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CHOLERA VACCINES AND THE EL TOR VIBRIO

BY

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Cholera is an acute infectious disease caused by a Gram-negative comma-shaped organism, the *Vibrio cholerae*, of which three subtypes are usually described—namely, the Inaba, the Ogawa, and the Hikojima. These three types share common O antigens, and hence these organisms may be regarded as representing O forms of a single serotype (Wilson and Miles, 1955).

Another vibrio organism, the El Tor vibrio, is also capable of causing a cholera-like infection, and the resulting disease has been called paracholera. Epidemiologically, the El Tor vibrio causes sporadic cases, but though the morbidity may be low the mortality may be high. This vibrio shares "H" and "O" antigens in common with the true cholera vibrios; it can, however, be differentiated from the latter vibrios by various tests —for example, haemolysis of sheep's red blood corpuscles, a positive Voges–Proskauer reaction, and resistance to cholera bacteriophages.

A widespread paracholera epidemic, caused by an El Tor vibrio, was reported in 1961 from diverse countries in the Far East, including the British colony of Hong Kong. Considerable quantities of cholera vaccine, prepared in the David Bruce Laboratories, were despatched at the request of the Far Eastern Land Forces Command. However, doubts have been cast on the protective value of cholera vaccines against infections caused by the El Tor vibrios (Mukerjee and Guha Roy, 1962); and it was remarked (*British Medical Journal*, 1962) that there is a clear need to determine whether the available vaccines are able to protect against paracholera.

Cholera vaccine has been used for the protection of personnel at risk for many years. The current David Bruce Laboratories "vaccinum cholerae" is prepared according to the recommendations laid down by W.H.O. (1959); it consists of a heat-killed and phenol-preserved suspension of 4,000 million Inaba and 4,000 million Ogawa organisms per ml. in 0.5% phenol-saline.

In view of the evidence that El Tor infections were widely spread, it was decided to undertake experimental studies on the virulence of this organism as compared with the true cholera vibrios and to show by *in vivo* experiments whether cholera vaccine would afford protection to laboratory animals against El Tor organisms.

Material and Methods

1. Animal Virulence Tests

These tests were done by challenging mice and guinea-pigs intraperitoneally with graded doses of El Tor vibrios; control animals were challenged at the same time and under the same conditions with *V. cholerae*.

(a) *Mice.*—(i) Groups of mice (C/57/Black strain—10 mice in each group) were inoculated by the following graded doses of the challenge organism, suspended in saline. No mucin was used. Mice were observed for a period of 72 hours. El Tor vibrios, 25, 50, 100, 200, and 300 million organisms/mouse; *V. cholerae* (Inaba), 250, 500, 750, 1,000 and 1,500 million organisms/mouse. (ii) As for (i) above, using Strong A albino mice as test mice. (iii) As for (i) above, using the following strains and challenge organism: C/57/black mice, 12.5, 25, and 50 million El Tor vibrios per mouse; Theiler mice, 12.5, 50, and 100 milion El Tor vibrios per mouse; C/3H/Brown mice, 12.5, 50, and 100 million El Tor vibrios per mouse; B/Alb/C mice, 12.5, 50, and 100 million El Tor vibrios per mouse; C/57/Black mice, 250, 500, and 1,000 million *V. cholerae* (Inaba) per mouse.

(b) *Guinea-pigs.*—Animals of the Dunkin-Hartley strain weighing 250–300 g. were injected intraperitoneally with the following doses of the challenge organism suspended in plain saline: El Tor vibrios 2,000, 4,000, and 6,000 million organisms/guinea-pig; *V. cholerae* (Inaba) 2,000, 4,000, and 6,000 million organisms/guinea-pig.

2. Active Mouse-protection Tests

(a) Groups of mice (C/57/Black strain—five mice in each group) were immunized by two subcutaneous graded doses (two 0.1 ml., two 0.2 ml., or two 0.5 ml.) of an El Tor vaccine (prepared from El Tor vibrio N.C.T.C. 10256) or the David Bruce Laboratories cholera vaccine Batch No. 396. The second immunizing dose was given five days after the first dose. The test mice, and control groups of mice, were challenged by the following organisms and doses, injected intraperitoneally five days after last immunizing dose—namely, El Tor vibrios, 200 or 300 million organisms per mouse; *V. cholerae* (Inaba) 1,000 or 1,500 million organisms per mouse.

(b) Four groups of mice (Strong A albino—10 mice in each group) were immunized by two 0.5-ml. sub-

cutaneous doses, of either the David Bruce Laboratories cholera vaccine Batch No. 389 or an El Tor vaccine (prepared from El Tor vibrio N.C.T.C. 10255), the two doses being separated by an interval of 10 days. Ten days after the last immunizing dose the appropriate groups were challenged by an intraperitoneal injection of the following organisms and doses—namely, El Tor vibrios, 200 or 300 million organisms per mouse; *V. cholerae* (Inaba), 800 or 1,200 million organisms/mouse. Groups of non-immunized mice were challenged at the same time and under the same conditions to estimate virulence of the challenge organisms.

3. Passive Mouse-protection Tests

(a) Immune sera were obtained from rabbits, which had been immunized by a course of five injections (the first two being given subcutaneously and the last three intravenously), given every third day; the vaccines used were an El Tor vaccine and the David Bruce Laboratories cholera vaccine Batch No. 398. Four days after the last immunizing dose the rabbits were bled, the serum was allowed to separate from the clotted blood, and the sera were pooled. Groups of mice (Strong A albino strain—five mice in each group) were passively immunized by the subcutaneous injection of graded doses of either of the pooled sera—namely, by 0.1 ml., 0.2 ml., or 0.5 ml. of El Tor serum or *V. cholerae* serum. Four hours after passive immunization the mice were challenged by one of the following doses injected intraperitoneally: El Tor vibrio (N.C.T.C. 10255) 200 or 300 million organisms/mouse. *V. cholerae* (Inaba) (N.C.T.C. 7260) 1,000 or 1,500 million organisms/mouse. Control groups of mice injected with 0.5 ml. of neutral rabbit serum were also challenged at the same time by 200 million El Tor vibrios, 1,000 million *V. cholerae* organisms, or 0.5 ml. of plain saline.

(b) The rabbits used in the above experiment 3(a) were bled again eight days after the last immunizing dose, and groups of mice (C/57/Black strain—10 mice in each group) were injected subcutaneously with a constant dose of 0.5 ml. of immune serum. The passively immunized mice were challenged on the following day (24 hours after the injection of serum) by one of the following graded doses injected intraperitoneally: El Tor vibrios, 25, 50, or 100 million organisms/mouse; *V. cholerae*, 1,000, 1,500, or 2,000 million organisms/mouse.

4. Titration of agglutinin antibodies, contained in the pooled sera used in experiments 3(a) and (b), were done to check the effectiveness of the immunization programme. Doubling dilutions of serum from 1/20 were made in total volumes of 1 ml. of saline; standard drops of Inaba, Ogawa, or El Tor from live suspensions were then added to each tube. The agglutination racks were shaken by hand, incubated at 37° C. for two hours, placed in a refrigerator overnight, and finally stood at room temperature for two hours before the titres were recorded. The final titre recorded was that dilution of serum in the tube showing agglutination at the limits of naked-eye visibility—namely, granular deposit with turbid supernatant.

5. The El Tor vibrio strains 10255 and 10256, obtained from the National Collection of Type Cultures, were isolated from patients in the recent 1961 Hong Kong epidemic.

Results

1. *Animal Virulence Tests.*—These tests show that the El Tor vibrio is a very virulent organism. It proved much more fatal to mice and to guinea-pigs than a strain of true cholera vibrio (Inaba, N.C.T.C. 7260); the latter strain being the most virulent cholera strain held at present in these laboratories, and used as a standard challenge organism in cholera experiments and vaccine potency tests. Table I shows the results of the mouse virulence tests.

TABLE I.—*Mouse Virulence Test. Showing Comparative Susceptibility of Various Strains of Mice to El Tor Vibrios and V. cholerae (Inaba) Injected Intraperitoneally. Challenge Doses in Millions of Organisms per Mouse*

Experiment 1(a)	El Tor vibrios					*V. cholerae* (Inaba)						Mouse Strain
	12·5	25	50	100	Viability	250	500	750	1,000	1,500	Viability	
i	—	8	8	10	52%	0	0	5	10	—	31%	C/57/Black
ii	—	8	10	10	52%	0	2	5	7	9	31%	Strong A albino
iii	0	0	2	—	42%	0	5	—	10	—	—	C/57 Black
	0	—	4	10	42%							Theiler
	0	—	0	8	42%							C/3H/Brown
	0	—	5	10	42%							B/A1b/C

Note: Numbers represent dead mice (after 72 hours) from each group of 10 mice tested.

2. *Active Mouse-protection Tests.*—(a) In the first test all the test mice (C/57/Black strain), immunized both by the cholera vaccine and even by the homologous El Tor vibrio vaccine, failed to withstand an intraperitoneal challenge by the very virulent suspension of El Tor vibrios; while complete protection was afforded to mice, immunized by two 0.5-ml. subcutaneous injections of a cholera vaccine or an El Tor vaccine, against challenge by an LD_{100} dose of *V. cholerae*. (b) In the second experiment it was found that a cholera vaccine protected Strong A albino mice against challenge by either a true cholera vibrio or by the El Tor vibrio; it was also observed that mice protected by an El Tor vaccine were susceptible when challenged by a true *V. cholerae*. Table II shows the results of the active mouse-protection tests.

3. *Passive Mouse-protection Tests.*—(*a*) In the first test the results obtained were similar to those observed in experiment 2(a)—that is to say, the very virulent challenge suspension of El Tor vibrios proved fatal to all the test mice which were passively immunized, including those mice injected with the homologous serum. On the other hand, mice given El Tor serum proved more resistant to an intraperitoneal challenge by *V. cholerae* than the mice protected with the homologous (*V. cholerae*) *serum.* (*b*) The second experiment was modified in the light of 3(a) above by giving reduced graded doses of the El Tor vibrio. It was observed in this experiment that mice passively immunized with an El Tor serum or a *V. cholerae* serum were equally protected against a homologous and a heterologous challenge by either the El Tor vibrios or the *V. cholerae*. The virulence control tests of the challenge organisms used in this experiment were done at the same time and under the same conditions as the virulence tests reported in experiment 1(a) (iii) above

(see Table I—Virulence tests), where 100 million El Tor organisms killed 8–10 mice out of 10 in the strains of mice tested; hence it is considered very likely that 100 million El Tor vibrios used in this experiment represent an LD_{100} dose. Table III shows titres of agglutinin antibodies of the pooled sera, and Table IV shows the results obtained in the passive mouse-protection tests using these pooled sera.

TABLE II.—*Active Mouse-protection Tests. Number of Mice Surviving an Intraperitoneal Challenge by the El Tor Vibrio or by V. cholerae (Inaba), After Immunization by Two Subcutaneous Doses of El Tor Vaccine or V. cholerae*

Exp. No.	Type of Vaccine and Dose	Intraperitoneal challenge Doses in Millions of Organisms per Mouse				Mouse Strain
		El Tor Vibrio		V. cholerae (Inaba)		
		200	300	1,000	1,500	
2a	El Tor					
	2×0·1 ml. s.c.i.	0/5	0/5	4/5	1/5	C/57/ Black
	2×0·2 ml. s.c.i.	0/5	0/5	4/5	2/5	
	2×0·5 ml. s.c.i.	0/5	0/5	5/5	1/5	
	DBL cholera :					
	2×0·1 ml. s.c.i.	0/5	0/5	3/5	2/5	
	2×0·2 ml. s.c.i.	0/5	0/5	4/5	0/5	
	2×0·5 ml. s.c.i.	0/5	0/5	5/5	2/5	
	Virulence control of challenge organisms: 25m. 50m. 100 million El Tor orgs./mouse. Viability 52%			500m. 750m. 1,000 million *V. cholerae* (Inaba) orgs./mouse. Viability 31%.		
		2/10	2/10	0/10	10/10	5/10	0/10	
		200	300	800	1,200	
2b	El Tor 2×0·5 ml. s.c.i.	8/10	4/10	3/10	0/10	Strong A Albino
	DBL cholera : 2×0·5 ml. s.c.i.	8/10	3/10	9/10	5/10	
	Virulence control of challenge organisms: 50m. 100m. 200 million El Tor orgs./mouse. Viability 30%.			250m. 500m. 750 million *V. cholerae* (Inaba) orgs./mouse. Viability 32%		
		5/10	3/10	0/10	10/10	3/10	0/10	

Note: Numerators represent survivors, denominators represent the number of mice in each test group.

Discussion

Virulence

Under the conditions in these laboratories, and set out in these experiments, the El Tor vibrio isolated from patients in the Hong Kong epidemic of 1961 has proved much more fatal to mice and guinea-pigs than the most virulent *V. cholerae* strain held here, and used by us as a standard challenge organism in cholera experiments on laboratory animals. Attention to the virulence of the El Tor vibrio had been drawn in the early years of the century by Kraus and Pribram (1905), who stated that this vibrio secretes a soluble haematoxin, as well as an exotoxin rapidly fatal to experimental animals. Thirty-four years later Takita (1939) confirmed that the El Tor vibrio produces a true thermolabile exotoxin, distinct from haemolysin, which proves fatal to mice on intraperitoneal injection.

TABLE III.—*Agglutinin Titre of Pooled Rabbit Sera Against Live Suspensions of Inaba, Ogawa, and El Tor Vibrios*

Exp. No.	Vaccine	Live Suspension		
		Inaba	Ogawa	El Tor
3a	El Tor	2,560	2,560	2,560
	D.B. Lab. cholera	10,240	2,560	2,560
	Neutral rabbit serum (controls)	0	0	0
3b	El Tor	2,560	2,560	1,280
	D.B. Lab. cholera	2,560	1,280	2,560

Note: Titre recorded as the lowest dilution of serum in the last tube showing granularity of deposit visible to the naked eye.

It is generally accepted that the true cholera vibrios do not secrete a true soluble exotoxin but contain endotoxins which are liberated on dissolution of the bacterial bodies; nevertheless recently Professor De (1961) has claimed that the true cholera vibrio produces an exotoxin—namely, an enterotoxin. Watanabe and Felsenfeld (1961) studied the toxins produced by El Tor vibrio, and summarized thus: "The present evidence suggests that the haemolytic and mouse lethal toxins are different, but the possibility of these two activities residing within the same molecule has not yet been ruled out completely. The separation of the two sections by chemical means represents a desirable goal in any attempt to reveal the possible significance of these two toxin actions in the pathology and immunology of para cholera."

Active Mouse-protection Tests

The first recorded experiments utilizing mice inoculated with cholera vaccine, and subsequently challenged by vibrios suspended in saline, are those of Fennel (1919), who showed that inoculated mice were protected against four or five lethal doses. During the second world war Ranta and Dolman (1943), considering the possibility that cholera vaccine might be needed for Canadian troops, worked out a method of preparing and standardizing cholera vaccine in bulk from suitable selected strains; in the course of carrying out this assignment they observed that vaccinated mice challenged with 2 M.L.D. of a saline suspension of *V. cholerae* died more quickly than unvaccinated con-

TABLE IV.—*Passive Mouse-protection Tests. Number of Mice Surviving an Intraperitoneal Challenge by El Tor Vibrio or V. cholerae (Inaba), After Passive Immunization by Pooled Rabbit Serum, Produced by El Tor Vaccine or DB Lab Cholera Vaccine*

Exp. No.	Type of Serum and Dosage	Intraperitoneal Challenge Doses in Millions of orgs./Mouse				Mouse Strain
		El Tor vibrio		*V. cholerae* (Inaba)		
		200	300	1,000	1,500	
3a	El Tor 0·1 ml. s.c.i.	0/5	0/5	1/5	2/5	Strong A albino
	El Tor 0·3 ml. s.c.i.	0/5	0/5	2/5	2/5	
	El Tor 0·5 ml. s.c.i.	0/5	0/5	3/5	2/5	
	Cholera 0·1 ml. s.c.i.	0/5	0/5	1/5	0/5	
	Cholera 0·3 ml. s.c.i.	0/5	0/5	0/5	0/5	
	Cholera 0·5 ml. s.c.i.	0/5	0/5	1/5	0/5	

Control groups of mice. Ten mice inoculated with 0·5 ml. rabbit serum and challenged by 200 million El Tor orgs.—0/10.
Ten mice inoculated with 0·5 ml. rabbit serum and challenged by 1,000 million Inaba orgs.—2/10.
Ten mice inoculated with 0·5 ml. rabbit serum and challenged by 0·5 ml. saline—10/10.

		25	50	100	1,000	1,500	2,000	
3b	El Tor 0·5 ml. s.c.i.	10/10	10/10	9/10	8/10	5/10	5/10	C/57/ Black
	Cholera 0·5 ml. s.c.i.	10/10	10/10	8/10	9/10	3/10	3/10	
3a	Virulence control of challenge organisms: 25 m. 50 m. 100 million El Tor orgs./mouse. Viability 52%. 2/10 0/10 0/10							
	250 m. 500 m. 750 m. 1,000 m. 1,500 million Inaba orgs./mouse.							
3b	10/10 8/10 5/10 3/10 1/10 Viability 31%.							
	12·5 m. 25 m. 50 million El Tor orgs./mouse. Viability 42%. 10/10 10/10 8/10							
	250 m. 500 m. 1,000 million Inaba orgs./mouse. Viability not recorded. 10/10 5/10 0/10							

Note: Numerators represent survivors, denominators represent the numbers of mice in the test group.

trols—that is, instead of being protected against intraperitoneal injections of living *V. cholerae*, the vaccinated mice became sick sooner and died more quickly when given a challenge dose than did control mice. They thought that vaccinated mice developed bacteriolysins, which caused accelerated lysis of the large number of vibrios introduced in the challenge dose, so that death occurred from acute toxaemia.

There are objections to the use of large numbers of vibrios for challenge doses in mouse-protection tests, and many workers in the last 20 years have followed the lead of Griffitts (1942), who showed that the virulence of the cholera vibrio on intraperitoneal injection in the mouse was markedly enhanced by suspension in 5% mucin. In our laboratories, however, it is our routine to suspend the challenge organism (of various genera and species) in physiological sodium chloride (0.85% NaCl); it will be appreciated, as Burrows *et al.* (1947a) pointed out, that the fatal infection of mice with *V. cholerae* suspended in mucin is a highly artificial one.

Our first experiment (2a) was inconclusive—that is, it did not give the answer whether a cholera vaccine would protect mice against El Tor vibrios. It is possible that the intervals of five days between the two immunizing doses, and between the second immunizing dose and the challenge dose, may well have been too short to allow the optimal effects of the two vaccines to be produced. In most of our work, not reported here, longer intervals are allowed (10–14 days), though Burrows *et al.* (1947b) state that active immunity develops rapidly in the mouse, and does not seem to increase appreciably after the fourth day after inoculation. It is felt that the challenge suspension of the very virulent El Tor suspension was comparatively overwhelming in relation to the protection afforded to mice by active immunization.

In the second experiment (2b) the test mice inoculated with either the cholera vaccine or the El Tor vaccine were protected against the El Tor vibrio, but against challenge by the cholera vibrio only the homologously protected mice survived in significant numbers.

Passive Mouse-protection Tests

Griffitts (1944a), having in mind the practical importance of the protection afforded by immunization for service personnel of the U.S. Armed Forces assigned to duty in regions where cholera is endemic, investigated the appearance and persistence of serum antibodies in man after injection with cholera vaccine. He showed that mice could be of definite value in demonstrating specific protective substances in serum against cholera organisms, and that these substances may be present in the absence of agglutinins for the vibrios.

Ideally when testing the relative merits of strains or different types of cholera vaccine, all available methods should be employed; of the methods available to the laboratory worker the passive mouse-protection test has been considered the most sensitive by Ahuja and Gurkirpal Singh (1948). It is worth noting that passive protection tests are subject to two objections: (1) these tests, requiring a source for immune serum, introduce a second animal variable, and (2) important factors contributing to the immune status of actively immunized animals are not subject to study by serum protection tests (Griffitts, 1944b).

In experiment 3(a) the test mice, passively immunized with El Tor serum or *V. cholerae* serum, were not protected against challenge by 200 million El Tor vibrios injected intraperitoneally; this is attributed to the comparative high virulence of the challenge organism (see experiment 2(a)). The El Tor serum, on the other hand, afforded protection to test mice against challenge by the cholera vibrio; indeed, it proved superior to the cholera serum in the degree of protection exhibited.

In experiment 3(b) no difference in protective power was seen between the two pooled immune sera, and, what is important, the *V. cholerae* serum protected test mice against a challenge by the El Tor vibrio.

The earliest literature on the subject of El Tor vaccine that came to hand while reviewing past work in this field of immunology is that of Kraus and Kovacs (1928), who showed that El Tor vaccines protected laboratory animals against El Tor vibrios and also against cholera vibrios; furthermore, antitoxins produced by El Tor vibrios neutralized toxins of El Tor and of cholera vibrios, such antitoxins having a prophylactic and a therapeutic effect.

Felsenfeld and Young (1945), trying out vaccines prepared by various methods with Inaba, Ogawa, and El Tor strains, found that the best results were achieved by the use of formol-killed Inaba organisms combined with formolized filtrates of El Tor strains.

Further work is proceeding in these laboratories; it may well be that the present vaccinium cholerae could be improved by making a trivalent vaccine, incorporating El Tor strains in addition to the recommended Inaba and Ogawa strains. This would finally have to be tried out under field conditions such as are found in countries of the Far East.

Summary

The El Tor vibrio causing the 1961 Hong Kong epidemic was investigated and compared with a true cholera vibrio. Virulence tests in mice and guinea-pigs, active mouse-protection tests, agglutinin production in rabbits, and passive mouse-protection tests were done.

It was shown that the El Tor vibrio is a very virulent organism; it was also shown that a cholera vaccine may give protection against El Tor vibrio, and that, similarly, a cholera serum may afford a certain degree of protection to passively immunized mice against the El Tor vibrio.

Further work is proceeding in the David Bruce laboratories. It is suggested that a trivalent cholera vaccine, incorporating Inaba, Owaga, and El Tor strains, may be an improvement on the present vaccine, but this can be settled only by field trials.

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APPARATUS FOR CONTINUOUS INFUSION CHEMOTHERAPY

BY

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Following the original use of intra-arterial nitrogen mustard by Klopp *et al.* (1950), several workers have developed arterial infusion techniques for the treatment of localized malignant disease (Sullivan *et al.*, 1959; Horwitz, 1960; Westbury *et al.*, 1962; Espiner *et al.*, 1962). It has been found that with the most commonly used antimetabolite, methotrexate, optimum results are obtained after at least two weeks of continuous infusion. This necessitated the development of simple and reliable apparatus for intra-arterial infusion. The related technique of continuous intravenous infusion of alkylating agents (K. A. Newton, personal communication 1960) requires somewhat similar apparatus.

In most cases a pressure of 170 mm. Hg (90 in. H_2O) is adequate for intra-arterial infusion, and this can easily be provided by gravity feed. However, the drip-infusion technique has three main deficiencies. In the first place, practical considerations limit its use in hypertensive patients who may require infusion pressures as high as 350 mm. Hg (190 in. H_2O). Secondly, it is difficult to control the volume of fluid infused to less than 1,500 ml. a day with a conventional drip-chamber, and this may be excessive in children and patients with cardiac failure or impaired renal function. Finally, since the hydrostatic pressure is constant there is no increase in pressure during incipient blockage of the catheter or increased resistance due to movement by the patient. Consequently there are frequent variations in flow rate and complete cessation sometimes occurs.

It is possible to control the infusion at a lower rate by the use of the micro-drip chamber shown in Fig. 1. This consists of a Pasteur pipette (A) with a silicone

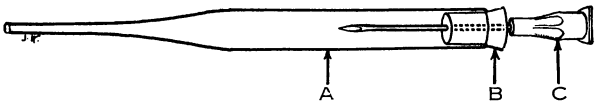

FIG. 1.—Micro-drip chamber.

rubber stopper (B) through which passes a 0.7-mm. hypodermic needle (C). With this simple refinement it is possible to control the infusion at a rate of approximately 500 ml. a day. The difficulties caused by variations in infusion rate have been alleviated by designing a drip-rate monitor and alarm. This device is operated by a special drip-chamber containing two platinum electrodes (Evans, 1955); each drop of fluid momentarily connects the electrodes, thus providing a signal to operate the monitor. The apparatus is driven by a 6-volt battery and provides visual indication of each drop. The drip rate is displayed on a meter calibrated from 0 to 50 drops a minute, and an alarm operates if the rate falls below 10 drops a minute for more than 1½ minutes.

These devices help considerably in the management of arterial drip infusions, but when the blood-pressure is so high as to preclude the use of this method or when

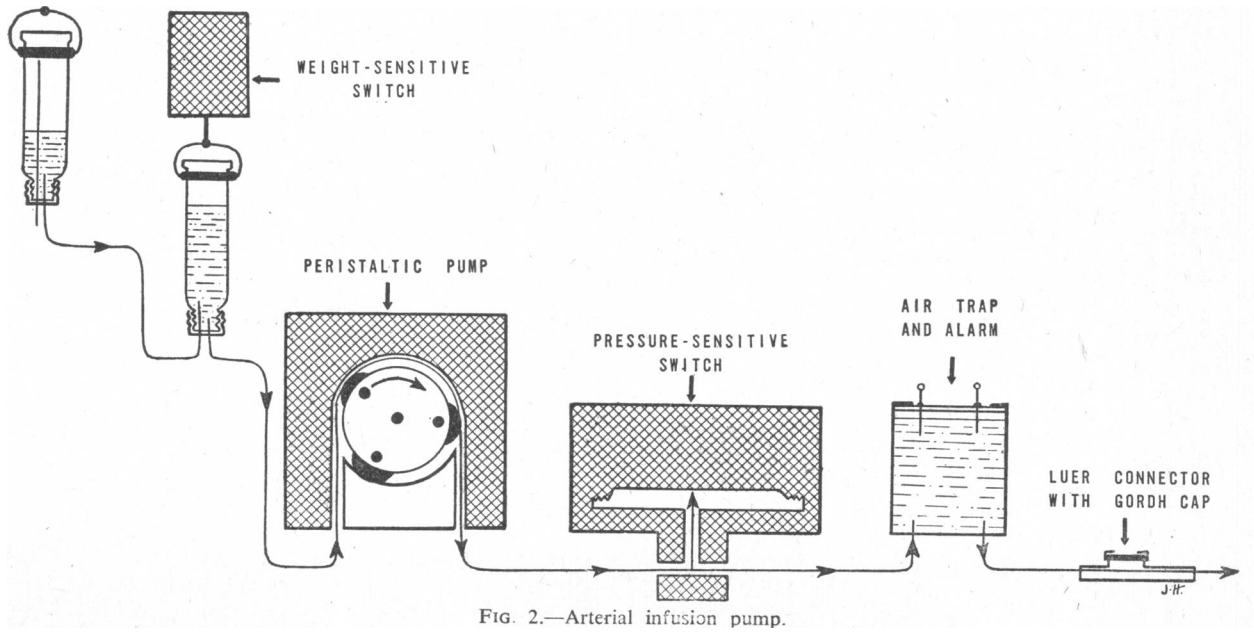

FIG. 2.—Arterial infusion pump.