad from a ^{60}Co source. After treatment the packets were left inside the tins in a cool room until required.

Sterilization of the germ-free tanks. Each tank was loaded through the top with all the heat-stable necessities for an experiment. These included two cages with equipment for food and water, thirty-six cans of drinking water, wet- and dry-bulb thermometers, scissors, can-opener, forceps, hook and chain for entry of the bags containing the eggs, cotton gloves to protect the rubber gloves, gauze swabs and cans or nylon bags for removal of waste materials. The plate-glass window was sealed in position (as described by Gustafsson, 1948, 1959), and the tank was loaded into an autoclave. The germicidal trap was left empty at this stage, but a rubber tube led into it from a small inlet on the door of the autoclave. The heater in the air sterilizer was turned on, to prevent damage to the electrical connexions by condensation of steam at the beginning of the autoclaving procedure. The autoclave was evacuated to a pressure of approximately 20 mm Hg. Steam was blown through the autoclave for 10 min, all outlet valves were closed, and the temperature was raised to 120° and held there for 30 min. The steam supply was then cut off, and air was passed in through the sterilizer. Meanwhile a container of germicide (see below), situated above the level of the tank, was heated by live steam to at least 80° and connected to the inlet on the autoclave door. When the pressure in the autoclave was still a little above atmospheric, the inlet was opened, and about 15 l. of germicide were allowed to run into the trap, thereby sealing the germ-free tank. The tank was left overnight in the autoclave, with air passing in through the sterilizer, to dry the interior. It was then taken into position in the laboratory and adjusted to the appropriate temperature and humidity.

Introduction of eggs and hatching of chicks. The procedure was essentially that described by Reyniers, Trexler, Ervin, Wagner, Luckey & Gordon (1949). All solutions used during the process, including the germicide in the traps, were maintained at 35° throughout. Clean eggs were candled after 18 days' incubation, and only those with sound shells containing a live embryo were selected. They were packed

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into Terylene net bags holding about seven eggs, immersed for 2 min in a detergent (2% v/v Lissapol, Imperial Chemical Industries Ltd) and massaged gently by an operator wearing sterilized rubber gloves. They were next passed into a 2% (w/v) solution of $HgCl_2$, and similarly massaged for 8 min. At this stage the eggs were ready to be passed into the tank; however, as there was frequently a chemical reaction between the germicides used in the trap and the residual $HgCl_2$ on the shells, they were rinsed rapidly in a separate batch of the germicide before being transferred to the trap. By means of the gloves fitted in the side of the tank a second operator drew in the bag of eggs with a hook and chain. The eggs were removed from the bag and laid on the floors of the cages to hatch. The total number of eggs introduced into a tank ranged from fourteen to twenty-one, according to the quantity available. After hatching, which took a further 3 days to complete, the required number of chicks was selected and the surplus killed by breaking their necks. The dead chicks were packed into cans or nylon bags together with the waste shells and unhatched eggs and passed out through the trap.

Eggs for the production of control chicks were submitted to the same sterilization procedure. After rinsing in the germicide, they were set in an ordinary commercial incubator and left to hatch in the usual way.

Introduction of materials into the tanks. During an experiment it was necessary to pass several objects, such as packets of diet or tubes of test media, aseptically into the tanks by way of the germicidal trap. The risk of introducing micro-organisms during this procedure was high, since the only available space for the tanks communicated closely with existing animal rooms. Stainless steel lids covered the traps to minimize the amount of dust falling into the germicide. To reduce the risk of surface contamination, all objects to be passed into the tanks were doubly wrapped and then sterilized. The containers of test media (see below) were brought to the tanks after autoclaving in two nylon covers. The outer one was removed at the trap, allowing the container with the inner cover still in place to sink into the germicide. The second cover was then removed under the surface. The tins of irradiated diet were similarly brought to the tanks before the lid was opened; a packet of diet was removed, the outer bag was slit open, and the inner packet of diet was slipped into the germicide. All objects were left immersed in the trap for at least $\frac{1}{2}$ h before being pushed up into the tank and taken in by means of the gloves fitted along the side. Throughout these procedures materials introduced into the tank were handled only by operators wearing sterile rubber gloves.

Germicidal trap solutions. Because of the need for passing objects through the germicidal trap, it is desirable that solutions used in it should be non-toxic, non-corrosive and non-volatile. The quaternary ammonium compounds used by Gustafsson (1948) fulfil these requirements, and for most experiments a solution of 0·1% (w/v) benzalkonium chloride with 0·25% (w/v) Na_2CO_3 was used. This concentration, at alkaline pH, is known to be effective against bacterial spores. Other solutions were tried with less success. An iodophor containing 200 p.p.m. I_2 provided an effective barrier against contamination, but was inconvenient because of its volatility. A 0·2% (w/v) solution of chlorhexidine afforded inadequate protection against moulds.

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Sterility checks. Sterility tests were made at about weekly intervals, usually beginning on the day the chicks hatched but before any diet had been introduced. The media and swabs for entry into the tank were autoclaved at 121° for 15 min. Four tubes of fluid thioglycollate, four tubes of Trypticase Soy Broth (B.B.L., Baltimore, Maryland, USA) and two tubes each containing seven cotton-wool swabs on wooden sticks were placed in a stainless-steel container with a lid sealed hermetically. The container was doubly wrapped in nylon and again autoclaved with the lid raised at 121° for 15 min. When cool, the can was dried at 100°. One can was prepared in this way for each tank, and an extra one was used as a control on the procedure.

When the can was cool, the lid was closed and the can was introduced into the tank as described above. The swabs were moistened in water and samples were taken from the vent of the chick and the droppings in the bottom of the cage. The tubes of media in the stainless steel containers were inoculated inside the tank and the remaining swabs subsequently used to inoculate in the laboratory 5% horse-blood agar and potato–dextrose agar plates. Two blood plates were incubated aerobically and two anaerobically at 37°. The potato–dextrose agar plates and some blood plates were incubated at room temperature. All test media were observed for 2 weeks after incubation. One swab was used to make a smear, which was stained by Gram's method to detect contaminants that might not grow on the media. The birds were judged to be germ-free only if all these tests were negative.

Experimental procedure. When hatching was complete in the germ-free tanks, the selected chicks were divided between the two cages so that the distribution of sexes was the same in both. From two to five birds were used in each cage, according to the number available. The germ-free chicks could not be weighed on hatching as there were no scales inside the tanks. They were therefore selected by appearance for uniformity of weight; it is unlikely that the mean initial weights of the groups differed by more than 2 g. One group in each tank was given the unsupplemented diet and the other received the diet with penicillin. The conventional chicks hatched in the incubator were allotted to cages in the controlled-temperature room so that the numbers of birds and distribution of sexes were the same as those in the cages in the germ-free tanks. There were four cages of control chicks for each tank; the irradiated diet with and without penicillin was given to two of the groups, and the remaining two received the corresponding unirradiated diets.

The experiments lasted for 4 weeks. All birds were weighed at the end of the experiment immediately after the tanks had been opened.

These experiments disclosed considerable differences in gain in weight among individual chicks within groups, which probably contributed much towards the marked variation between groups on any one treatment and between the overall results of different experiments. It seemed possible that the social order usually established in groups of birds housed together might be leading to differences in food consumption and consequently in weight gains. To test this supposition, four tank runs were done to compare the variability of chicks housed individually and in groups. On these occasions only the basal diet was used. One cage in each tank was divided longitudinally into three compartments by means of wire netting, and a

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separate food and water container was provided in each. One chick was kept in each of the three compartments for the whole 4-week test period, and three similar chicks were reared together in the other cage.

RESULTS

Maintenance of sterility. In all, twenty-two tank runs were done by the technique described above, and of these eighteen remained sterile throughout the whole experimental period. An analysis of the contaminations is shown in Table 1. Three runs were found to be contaminated at the first sterility check, which on two out of the three occasions was done after the chicks had hatched but before any food was taken into the tank, and contamination from the egg-shells was suspected. On all three occasions eggs had been taken from hens on range; eggs from battery hens did not, as far as could be judged, give rise to any contamination. No cause was ascertained for the remaining contamination.

Table 1. *Analysis of contaminations in experiments in germ-free tanks*

No. of tanks	Days after entry of eggs	Weekly sterility check no.	Contaminant	Probable cause of contamination
18	30	5	None	—
1	30	5	Gram-positive spore former	Unknown
1	6	1	Gram-negative rod	Egg-shells
1	3	1	Gram-negative rod	Egg-shells
1	3	1	*Pseudomonas* sp.	Egg-shells

Hatchability of eggs. The sterilization procedure resulted in a slightly lower hatch than would be expected from normal eggs selected after 18 days' incubation. Hatchability ranged from 70 to 85 %, and the percentage of chicks hatched in the tanks was similar to that in the incubator, provided that temperature and humidity were properly controlled.

Effect of irradiation on the nutritive value of the diet. Chemical and microbiological measurements of the vitamin content of the diet before and after sterilization were done by our colleagues Drs J. E. Ford, M. E. Gregory and S. Y. Thompson, by methods essentially the same as described elsewhere (Ford, Gregory & Thompson, 1962). The results of their vitamin assays on diet irradiated in air or under reduced pressure are given in Table 2. Although the irradiation treatment reduced the amount of some of the vitamins, the losses were comparatively small, and the quantities remaining were of the order generally accepted as adequate for young growing chicks. There was evidence that less loss of the fat-soluble vitamins occurred in the vacuum-packed diets than in the air-packed diets, and the vacuum pack was adopted as routine. Further investigations showed that a 'stabilized' preparation of vitamin A (Rovimix, Roche Products Ltd) suffered less destruction on irradiation than did the vitamin A acetate hitherto used; hence in the later experiments reported here Rovimix stabilized vitamin A was substituted for the oily solution. A satisfactory method for determining vitamin K in the diet was not found. At the end of one experiment crude blood-

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clotting times were determined by the method of Macfie, Bacharach & Chance (1939). The mean values with their standard errors (min) for groups of six to eight chicks were: germ-free without penicillin $3{\cdot}2 \pm 0{\cdot}51$; germ-free with penicillin $2{\cdot}8 \pm 0{\cdot}84$; conventional without penicillin $3{\cdot}4 \pm 0{\cdot}79$; conventional with penicillin $2{\cdot}9 \pm 0{\cdot}53$. As these values were well within the normal range, it was assumed that the vitamin K content of the diet was adequate.

The nutritive value of the dietary protein as measured by *Streptococcus zymogenes* (Ford, 1960) was not affected by the irradiation process either in air or under reduced pressure. Similarly, there was no detectable loss in penicillin content of the supplemented diet.

Table 2. *Effect of γ-irradiation at 5 Mrad on the vitamin content (μg/g) of the chick diet*

	Air-packed diet		Vacuum-packed diet	
Vitamin	Control	Irradiated	Control	Irradiated
Vitamin A	1·25	0·90	1·28	0·83
β-Carotene	3·0	1·5	3·3	2·0
Vitamin E	20·3	9·9	18·1	16·3
Thiamine	5·7	4·1	5·5	3·6
Riboflavin	2·8	2·8	2·6	2·8
Vitamin B_6 (as pyridoxal)	4·5	3·3	4·6	3·6
Nicotinic acid	34	35	33	32
Pantothenic acid	4·9	5·4	5·4	5·4
Folic acid	4·4	5·2	5·2	5·3
Vitamin B_{12}	0·008	0·008	0·008	0·008
Biotin	0·05	0·05	0·06	0·05

Growth of chicks and response to penicillin. The weights at 4 weeks of age of germ-free and conventional chicks with or without dietary supplements of penicillin are shown in Table 3. Analysis of variance of group means revealed no significant difference between the weights of conventional birds given sterilized or unsterilized diet whether or not it contained the antibiotic. The conventional chicks given penicillin grew better than their controls not given the supplement, and the improvement in growth was of the order usually observed in this laboratory. When the results with both sterilized and unsterilized diets were pooled the increase in weight due to penicillin reached significance ($P < 0{\cdot}05$). The germ-free chicks did not respond to penicillin, but grew significantly better ($P < 0{\cdot}001$) than the conventional birds, even those that had received the antibiotic.

In the experiments on individually caged birds, statistical analysis showed that the variance within groups of birds kept singly was greater than that in groups running together. Although this experiment was only on a small scale, the results suggested no advantage of individual housing; as it increased the labour of feeding and watering the chicks, it was abandoned.

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Table 3. *Mean body-weights (g) at 4 weeks of age of chicks with and without penicillin in the diet in germ-free and conventional environments*

		Germ-free birds Sterilized diet		Conventional birds Sterilized diet		Conventional birds Unsterilized diet	
Expt no.	No. of birds/group	Without penicillin	With penicillin	Without penicillin	With penicillin	Without penicillin	With penicillin
1	5	326	339	314	308	301	299
	5	362	317	275	309	294	319
2	5	249	329	249	271	285	340
	3	283	306	279	316	243	305
3	3	253	260	282	302	257	299
	2	306	299	242	280	270	299
4	4	308	275	236	270	256	278
	4	286	245	248	223	255	276
	4	281	283	190	231	234	232
5	4	301	235	272	274	260	222
	4	321	357	278	263	271	260
6	3	325	258	198	228	206	231
	4	302	294	265	232	245	259
Mean		300	292	256	270	260	278

Each line of values represents one replicate in which the two groups of germ-free birds were from a single tank.

The standard error of the difference between two means of thirteen observations = 10·1 (42 df).

DISCUSSION

It is apparent that the Gustafsson germ-free apparatus, originally designed for rats, can with small modifications be adapted for chicks. It is also clear that with a careful aseptic routine this type of apparatus, including a germicidal trap for passage of sterile material into the interior, can be maintained germ-free even in a comparatively unfavourable environment.

In our experiments the chief cause of contamination appeared to be eggs taken from hens on range, and it seems likely that, under such conditions, particularly in wet weather, organisms might become so deeply lodged in the shell as to survive the disinfection process. There was never any reason to suspect that contaminants were introduced with eggs from artificially inseminated battery hens. The latter form of management is expensive of time and labour, and other types of housing are under investigation in an attempt to find a simpler method of producing suitable clean eggs.

One tank failed to pass the test for sterility after it had been germ-free for about 3 weeks; it seems likely that contamination occurred either during the introduction or the sterilization of diet or test media. Since in the eighteen tanks that remained germ-free for 4 weeks at least 150 kg diet were used, irradiation at 5 Mrad seems an effective method of sterilization. Horton & Hickey (1961) successfully reared germ-free guinea-pigs on a diet irradiated at 2 Mrad. Our results showed that the damage to the vitamins caused by the irradiation at 5 Mrad was small; although the weight

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gain of conventional chicks seemed slightly less on the sterilized than on the unsterilized diet, the difference was not significant; in any event the irradiated diet was nutritionally adequate to support a much higher level of gain in the chicks in the germ-free tanks. Experiments in progress with conventional chicks housed in brooders support the suggestion that growth is slightly less than optimal on a diet sterilized at 5 Mrad, although it is normal on the diet sterilized at 2 or 3 Mrad. In the light of the experience of Horton & Hickey (1961), it is possible that the level of irradiation used by us could be lowered. More extensive trials in the germ-free tanks are planned to determine whether or not sterility can be maintained with diets treated at lower levels of irradiation.

When penicillin was included in either the sterilized or the unsterilized diet of conventional chicks, they gained weight better than the controls not given the supplement. In the germ-free birds, however, the antibiotic did not affect growth. These results confirm the findings of Forbes & Park (1959) and lend further support to the belief that antibiotics in the diet suppress some microbial retardation of growth. *Clostridium welchii* is an example of an organism that, when implanted in the gut of germ-free chicks, caused growth depression reversible by penicillin (Lev & Forbes, 1959). As can be seen from Table 3, the germ-free chicks grew better than the conventional birds given supplements of penicillin, a fact also observed by Forbes & Park (1959). It is unlikely that the better growth in the germ-free tanks was a result of physical environment; conditions of housing, lighting and temperature were much the same for both germ-free and conventional chicks, whereas ventilation and humidity were somewhat more favourable in the conventional chick room than in the germ-free tanks. The greater weights of the germ-free compared with the conventional birds therefore suggest that there must be other microbial components of the normal environment that interfere with growth but are resistant to penicillin. Experiments on chicks with a defined and controlled gut flora are needed to elucidate this problem.

As in most experimental work with chicks, a major hindrance in these experiments was the large variation between individual birds on any one treatment. Previous attempts in this laboratory have failed to reduce this variance by selective breeding, and in the small series of tests here described there was no indication that chicks housed individually responded any more uniformly than those kept in groups. It appears that the basic biological variation between chicks is high, and that it is no less in germ-free than in conventional birds. Considerable replication is therefore necessary before valid conclusions can be drawn, particularly when relatively small effects, such as the growth response to antibiotics, are under investigation.

SUMMARY

1. After small modifications the Gustafsson germ-free apparatus proved suitable for experiments with young chicks.

2. A practical chick diet of natural ingredients maintained satisfactory gain in weight and sterility after treatment by γ-irradiation at 5 Mrad.

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3. Addition of 45·5 mg procaine penicillin/kg diet improved the weight gain of conventional but not of germ-free chicks.

4. Germ-free chicks gained more in weight than their conventional controls, even than those that had received penicillin.

5. Variance between individuals was as great in germ-free as in conventional chicks.

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EXPLANATION OF PLATE

Two views of the modified Gustafsson apparatus for germ-free animals. *A*, rubber sleeves with gloves for manipulations inside the apparatus; *B*, trap for germicide; *C*, air sterilizer; *D*, heating lamps; *E*, control for thermostat; *F*, fluorescent lamp holder.

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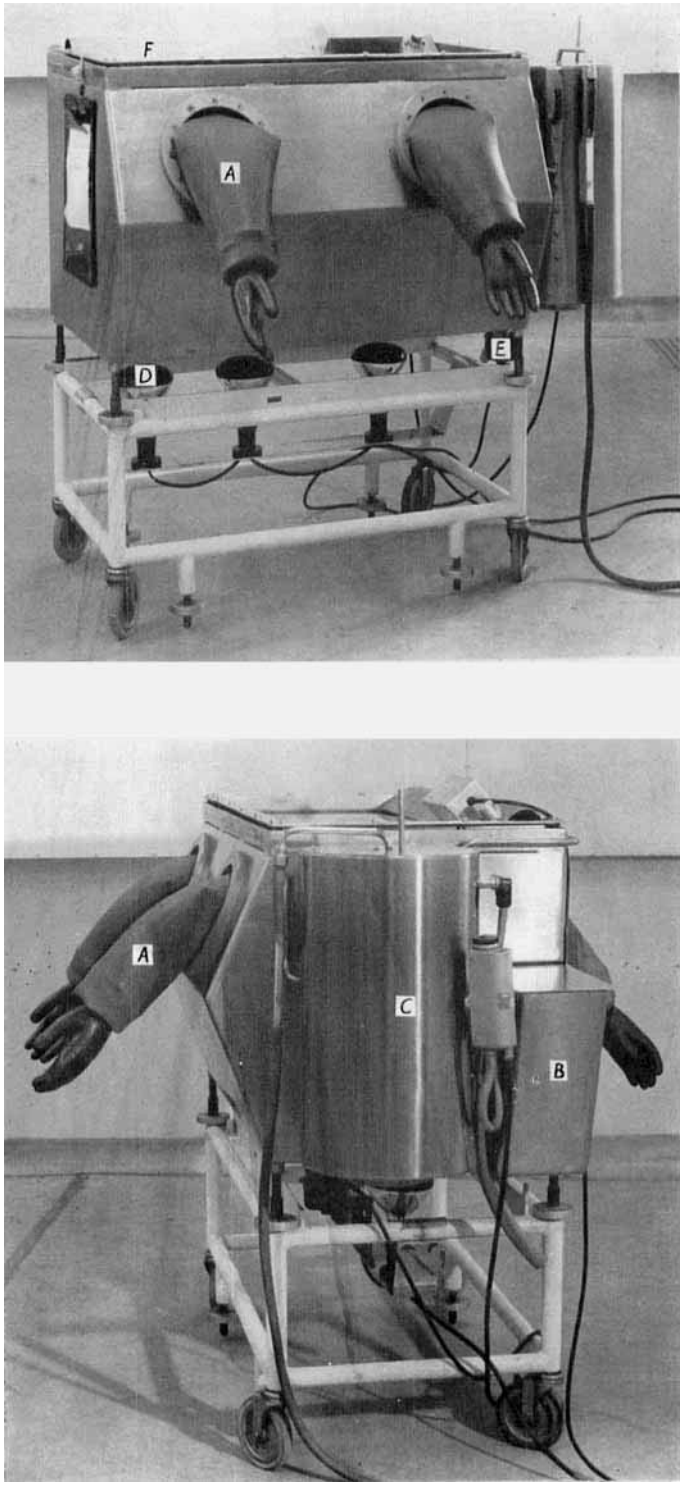

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