

CHOLERA

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Introduction

THERE have been in the past six pandemics of cholera recorded in medical history—1817, 1829, 1852, 1863, 1881, and 1899 (Pollitzer 1959a). The sixth pandemic ended in 1921—the year when I was born. Consequently I grew up, and in due course passed my medical apprenticeship, in an atmosphere where cholera was considered in medical circles as a dead duck!

It had been pushed back, or should I say more accurately it had retreated to its putative homeland, namely the deltas of the Ganges and Brahmaputra, and probably the Yangtze in the far, far east; the cholera problem was swept under the carpet; and the human species in the twentieth century could pat itself on its sapient head and congratulate itself as being the outright winner against its bacterial opponent—Never again! It shall not pass!

That I had not been singularly unfortunate in my ignorance of this medical ‘time-bomb’ was corroborated by the editor of the *Lancet* who commented less than three years ago:

“To most medical students in Europe and the United States cholera is a name and no more; and their examiners have not much experience of the disease and are unlikely to ask questions about it.”

Lancet 1971.

But is there a physician with soul so dead that in his life-time could truly say that man has verily licked and eradicated any communicable (nee infectious) disease?

“All micro-organisms are subject to mutation, presenting the reticuloendothelial artificial vaccine production with fresh unexpected antigen/antibody problems. Thus nothing potentially remains static and methods of established treatment or protection can be upset. No matter what we do, or how successfully, living organisms cannot be eliminated in global terms, and they fight back to live, and even new pathogenic species can be expected.”

J. Macrea (1972).

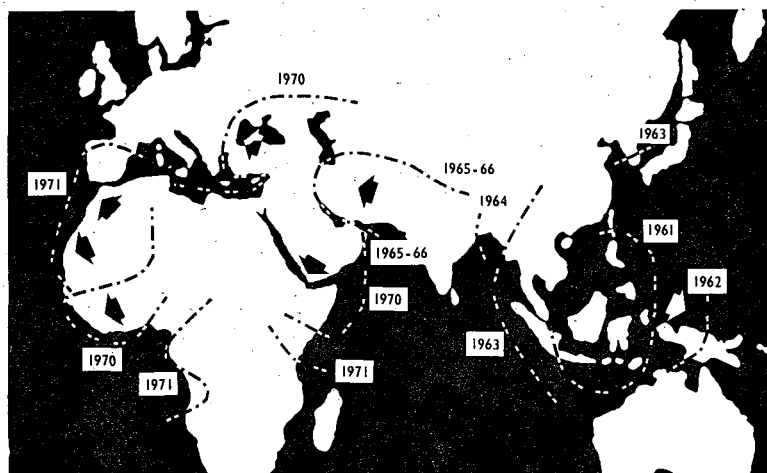
The sixty-four dollar question on Cholera asked in my earlier days was:

“Since the disease has receded and disappeared completely from regions which have been invaded more than once and which have shown epidemic outbreaks in consecutive years, why does it persist in India and a very few of its contiguous countries? Why is cholera endemic in this area?”

A. M. Kamal (1963)

In the event my own innocent choleralogic virginity was rudely shattered on my very first overseas posting in the army medical service—Cholera in Egypt 1947, which was the prelude (and with the wisdom of hindsight not the only one: the prior epidemic in the Celebes of 1937 and the later Thai epidemic of 1958) to the present and seventh pandemic which in the past fourteen years has undoubtedly surpassed itself and succeeded

in reaching countries not visited by cholera in the preceding episodes. The global spread from 1961 to 1971 is shown in Figure 1.



(Reproduced by the Department of Medical Illustration Manchester University)

Fig. 1. Global spread of cholera 1961-1972 (W.H.O.)

“After remaining relatively quiescent in 1972 and the first part of 1973 cholera came into prominence when it invaded Italy and several imported cases occurred in four other European countries. It also caused concern in the drought-affected areas of West Africa. Mozambique and Malawi, which had not previously recorded the presence of cholera in this pandemic reported cases in September and October.

The global cholera situation reports for the first five months of 1973 showed that six countries in Africa and nine in Asia were affected, but by the 24 October 1973, thirteen countries in Africa and fourteen in Asia and one in Europe (besides imported cases in France, Germany, Sweden and the United Kingdom) were being hit by cholera”.

World Health Organisation (1973a)

Indeed cholera poses an awful threat to the Central countries of the American continent, and once it established a bridgehead in those countries, cholera could spread as disastrously as did the epidemic of shigellosis three years ago. The Latin American countries have been warned, and alerted.

“In view of the possibility of the extension of cholera to areas with poor sanitation in Latin and Central America, the W.H.O. has assisted Member States in that region to strengthen their surveillance activities and treatment facilities by training the public health personnel through formal courses and seminars”.

World Health Organisation (1973b)

Bacteriology: The ABC of cholera

“There are more vibrios in heaven and earth, O Robert Koch, than are dreamt of in your philosophy!”

a. Bacteriologists are aware that there are dozens of vibrios, halophilic, phosphorescent, micro-aerophilic, etc. and have tried (and for that matter are still trying) to put their house in order. The vibrios are motile bacilli with a tail, hence they possess H (flagellar) and O (somatic) antigens; the H antigens, unlike the H phase 1 antigens of the *Salmonellae* are not of great importance in identification, whereas the O antigens, as in the *Shigellae* organisms are very useful. Thus by means of six diagnostic sera prepared from the somatic O antigens, the vibrios can be classified in 6 groups from I to VI; the cholera vibrio (hereafter called VC) as befits its importance was placed in Gp 0-1 (Gardner and Venkatraman 1935).

Japanese workers further sub-divided the VC in 3 serotypes mainly on the basis of the two somatic antigens which they called B and C (one is somehow reminded of the *Brucella* species classification on the basis of the *Brucella* A and M antigens) thus at present we have

AB—Ogawa. AC—Inaba. ABC—Hikojima.

the A somatic antigens being a common antigen to the three serotypes. More recent work in Japan suggests that the Inaba type may well be the progeny of the Ogawa—Hikojima complex, the latter two types differing essentially only quantitatively (Sakazaki and Tamura 1971a).

American and French workers in the 1940's detected further antigens D, E and so on up to M, but these somatic antigens have not come in general use, and will not be further discussed (Burrows et al 1946, Gallut 1949).

It is of interest to record that Ogawa and Inaba serotypes were so named after the Japanese patients from whom these types were first isolated, whereas Hikojima was isolated from a carrier detected at that quarantine station in Northern Kyushu in Japan (De 1967).

The other quarantine station vibrio which is causing the 7th cholera pandemic is the El Tor (the West Sinai Quarantine Station) *Vibrio* which since the mid-1960's has been accorded full international status as a VC after a full half century of its discovery by Gotschlich (1906), Feeley (1966). As a true VC it falls by definition in the same somatic antigenic classification described above. However bacteriologists would not let well alone, and by means of laboratory tests can distinguish the colours of its coat from those of the classical VC (Table I). It is therefore known as a biotype and even in

Table I
Vibrio El Tor biotype

Phage IV (Mukerjee)	Resistant	Mukerjee, S. 1970
Haemolysin	Positive	Greig, E. D. W. 1914
Haemagglutination	Positive	Finkelstein and Mukerlee 1963
Voges—Proskauer	Positive	Gan and Tsia 1963
Polymyxin B	Resistant	Powell and Finkelstein 1964

this green and pleasant land we have become accustomed during the last decade to read bacteriology forms reporting that from a patient's stools the El Tor biotype, Ogawa

serotype of the *Vibrio cholerae* has been isolated or *Vibrio cholerae*, serotype Inaba, El Tor biotype as the case may be—depending on the particular strain prevalent at a particular time in a particular locality; thus while in East Africa the prevalent type is Inaba, the corresponding El Tor vibrio in the West African countries has been serotyped as an Ogawa (De Maeyer-Cleempoel 1972). The inexorable spread of El Tor by sea and fresh water routes in these African countries for the first time in history is a very ominous feature of the present pandemic (Barua 1972).

The El Tor may well be poised to spring next upon the Central American states, as has been remarked above. The attention of the reader is drawn to the fact that this particular biotype is naturally (*sensu stricto*) credited with certain characteristics that tend to favour its spread and its persistence in the environment once it is deposited there (Felsenfeld 1965, Miyaki et al 1967, Yefimtseva et al 1972).

“As compared with the classical biotypes, the El Tor

1. Is hardier. 2. Remains viable much longer in water. 3. Is shed by patients over a longer period of time (note the counterpart to Typhoid Mary namely Cholera. Dolores (Azurin et al 1967a). 4. Produces a lower incidence of clinically apparent disease”.

Sommer and Woodward (1972)

b. In the perusal of literature in this field one comes across such acronyms as NAG, NCV and NVC (Spaun et al 1970, Dutta, Panje and Jhala 1963, McIntyre et al 1965).

Non-agglutinable vibrios (NAG) are in actual fact not non-agglutinable, that is to say these vibrios can be agglutinated by an appropriate homologous serum hence this term should be dropped.

Non-cholera vibrios (NCV) are vibrios isolated on occasions from patients suffering from diarrhoea but which do not conform to the serological classification stated for the true VC; some authors apply this term to vibrios which are isolated from the bottom of one's garden.

Non-vibrio cholera (Linbenbaum et al 1965) occurs where a physician firmly diagnoses cholera and hurriedly informs the area community medicine specialist (nee local Ministry of Health) that there is cholera in the district; there then ensues a Brouhaha but two or three days later the unlucky bacteriologist produces an organism other than a vibrio. As can be seen this subject is rather a nagging problem for the microbiologist.

“During a flight from London to Sydney, via Frankfurt, Bahrain and Singapore, about 60 people suffered from abdominal pain, vomiting and diarrhoea.

It became known later that a non-agglutinable cholera vibrio (not *Vibrio parahaemolyticus*) had been isolated.

Non-cholera vibrios, indistinguishable from cholera vibrios by routine biochemical tests are not agglutinated by cholera antiserum. They have for long been considered of doubtful pathogenicity. But there are many accounts of outbreaks of gastro-enteritis that have been associated with these vibrios. Some strains have been shown to produce a toxic factor very similar to, if not identical with, that of the true cholera vibrios”.

British Medical Journal (1973)

c. The reader must be reminded that the ad-hoc classification of vibrios (and other organisms) is man-made and artificial.

No one has informed the VC of this pidgeon-holing so that the VC takes enormous pleasure to change from Inaba, to Ogawa, to NCV and to NAG at its own will and leisure—just to cut *Homo sapiens* to size, I suspect, and who are we to criticise the genetic arrangements of a fellow organism co-habiting on Planet Earth? (Sakazaki and Tamura 1971b, Sack and Miller 1969, Gangarosa et al 1967, Miller et al 1972).

It may well be that a New Look is needed to up-date the classification of vibrios. One such suggestion from Japan proposes that the VC (both classical and El Tor) should be known as *Vibrio cholerae*, serotype 1 and other vibrios should be reclassified as *Vibrio cholerae*, serotype 2, serotype 3 and so on, that is to say the term *Vibrio cholerae* should not be restricted to the classical/Et Tor biotypes, but should include NCV/NAG (Sakazaki et al 1970). From the United States of America on the other hand it has been tentatively suggested that NAG and NCV should be renamed as a new species *Vibrio enteritidis* to include all strains associated with diarrhoea in man but not agglutinated by cholera serum thus affecting a compromise between the demands of taxonomy and the needs of epidemiology (Finkelstein 1973).

I cannot help but extend my sympathy, and admiration, to the bold anonymous member of the First Expert Committee on Cholera held in New Delhi 1951 who proposed that:

“actually all descendants of a vibrio which caused cholera in man are capable of doing so are entitled to that name, irrespective of their shape, size, cultural and biochemical reaction, chemical and antigenic constitution, or pathogenic and epidemic behaviour, unless they have undergone permanent mutation”

World Health Organisation (1952)

Alas he was over ruled—nem. pro!

The toxins of cholera

“It would seem that the cholera poison when reproduced in sufficient quantity, acts as an irritant on the surface of the stomach, and intestines, or what is still more probable, it withdraws fluid from the blood circulating in the capillaries, by a process analogous to that by which the epithelial cells of the various organs abstract the different secretions of the healthy body”.

John Snow (1855)

It is characteristic in cholera that bucketfuls of fluids pour out of the unfortunate patient from either end—the fluid which is sluiced out from the nether region is described as a ‘rice-water’ stool. During the 1940’s in my undergraduate days the rice-water stool, which looked like dirty water containing flakes of ‘material’, was attributed quite plausibly to the mucinolytic enzymes which peeled off the intestinal epithelium, the denudation and desquamation of which thus allowed the body fluids to surge out (Bannerjee 1939, Burnet and Stone 1947).

Subsequently in the 1950’s when I was preparing for the D.T.M.&H. the explanation then in vogue carried in all textbooks of tropical medicine was that the endotoxins of the VC interfered with the Sodium pump mechanism—that is to say the process by which the intestinal cell pulls in actively the sodium ion from the lumen back into the plasma, and water molecules followed this shift.

"The theory that the cholera stool is a sort of transudate which results from the sloughing of the intestinal mucosa has been disproved.

A theory has been propounded to explain the very copious water and electrolyte loss in this disease. This theory involves the elaboration by the cholera vibrio of an inhibitor of the active sodium transport by the mucosal cell.

Such a sodium pump inhibitor has been found repeatedly in the stools of patients suffering from classic Asiatic cholera and from disease due to the El Tor Vibrio."

Phillips (1963)

Alas this explanation has also been found wanting, during the great stimulus to research on intestinal physiology and pathology for which the present pandemic of cholera has been the prime mover (Furhman and Furhman 1960, Fuhrman, Fuhrman, and Burrows 1962).

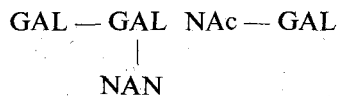
To bring some order out of the chaos, from the University of Chicago Burrows (1968) proposed the following classification of the cholera toxins at least as a sort of spring board from which further progressive steps could be undertaken (Table II).

Burrow's Type 1 Toxin corresponds to the somatic antigens, it consists of lipopolysaccharides and it is found in the bacterial cell wall and body of the bacteria. Whatever damage as a Gram-negative endotoxin it may do to the patient it is not the primary cause of the flux in cholera which is now known definitely to be caused by the Type 2 Toxin (cholera toxin, enterotoxin, exo-enterotoxin). The Type 2 is found free in the supernatant and/or in the filtrate of a bacterial culture. There is still much speculation as to whether it consists of one factor giving rise to appropriate effects according to the target cells or whether it is multifactorial in composition. Thus one can demonstrate a Diarrhoeal Factor (DF) which causes the intestines to hypersecrete ions and water, and a Permeability Factor (PF) which dilates blood vessels (Craig 1965). The toxin also has an effect on fat cells causing lipolysis, and so on (Vaughan, Pierce and Greenough 1970, Zieve, Pierce and Greenough 1970, Bourne Lichtenstein and Henney 1972). In relation to the diarrhoea characteristic of cholera the DF is the factor which is of the greatest importance. This toxin has been obtained in a Purified crystalline state by Finkelstein and LoSpalluto of the University of Dallas, in Texas, United States of America (Finkelstein and LoSpalluto 1969, 1970, 1972, LoSpalluto and Finkelstein 1972).

To the third toxin, Burrow's Type 3 Toxin, was attributed formerly the seizing up of the Sodium Pump—however as has been mentioned above the bucketfuls of the cholera evacuations are no longer attributed to the interference with this mechanism, in fact one can hardly call this type a toxin in the usual manner of speaking—VC filtrates free from ammonium ions, which may have caused erroneous results reported in earlier work, are still capable of inducing a typical cholera diarrhoea (Grady et al 1967, Norris et al 1967, Leitch, Burrows and Stolle 1967, Grady et al 1968).

Furthermore the VC also produces Mucase and Receptor destroying enzyme (RDE) neuraminidase, sialidase, and other enzymes as well (Krishna Murti 1968, Datta and Mitra 1970, Leitch 1972). It is very tempting to assume that the possession of these active substances must confer some benefit to the VC either as helping it in its attack on its victim-host or as a means of defence, the former alternative being the more probable. A reasonable working hypothesis that comes to mind is that the mucase

removes the film of defensive mucus that shields the cells of the intestinal mucosa and possibly helps to keep down the numbers of bacterial flora in the upper part of the small intestine, and thus enables the VC to get intimately adsorbed to the intestinal cells while the neuraminidase provides more receptor sites with which the cholera toxin can combine. It is thought that the absorption of the VC to the epithelial cells is a vital step in the pathophysiology of cholera since the VC can then deposit its Type 2 Toxin in direct proximity to the cell membrane of the epithelial cells, especially the active region of the ganglioside Gm1. (Freter, Smith and Sweeney 1961, Peterson, Lospalluto and Finkelstein 1972, Van Heyningen et al 1971, King and Van Heyningen 1973, Pierce 1973, Holmgren, Lonnroth and Svennerholm 1973a, Lonnroth and Holmgren 1973, Cuatrecas 1973a and b). This critical region in Gm1 postulated by Holmgren, Lonnroth and Svennerholm 1973b, as the receptor site for cholera toxin is the portion:



where NAN = N-acetylneuraminic acid, GAL NAc = N-acetylgalactosamine, and GAL = Galactose.

It is possible that neuraminidase production may facilitate entero-toxicity by uncovering more receptors (this enzyme can convert many gangliosides to Gm1 and so helping the toxin to find more binding sites though not being strictly essential for the DF action of the cholera toxin.

Raison d'être of the cholera toxin?

"There is no clear reason for the production of exotoxin by the VC—as far as it is known no useful function has been accorded to the cholera toxin in the vibrio's own metabolism"

Banwell and Sherr (1973)

The most attractive hypothesis that I have come across is the one put out by that scientific (yet practical) American R. A. Finkelstein who suggests that the vibrio makes use of its human host-victim to serve as its mail-man (postman) in disseminating far and wide the teeming millions of its descendants, and thus give this none-too-hardy micro-organism the best chance for the continued survival of its species.

The Thiry-Vella loop and other animals

"Man is a wonderful piece of portable plumbing".

Though experiments on human volunteers and tests on patients have been used successfully and fruitfully in modern Choleraology, the supply of these live and co-operative agents is inadequate and erratic and recourse had to be taken to the utilisation of inferior (sic) animals.

"The feasibility of establishing chronic intestinal loops which retain normal absorptive function over long periods of time, has allowed for serial studies on an animal which had not been recently subjected to surgical procedure and anaesthesia, thus approaching more satisfactorily the conditions of nature. Chronic Thiry-Vella loops of 60-70 cm segments of various sections of the small intestine were found to respond to intra-luminal instillation of enterotoxin with less variability than the entire intact small bowel. Furthermore once installed such loops remained functional several months.

The development of the canine model has therefore made a major contribution to the investigation of cholera and cholera toxins and provided an important stimulus to studies which, in other animal models, could not be attempted "

Craig (1971)

Take a dog, slit the abdominal wall, isolate a loop of intestine keeping its vascular supply intact and bring out the two ends to open on the exterior of the abdominal wall; restore the continuity of the alimentary tract by an end-to-end anastomosis, suture the abdominal incision and you are in business (Fig. 2).

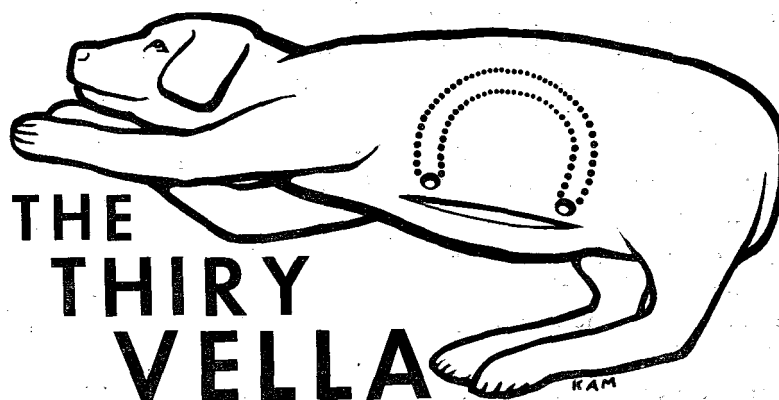


Fig. 2. The Thiry-Vella loop.

What can one do? The possibilities are multiple and varied:

a. A bacterial culture of VC can be placed in the loop, and one can observe the reaction of the intestinal loop, in a way reminiscent of the American Army Surgeon William Beauchamp and his patient the Canadian trapper Alexis St. Martin with his epoch making gastric physiology demonstration.

b. One can show the existence and the toxicity of a supernatant/filtrate. Indeed one does not need the whole bacterial culture as such—the supernatant or filtrate from a VC culture will produce the cholera effect thus showing the factual presence of an exotoxin. Various batches of toxin produced in different media, under different cultural conditions, from various strains of the VC can be compared and titred as regards toxicogenicity.

c. By estimating the volume and electrolyte composition of the fluid accumulating in the loop, and comparing the biochemical values with those of the plasma one can study the fascinating pathophysiology of cholera.

d. The animal can be treated with antitoxin, drugs or vaccines and the effects of these or the action of the cholera toxin can be evaluated.

As stated above in this experimental live model the animal can be kept alive and well, and therefore can be made the subject of more than one successive experiment. Animals other than the dog can be used, and moreover the Thiry-Vella loop technique can be utilised for other organisms (Bywater 1973); the increasing number of Entero-

pathogenic coliform organisms which are capable of producing an enterotoxin can be identified by this technique, the hitherto commonly used and still practised serological typing of these EPCO's is not entirely adequate to identify the exotoxin-producing strains of these intestinal pathogens. Other organisms producing exotoxins detected are *Clostridium welchii* (perfringens), *Pseudomonas aeruginosa*, *Shigella dysenteriae* 1, and the more recently investigated *Vibrio parahaemolyticus* (sea food) and *Bacillus cereus* (Chinese restaurants).

In fairness to my reader I must not leave him or her under the impression that the Thiry-Vella loop is the only animal model technique, or even the most commonly used in cholera research—ligated intestinal segments in which portions of the intestines of the desired length are tied in segments and replaced in the abdominal cavity without interrupting the physical continuity of the intestinal wall, and also suitably chosen and prepared 'intact' animals not subjected to surgical procedures are utilised to a greater extent (Finkelstein, Norris and Dutta 1964, Swallow, Code and Freter 1965, Kasai and Burrows 1966, Sack et al 1966, Aziz et al 1968, Pal, Pandit and Raghavan 1968, Basu and Pickett 1969, Heckly, Wolochow and Christiansen 1969, Bhattacharya, Bose and Ghosh 1971, Spira and Goeffert 1972, Chaicumpa and Rowley 1972).

I must add (with regret) that to the best of my knowledge I have no spiritual affinity or consanguinity with the Vella of the Thiry-Vella Intestinal loop!

The Big Deal molecule

"It is our business to enquire—whence comes the fluid which is poured out into the intestine during the attacks, and in what way, by means of what mechanism is it poured out?"

Cohnheim (1899)

One of the world's happiest men in 1971 must have been Earl W. Sutherland (Brit. med. J. 1971, Lancet 1970), Professor of Physiology, Vanderbilt University, Tennessee) when he was informed from Stockholm that he had been awarded the coveted Nobel Prize in Physiology and Medicine. This investigator had been interested in finding out how adrenaline liberated from the adrenal gland induced the hepatic cell to transform glycogen into glucose. His solution to the problem may be visually expressed by Figure 3, from which it can be seen that the Endocrine Glands liberate hormones which stimulate the enzyme Adenyl Cyclase sitting in the cell membrane to produce cyclic 3, 5,—adenosine monophosphate (cAMP) utilising the abundant supply of adenosine triphosphate (ATP) sited on the inner side of the cell membrane. The cAMP is then free to diffuse throughout the cell where it acts as the intracellular mediator of the exciting extracellular hormone and directs the cell to do its thing (sic!) that is, the thyroid cell secretes thyroxine, the adrenal cell—adrenaline, and so on. In modern parlance the hormone is spoken of as the first (or extracellular) messenger, and the cAMP as the second (or intracellular) messenger, so that while hormones are distinct substances produced in specialised glands they appear to express their individual potentiality indirectly by a common intermediary, namely cAMP, which explains why the latter substance is known affectionately by endocrinologists and biochemists as the Big Deal Molecule (Table III) (Robison, Dutch and Sutherland 1968).

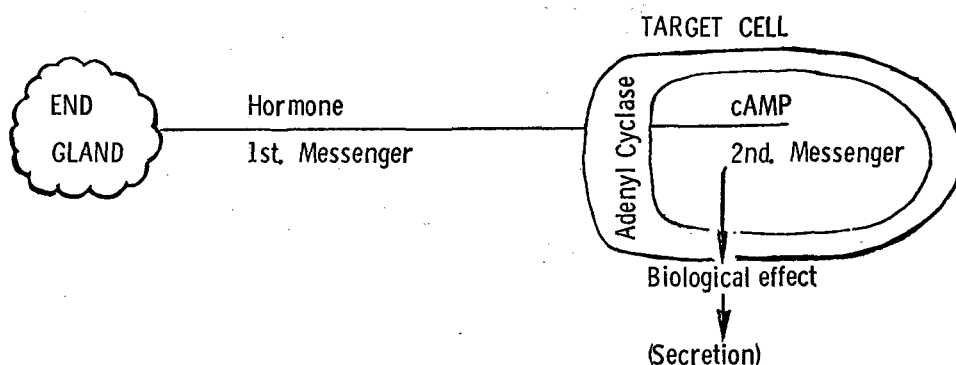


Fig. 3. Earl Sutherland's camp.

Table II
Burrows: The toxins of VC

Type	Heat	Dialysable	Constituent	Tested by
1	Stable	No	Lipopolysaccharide	Mouse lethal Chick embryo
2	Labile	No	Lipoprotein (DF/PF)	Suckling mice Rabbit/dogs Cell cultures
3	Stable	Yes	NH ₃	Anuran epithelium Pah kidney uptake

Table III
cAMP mediator for

ACTH — PITUITARY	GLUCAGON	PANCREAS
LH — PITUITARY	CATECHOLAMINES	— ADRENAL
TSH — PITUITARY	TRIIODOTHYRONINE	— THYROID
MSH — PITUITARY	SEROTONIN	— PINEAL BODY
INSULIN — PANCREAS	PROSTAGLANDIN	— VARIOUS TISSUES

In the context of this paper the cholera toxin (the more strictly descriptive name of VC adenyl cyclase activator or activase has already been suggested (Finkelstein 1972) is thought to act like a hormone by activating the adenyl cyclase—cAMP pathway in the intestinal mucosal cell (Pierce, Greenough and Carpenter 1971a, Chen, Rohde and Sharp 1971 and 1972, Sharp et al 1973).

We have seen how the cholera toxin (hereinafter abbreviated to CT) is liberated by adsorbed vibrios (the mechanism by which the VC adheres to the intestinal epithelial surface is unknown—W.H.O. 1972a. This important phenomenon of attachment and adhesiveness is also seen in other enteropathogens, for example, *Escherichia coli* where the mechanism is attributed to the possession of the antigen K-88 thus,

"Our results show that K88 antigen is responsible for the attachment of K88-positive bacteria to the wall of the small intestine and that adhesion is essential for the virulence of K88-positive bacteria".

Jones and Rutter 1972)

directly on the surface of the lining cells, and stimulates active anion transport plasma-to-lumen; this can be well demonstrated experimentally *in vitro* by the utilisation of an Ussing chamber using a rabbit intestinal mucosa preparation where the exposures of the mucosa to cAMP or CT causes a large increase in short circuit current, and increased secretion of chlorides and bicarbonates, and decrease in sodium transport (Field 1971; Field et al 1972).

En passant to place the vexed matter of the sodium transport in its true perspective to the reader the following summarised extract is placed before him for his own judgement.

"With respect to sodium handling there is evidence for

- a. decreased lumen to plasma sodium movement (Leitch, Burrows and Stoll 1967).
- b. decreased lumen to plasma, and plasma to lumen sodium movement with the former being the greater change (Love et al 1972).
- c. for no change in unidirectional lumen to plasma sodium flux (Iber et al 1969, Love 1969).
- d. and of course for increased plasma to lumen sodium movement (Love 1965)".

Sharp (1973)

In the event the hypersecretion of fluid and electrolytes which follow overwhelm the not-unlimited re-absorptive capabilities of the lower reaches of the alimentary tract, and the balance of non-absorbed fluids and electrolytes is the flux seen in cholera diarrhoea.

This biochemical explanation of the CT has aroused great interest—the practical goal being possible effective and timely drug therapy and chemoprophylaxis of cholera, in addition to the intrinsic light it has shed on common physio-pathological processes. In many laboratories research workers are investigating the exact details and sequence of events involved which of a necessity have been sketched only briefly above.

Thus it is known that the CT is very rapidly absorbed to the cell membrane; after this rapid binding (1-5 minutes) no amount of washing, indeed not even a high level of antitoxin in the intestinal lumen itself, much less in the serum of an actively immunised animal will have any effect at all on the subsequent train of events leading inexorably to the voluminous fluid secretion (Curling et al 1968, McGonagle et al 1969, Mosley and Ahmed 1969, Pierce, Greenough and Carpenter 1971b, Goodgame, Banwell and Hendrix 1972).

And yet one curious and intriguing feature has been recognised, namely there is a lag period between the application of CT to the cell membrane and the expression of its effect; the net effect of the application of cAMP and CT to ileal strips *in vitro* is the same—the movement of chloride and bicarbonate from serosa to mucosa is increased, and the movement of sodium from mucosa to serosa is decreased, but although the action of cAMP is prompt that following CT is delayed (Kimberg et al 1971, Guerrant, Chen and Sharp 1972).

To explain this lag period the hypothesis has been put forward that CT is bound to ganglioside (glycolipid) receptors of the cell membrane; the resulting complex is however

inactive at first and time is required for it to become active either by a change in its configuration or by its re-location within the cell membrane. The resulting alteration in the dynamics of the cell membrane activates the Adenyl Cyclase by direct stimulation or possibly by repressing some inhibitor of that enzyme, and so the chain reaction is started. The CT-Ganglioside complex is thought to be fairly stable hence once it is formed its effects are expressed for many hours before the activity fades off by its degradation into inactive cAMP (adenosine monophosphate) by phosphodiesterases (Hardman, Robinson and Sutherland 1971).

Adenyl cyclase is not the only enzyme affected by CT; the activity of sodium and potassium dependent adenosine triphosphatases of the intestinal cell membranes is reduced by as much as 60 per cent, but the pathophysiological significance of this observation is not yet understood.

It is highly providential that CT is not absorbed into the general circulation, at least to any great extent to cause systemic effects, otherwise as a stimulant of cAMP its results would be wide, varied, weird and disastrous (Williams and Dohadwalla 1969, Dohadwalla and Vaughan 1969, Schaeffer, Sun and Walker 1972, Gorman and Bitensky 1972, Peirce et al 1972a, Donta, Kong and Sloper 1973, Henney et al 1973, Sultzer and Craig, 1973).

In the cause of studies in this area various substances have been found to inhibit the action of CT such as ethacrynic acid, cycloheximide, aspirin and phenylbutazone, prednisone and dexamethasone, insulin and alloxan—these findings offer some future hope that a drug may be found suitable for drug treatment or prophylaxis of cholera diarrhoea (Lizzy, Narasimha and Puttaswamy 1968, Carpenter, Curlin and Greenough 1969, Norris, Curran and Schultz 1969, Serebro 1969, Jacoby and Marshall 1972, Fink and Katz 1972, Kimberg et al 1973, New Scientist 1973, Strombeck 1973).

“A drug might seize and deactivate the toxin (as an antibody does) before it arrives at the cell surface, or it might occupy the receptor site on cells the toxin could attack, or it might directly inhibit the stimulation of adenyl cyclase.

As an answer to cholera it may come about some day that at the first sign of the disease a poor river fisherman in Asia, or the accidentally exposed population of a modern city will be able to simply to take a pill and go back to work.”

Hirschorn and Greenough (1971)

Vaccination

“To derive the maximum benefit from the cholera vaccines, which are of limited efficiency for a short period, it is essential that they be used properly and at the right moment. In the past, vaccination has been greatly overrated as a preventive measure”.

World Health Organisation (1971)

“Where population density is low, transportation difficult, and public awareness low, mass vaccination given just prior to the epidemic season can prevent an estimated 50 per cent of cases in the following season”.

McBean (1971)

It is expected of me in this day and age to talk about cost-benefit and cost-effectiveness of vaccination, especially as applied to the developing countries which are beset by

many problems, other than community medicine ones, and the expected answers (candidates at examinations please note) are:

Environmental sanitation—yes. Treatment centres—probably yes. Chemoprophylaxis—possibly yes. Vaccination—no.

(W.H.O. 1972d, Mosley, Bart and Sommer 1972a, Abel-Smith 1973, Grundy and Reinke 1973, Sommer and Mosley 1973). Various aspects of this debatable point are well brought out in the following extract from a speech by the Chief Medical Officer (Bacterial Diseases) of the World Health Organisation:

“We have developed mathematical models which enable public health administrators to simulate various situations and select the most effective and least expensive immunisation programmes.

Cost-benefit analysis of various control programmes shows that sanitation is the most effective and least expensive control measure.

It seems that cholera vaccine used indiscriminately and at considerable cost gives little benefit in return. Vaccination does not therefore play a primary role in the control of cholera, contrary to the belief of many ill-informed physicians and laymen”.

Cvjetanovic (1971)

But at the present rate of population growth (out-pacing environmental sanitation facilities, which are non-existent in many places anyway) and taking into account such important factors as money, education, geography and so on, one has to preach and practise that which is practicable to do—and I have never heard as yet of a country which threatened by the appearance of a cholera epidemic in its immediately adjacent neighbouring countries did not make available vaccines for its population. The odd imported case appearing in Northern European countries and in the North American countries which is quickly hospitalised, rapidly diagnosed and expertly treated does not alter this fundamental fact. If by chance, accident or design an explosive outbreak of cholera diarrhoea occurred in one of our major cities, say Aberdeen—would we vaccinate or not? (Lancet 1973).

“The public health worker has to do his work within a framework in which socio-political considerations exist.

a. The public knows that a vaccine against cholera exists. b. The public has a historically well-founded fear of cholera. c. Therefore a demand for vaccination naturally arises when cholera breaks out”.

Mackay (1973)

Let us therefore take stock of our vaccine armamentarium and not go over well-trodden ground the reader's attention is with due diffidence drawn to Vella (1972a).

a. Presently available—the vaccine at present in world-wide use consists of 8,000 million dead bacilli per largest dose. This type of vaccine has been proved in field trials to give 30 to 70 per cent protection for three to six months (note however, Azurin and Alvero 1971). This type of vaccine produces in vaccines and anti-bacterial antibodies, such as vibriocidal antibodies which show a degree of correlation with protection against cholera (Mosley et al 1972b).

But hark! “At the moment none of the parenteral vaccines against enteric bacterial infection is wholly satisfactory:

These vaccines are lacking in efficiency. The duration of the immunity is inadequate. They tend to cause side reactions. The cost of using them to immunise the population most at risk is excessive”.

World Health Organisation (1972b)

b. Immediate future—the protection afforded by present vaccines can be enhanced by adjuvants such as adsorbed vaccines with aluminium salts, one such vaccine developed with W.H.O. support by Hungarian workers is stated to be more potent and less reactogenic than conventional vaccines in animal and human experimental work (Joo 1972, Joo et al 1972).

c. Near future—to get protection against Type 2 (Exotoxin) Toxins, a toxoid vaccine has been prepared and is being evaluated:

“Recent experiments employing laboratory models have shown parenteral administration of cholera toxin or toxoid protects animals against challenge with virulent bacilli. Because of such results much effort is now being expended to prepare a toxoid from cholera enterotoxin which is acceptable for use in human beings.

One can only hope that such a product will prove to be a significantly better immunising agent than vaccines currently in use.”

Formal, Dupon and Hornick (1973)

Formalin—inactivated toxoid shows a tendency to reversion to toxicity (Pierce et al 1972b, Northrup and Chisari 1972), fortunately the VC in addition to active CT also elaborates a non-pathogenic moiety, called choleraegenoid (Finkelstein, Peterson and LoSpalluto 1971) which is in effect a natural toxoid; it has however been shown to cause too much reaction in monkey and guinea-pigs. Adjuvanted—gluteraldehyde toxoids show good prospects (W.H.O. 1973c).

A combined vaccine is also possible by suspending bacterial cells (or even possibly antigenic factors, extracts or sub-units of the bacterial cell) in toxoid. Such a vaccine would be expected to give rise to both antibacterial antibodies and antitoxic immunoglobins (Pierce, Kaniech and Northrup 1972c).

“Because the killed vibrio vaccine does confer some (although inadequate) protection against cholera, there is reason to expect that a vaccine containing both somatic antigen of *V.cholerae* and toxoid might be superior to a vaccine consisting of one of the other alone. Thus it is quite possible that the ultimate vaccine will be an impure preparation of toxoid which contains both antigens. Such a vaccine would not be difficult to make on a large scale. If immunity can be maintained in man, by oral boosters after initial vaccination by injection, it should be feasible to vaccinate and protect large numbers of people against cholera in areas of the world where the disease is either endemic or epidemic.

Adams (1973)

d. Distant future—It would appear highly desirable to provide local protection for the intestine since this is the organ under attack by the VC, which is not able to effect a bacteraemic or septicaemic state. This can be done by oral vaccine made up of dead bacilli, or better still live organisms—either harmless or attenuated vibrios or some common intestinal saprophyte to which the antigen properties but not the pathogenicity of the VC have been transferred genetically by natural or artificial means. This would give rise to both humoral and copro-antibodies; cell-mediated immunity in cholera has

not been given much attention (Freter and Gangarosa 1973, Mukerjee 1963, Freter 1965, Bhaskaran and Sinha 1967 and 1971, Sanyal and Mukerjee 1969, Felsenfeld et al 1970, Hornick et al 1971, Howard 1971, Sinha and Bhaskaran 1972, Agarwal and Ganguly 1972, Edsall et al 1972).

“Oral prophylaxis is likely to have many advantages and could ultimately become the most desirable method of immunisation. However it is all too obvious that the information currently available is too fragmentary to allow such a prediction to be made with confidence and conviction.

Until this information is to hand the fundamental questions regarding the use of oral vaccines in human populations cannot be answered specifically.”

World Health Organisation (1972c)

Questions and answers

The subject of vaccines bristles with open questions, which I can hear in my mind's ear the reader would want to pose if this paper were a talk (which it was) and not a written version.

Question. Should one give a classical vaccine prepared from Inaba/Ogawa serotypes (CHO/VAC BP) or one prepared from the biotype causing the present pandemic (El Tor/Vac BP) or a combined El Tor—classical vaccine (Mixed CHO/Vac BP).

Answer. Many indeed most authorities, on the good and solid grounds that the serotypes of the Inaba/Ogawa and El Tor biotypes are identical see no real need for a combined (Mixed CHO/Vac) vaccine. I myself have a slight cautious reservation and I am still of the opinion expressed two years ago that “obviously however nothing is lost by the inclusion of an El Tor component” (Vella 1971, Griffith 1972). I persist in this view, albeit fighting in what seems a forlorn cause, when

a. I view such aspects of the cholera situation as the fact that the present 7th pandemic is caused after all by the El Tor biotype.

b. Again there is the difference between El Tor and the classical vibrio. This can be shown by many more tests than the mighty five presented in Table I, for example see Ray Chaudhuri and Chatterjee 1969 and Adhikary and Chatterjee 1969.

c. Admittedly with personal bias I take encouragement from a W.H.O. expert Committee which states *inter alia*: “cholera vaccine that conforms to these requirements may, however, contain *Vibrio cholerae* biotype El Tor in addition to the classical biotype. Should countries wish to manufacture vaccines containing solely the El Tor biotype for their own national use these requirements are generally applicable. Some information on the El Tor strain for vaccine production is given in the Appendix”. (W.H.O. 1969a).

d. “The immunity conferred by the classical *V. cholerae* vaccine waned after three to four months, that of the El Tor vibrio vaccine after six months” (Azurin et al 1967b), and

e. Kumar and Dutta (1971): “Cholera—El Tor vaccine (Haffkine) is a quadrivalent vaccine used currently in the prophylaxis of cholera disease”.

Question. Should one administer the vaccine by subcutaneous or by the intradermal route?

Answer. The present climate of opinion is veering towards the former alternative—thus speak McBean et al (1971):

"The subcutaneous method is superior to the intradermal method. The subcutaneous route of vaccine administration should be used where vaccination is decided to be in the best interests of the individual person or the population", and in a similar vein Gateff et al 1971:

"The subcutaneous route has consistently given a higher mean antibody level than the intradermal route. The latter technique has the additional disadvantage of not giving constant and reproducible results".

If I may offer a strictly personal suggestion to the reader, perhaps the best solution would be to reserve the intradermal method for booster doses and for patients who complain of unpleasant reactions after subcutaneous injections.

Question. Can the needleless injectors be relied upon to introduce the vaccine in the recommended dose at the desired site?

Answer. This is not absolutely certain—it is especially so I think when dealing with the small volumes necessitated by the intradermal route of vaccination where the desired stimulating antigenic effect depends on the antigenic mass being delivered at the appropriate site—the reader will readily discern that this is not quite the case with live vaccines where one expects that the injected viable viruses or bacteria will take root, increase and multiply (Vella 1972b).

Question: Does one give one or two doses?

Answer. It is true that the W.H.O. allows the authentication of the International Certificate against Cholera after one dose, but when dealing with inactivated vaccines I have always been inclined to feel and so advise that two doses, like two heads, are better than one—after all this is elementary immunology, but see also Vella (1970).

"A single dose of antigen may be effective under emergency conditions or in other mass vaccination programmes, but a second dose given 7-10 days later should ensure a good immune response in practically all persons.

In children under 5 years of age, the administration of a second dose may be of considerably greater importance than in adults".

Watanabe and Verwey (1970)

Question. Are combined vaccines such as TAB/Cholera or Cholera/Tetanus recommended and/or available?

Answer: The W.H.O. expert advisers observe

"The routine use of a cholera vaccine combined with tetanus (or perhaps diphtheria) toxoid would no doubt be of great public health value in developing countries, since both tetanus and diphtheria are highly prevalent in cholera endemic areas".

World Health Organisation (1969b)

For the increasing number of holidaymakers and business men going overseas,

"A combined Typhoid Paratyphoid A and B/Cholera vaccine may be issued or primary immunisation for travellers".

Emmond (1972)

Question. What about the cross—antigenic relationship between the VC and the Brucella, and other genera which may give rise to false positive serologic tests in diagnosis and epidemiological surveys.

Answer. As a general rule one can safely assume from basic bacteriological principles that there is no bacterial genus which is an island unto itself (Leminor, Chalon and Veron 1972). The reader will therefore be conditioned to accept without much difficulty that there are cross-antigenic relations between the VC and *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, and lastly but not least *Brucella*. Thus diagnosis of past cholera infections in serologic mass surveys may be hampered by 'false' positive results due to other organisms, while on the other hand the administration of cholera vaccines may produce and/or boost up antibodies against such as *Brucella* organisms (Pollitzer 1959c, Feeley 1969, Gangarosa et al 1970, Joshi and Prakash 1971, McAlack, Cerny and Freedman 1971, Barua and Watanabe 1972, Winkle, Refai and Rohde 1972, Minden, McLatchy and Farr 1972).

Question. Would the speaker say something about the disturbing rumours that in areas where millions of inhabitants of this world live—referring to the Yellow Fever Belt—the cholera vaccine and the yellow fever vaccine appear to be mutually antagonistic!

Answer. The Yellow Fever Belt is that area in the Americas and Africa about 15° north and south of the Equator, but if cholera vaccine acts antagonistically to yellow fever antibodies, it may possibly also be antagonistic to other arbovirus antibodies and hence will also affect the Asian continent (Gateff 1972, Felsenfeld et al 1973a).

"The lowering of the yellow fever neutralising titres in man after cholera vaccination may be creating a problem. The reason for the bilateral antagonism between cholera vaccine and yellow fever vaccine is not yet known".

Felsenfeld, Wolf and Dutta (1973b)

Conclusion

"In an off-beat sort of way we should be grateful to cholera—for it was the terror caused by its great pandemics that forced international co-operation in public health. It was the principal disease covered by the first international health conventions, and headed the list of quarantinable disease—and with good reason. This scourge has through the ages proved one of the fleetest and deadliest of the Horsemen of the Apocalypse".

John Miles (1972)

REFERENCES

- ABEL-SMITH, B. (1973). *Chron. Wld Hlth Org.* **27**, 407.
 ADAMS, Mary M. E. (1973). *Science* **179**, 552.
 ADHIKARY, P. and CHATTERJEE, S. N. (1969). *Bull. Calcutta Sch. trop. Med.* **17**, 6.
 ARGARWAL, S. C. and GANGULY, N. H. (1972). *Inf. Immun.* **6**, 17.
 AZIZ, K. M. S., MOHSIN, A. K. M., HARE, W. K. and PHILLIPS, R. A. (1968). *Nature (Lond.)* **220**, 814.
 AZURIN, J. C. and ALVERO, M. (1971). *Bull. Wld Hlth Org.* **44**, 815.
 AZURIN, J. C., KOBARI, K., BARUA, D., ALVERO, M., GOMEZ, C. Z., DIZON, J. J., NAKANO, E., SUPLIDO, R. and LEDESMA, L. (1967a). *Bull. Wld Hlth Org.* **37**, 745.
 AZURIN, J. C., CRUZ, A., PERSIGAN, T. P., ALVERO, M., CAMENA, T., SUPLIDO, R., LEDESMA, L. and GOMEZ, C. Z. (1967b). *Bull. Wld Hlth Org.* **37**, 703.
 BANNERJEE, D. N. (1939). *J. Indian med. Ass.* **8**, 391.
 BANWELL, J. G. and SHEER, H. (1973). *Gastroenterology* **65**, 467.
 BARUA, D. (1972). *Proc. roy. Soc. Med.* **65**, 423.
 BARUA, D. and WATANABE, Y. (1972). *J. Hyg. (Lond.)* **70**, 161.
 BASU, S. and PICKETT, M. J. (1969). *J. Bact.* **100**, 1142.
 BHATTARCHARYA, S., BOSE, A. D. and GHOSH, A. D. (1971). *Appl. Microbiol.* **24**, 341.
 BHASKARAN, K. and SINHA, V. B. (1967). *J. Hyg. (Lond.)* **65**, 135. (see also Bhaskaran and Sinha 1971. *Curr. Sci.* **40**, 281).

- BOURNE, H. R., LICHTENSTEIN, L. M. and HENNEY, C. S. (1972). *J. clin. Invest.* **51**, 12 (as abstract No. 39).
- BRITISH MEDICAL JOURNAL (1971). *Brit. med. J.* **iii**, 188.
- BRITISH MEDICAL JOURNAL (1973). *Brit. med. J.* **iv**, 117.
- BYWATER, R. J. (1973). *Res. Vet. Sci.* **14**, 35.
- BURNET, F. M. and STONE, J. D. (1947). *Aust. J. exp. Biol. med. Sci.* **25**, 219.
- BURROWS, W. (1968). *Ann. Rev. Microbiol.* **22**, 245.
- BURROWS, W., MATHER, A. N., MCGANN, V. V. and WAGNER, S. M. (1946). *J. infect. Dis.* **79**, 168.
- CARPENTER, C. C. J., CURLIN, G. T. and GREENOUGH, W. B. (1969). *J. infect. Dis.* **120**, 332.
- CHACUMPA, W. and ROWLEY, D. (1972). *J. infect. Dis.* **125**, 480.
- CHEN, L. C., ROHDE, J. E. and SHARP, G. W. (1971). *Lancet* **i**, 939.
- CHEN, L. C., ROHDE, J. E. and SHARP, G. W. (1972). *J. clin. Invest.* **51**, 731.
- COHNHEIM, J. F. (1890). Lectures on General Pathology—A Handbook for Practitioners and Students. vol. 3. New Sydenham Society. London. p. 954.
- CRAIG, J. P. (1965). *Nature (Lond.)* **207**, 614.
- CRAIG, J. P. (1971). Cholera Toxins. In *Microbial Toxins*. ed. S. Kadis, T. C. Montie, and S. S. Tajl. Academic Press. New York. p. 189-284.
- CUATRECAS, P. (1973a). *Biochemistry* **12**, 3547.
- CUATRECAS, P. (1973b). *Biochemistry* **12**, 3558.
- CURLING, G. T., SUBONG, A. E., CRAIG, J. P. and CARPENTER, C. C. J. (1968). *Trans. Ass. Amer. Physns* **81**, 314.
- CVJETANOVIC, B. (1971). Vaccination against Cholera. Seminar on Immunisation in Africa. Kampala Dec. 7-10 1971. Centre Internationale de L'Enfance. Merieux Foundation. p. 168.
- DATTA, B. C. and MITRA, S. (1970). *Indian J. med. Res.* **58**, 1505.
- DE, S. E. (1961). *Bull. off. int. Hyg.* **25**, 72.
- DOHADWALLA, A. N. and VAUGHAN, W. E. M. (1969). *J. infect. Dis.* **120**, 658.
- DONTA, S. T., KING, M. and SLOPER, K. (1973). *Nature New Biol.* **243**, 246.
- DUTTA, N. K., PANJE, M. V. and JHALA, H. I. (1963). *Brit. med. J.* **i**, 1200.
- EDSALL, G., HORNICK, R. B., JOO, I. and Ogonuki, H. (1972) Improvement of Cholera Vaccines. WHO/BD/Cholera/72.24. p. 3. (unpublished).
- EMMOND, R. (1972). Immunisation. Infectious Diseases Medicine 5. p. 354. Publ. Med. Educ. (Inter.) Ltd.
- FEELEY, J. C. (1966). *Int. J. system. Bact.* **16**, 135.
- FEELEY, J. C. (1969). *J. Bact.* **99**, 645.
- FELSENFIELD O. (1965). *Bull. Wild Hlth Org.* **33**, 725.
- FELSENFIELD, O., STETCHER-BARRIOS, A., ALDOVA, E., HOLMES, J. and PARROTT, M. W. (1970). *Appl. Microbiol.* **19**, 463.
- FELSENFIELD, O., WOLF, R. H., GYR, K., GRANT, L. S. and DUTTA, N. K. (1973a). *Lancet* **i**, 457.
- FELSENFIELD, O., WOLF, T. H., and DUTTA, N. K. (1973b). *Proc. Soc. exp. Biol. (N.Y.)* **143**, 548.
- FIELD, M. (1971). *New. Engl. J. Med.* **284**, 1137.
- FIELD, M., FROMM, D., AL-AWQATI, Q. and GREENHOUGH, W. B. III (1972). *J. clin. Invest.* **51**, 786.
- FINK, A. D. and KATZ, R. L. (1972). *Nature (Lond.)* **238**, 270.
- FINKELSTEIN, R. A. (1972). *Toxicon* **10**, 441.
- FINKELSTEIN, R. A. (1973). *Microbiology* **2**, 553.
- FINKELSTEIN, R. A. and MUKERJEE, S. (1963). *Proc. Soc. exp. Biol. (N.Y.)* **112**, 355.
- FINKELSTEIN, R. A. and LoSPALLUTO, J. J. (1969). *J. exp. Med.* **130**, 185.
- FINKELSTEIN, R. A. and LoSPALLUTO, J. J. (1970). *J. infect. Dis.* **121**, (suppl.) 563.
- FINKELSTEIN, R. A. and LoSPALLUTO, J. J. (1972). *Science* **175**, 529.
- FINKELSTEIN, R. A., NORRIS, H. T. and DUTTA, N. K. (1964). *J. infect. Dis.* **114**, 203.
- FINKELSTEIN, R. A., PETERSON, J. W. and LoSPALLUTO, J. J. (1971). *J. Immunol.* **106**, 868.
- FORMAL, S. B., DUPON, H. L. and HORNICK, R. B. (1973). *Ann Rev. Med.* **24**, 103.
- FRETER, R. (1965). Copro-antibody and Oral Vaccines. Proceedings of the Cholera Research Symposium. Hawaii. Jan 24-29. United States Publ. Hlth Ser. Publ. No. 1328. p. 222.
- FRETER, R. and GANGAROSA, E. J. (1963). *J. Immunol.* **91**, 724.
- FRETER, R., SMITH, H. L. and SWEENEY, F. J. (1961). *Tex. Rep. Biol. Med.* **27**, (suppl. 1) 299.
- FURHMAN, G. J. and FUHRMAN, F. A. (1960). *Nature (Lond.)* **188**, 71.
- FURHMAN, F. A., FURHMAN, G. J. and BURROWS, W. (1962). *J. infect. Dis.* **111**, 225.
- GAN, K. H. and TJIA, S. K. (1963). *Amer. J. Hyg.* **77**, 184.
- GANGAROSA, E. J., SANATI, A., SAGHARI, H. and FEELEY, J. C. (1967). *Lancet* **i**, 646.
- GANGAROSA, E. J., DeWITT, W. E., FEELEY, J. C. and ADAMS, M. R. (1970). *J. infect. Dis.* **121**, (suppl.) 536.
- GARDNER, A. D. and VENKATRAMAN, K. V. (1935). *J. Hyg. (Lond.)* **35**, 262.
- GATEFF, C. (1972). *Bull. Soc. Path. exot.* **65**, 784.
- GATEFF, C., DeWITT, W. E., LEMARINIER, G., BRADLEY, M. and LABUSQUIERE, R. (1971). Anticholera Antibodies after Vaccination by Injection under Pressure without a Needle. Seminar on Immunisation in Africa. Kampala. Dec 7-10. 1971. P. 159-166. Centre Internationale de L'Enfance. Merieux Foundation.
- GOODGAME, J. T., BANWELL, J. G. and HENDRIX, T. R. S. (1972). *Fed. Proc.* **31**, 259. (abs. No. 264).
- GORMAN, R. E. and BITENSKY, M. W. (1972). *Nature (Lond.)* **235**, 439.
- GOTSCHELICH, F. (1906). *Z. Hyg. Infekt.-Kr.* **53**, 281.
- GRADY, G. E., MADOFF, M. A., DUHAMEL, R. C., MOORE, E. W. and CHALMERS, T. C. (1967). *Gastroenterology* **53**, 737.
- GRADY, G. E., MADOFF, M. A., DUHAMEL, R. L., MOORE, E. W. and CHALMERS, T. C. (1968). *J. infect. Dis.* **118**, 263.

- GREIG, E. D. W. (1914). *Indian J. med. Res.* **2**, 623.
- GRIFFITHS, A. H. (1972). *Publ. Hlth (Lond.)* **87**, 17.
- GRUNDY, F. and REINKE, W. A. (1973). *Wld Hlth Org. Publ. Hlth. Pap. No.* **51**, 147.
- GUERRANT, R. L., CHEN, L. C. and SHARP, G. W. C. (1972). *J. infect. Dis.* **125**, 377.
- HARDMAN, J. G., ROBINSON, G. A. and SUTHERLAND, E. W. (1971). *Ann. Rev. Biochem.* **41**, 311.
- HECKLY, R. J., WOOLCHOW, H. and CHRISTIANSEN, C. (1969). *J. Bact.* **100**, 1140.
- HENNEY, C. S., LICHTEN, L. M., GILLESPIE, Elizabeth and ROLLEY, R. T. (1973). *J. clin. Invest.* **52**, 2853.
- HIRSCHORN, N. and GREENHOUGH, W. B. (1971). *Scientific American* **225**, 21.
- HOLMGREN, J., LONNROTH, I. and SVENNERHOLM, L. (1973a). *Scand. J. infect. Dis.* **5**, 77.
- HOLMGREN, J., LONNROTH, I. and SVENNERHOLM, L. (1973b). *Inf. Immun.* **8**, 208.
- HORNICK, R. B., MUSIC, S. I., WENZEL, R., CASH, R., LIBONATI, J. P., SNYDER, M. J. and WOODWARD, T. E. (1971). *Bull. N.Y. Acad. Med.* **47**, 1181.
- HOWARD, B. D. (1971). *Nature (Lond.)* **230**, 77.
- JACOBY, H. I. and MARSHALL, C. H. (1972). *Nature (Lond.)* **235**, 163.
- JONES, G. W. and RUTTER, J. M. (1972). *Inf. Immun.* **6**, 912.
- JOO, I. (1972). *Zbl. Pharm.* **111**, 235.
- JOO, I., CSIZER, R., JUHA, S. Z. and VERA, P. (1972). *Progr. immunobiol. Standard* **5**, 350.
- JOSHI, D. V. and PRAKASH, O. (1971). *Indian J. med. Res.* **59**, 26.
- KAMAL, A. M. (1963). *Bull. Wld Hlth Org.* **28**, 272.
- KASAI, G. J. and BURROWS, W. (1966). *J. infect. Dis.* **116**, 606.
- KIMBERG, D. V., FIELD, M., JOHNSON, J., HENDERSON, A. and GERSHON, Elaine (1971). *J. clin. Invest.* **50**, 1218.
- KIMBERG, D. V., FIELD, M., GERSHON, Elaine, SCHOOLEY, R. T. and HENDERSON, Antonia (1973). *J. clin. Invest.* **52**, 1376.
- KING, C. A. and VAN HEYNINGEN, W. E. (1973). *J. infect. Dis.* **127**, 639.
- KRISHNA MURTI, C. R. (1968). *J. Sci. Ind. Res.* **27**, 430.
- KUMAR, S. and DUTTA, N. K. (1971). *Indian J. med. Res.* **59**, 1375.
- LANCET (1970). *Lancet* **ii**, 119.
- LANCET (1971). *Lancet* **i**, 869.
- LANCET (1973). *Lancet* **ii**, 601.
- LEITCH, G. J., BURROWS, W. and STOLLE, L. C. (1967). *J. infect. Dis.* **117**, 197.
- LEITCH, G. J. (1972). *Exp. molec. Path.* **17**, 198.
- LE MINOR, L., CHALON, A. M. and VERON, M. (1972). *Ann. Inst. Pasteur* **123**, 761.
- LINDENBAUM, J., GREENOUGH, W. B., BENENSON, A. J., OSEASOHN, R., RIZVI, S. and SAAD, A. (1965). *Lancet* **i**, 1081.
- LIZZY, K. S., NARASIMHA RAO P. L., and PUTTASWAMY, T. L. (1968). *Indian J. exp. Biol.* **6**, 168.
- LONNROTH, I. and HOLMGREEN, J. (1973). *J. gen. Microbiol.* **76**, 417.
- LOSPOLLUTO, J. J. and FINKELSTEIN, R. A. (1972). *Biochim. biophys. Acta (Amst.)* **257**, 158.
- MCALACK, R. F., CERNY, J. and FREEDMAN, H. (1971). *J. Immunol.* **107**, 1752.
- MCBEAN, A. M. (1971). Cholera Vaccine for Africa. Seminar on Immunisation in Africa. Kampala. Dec 7-10 1971. Centre Internationale de L'Enfance. Merieux Foundation.
- MCBEAN, A. M., AGLE, A. N., CAMPAORE, P. and McCORMACK, W. M. (1971). Comparison of the Intradermal and Subcutaneous Routes of Cholera Vaccine Administration. Seminar on Immunisation in Africa. Kampala. Dec 7-10 1971. Pp. 152-158. Centre Internationale de L'Enfance. Merieux Foundation.
- MCGONAGLE, T. J., SEREBRO, H. A., IBER, F. L., BAYLESS, T. M., and HENRIX, T. R. (1969). *Gastroenterology* **57**, 5.
- MCINTYRE, O. R., FEELEY, J. C., GREENOUGH, W. B. III, BENENSON, A. S., HASSAN, S. I. and SAAD, A. (1965). *Amer. J. trop. Med. Hyg.* **14**, 412.
- MACKAY, D. M. (1973). *Lancet* **ii**, 1439.
- MACRAE, J. (1972). *Practitioner* **209**, 507.
- MAEYER-CLEEMPOEL, S. (1972). *Arch. belges Med. soc.* **30**, 380.
- MILES, J. (1972). History of Medicine. Spring 72. p.20.
- MILLER, C. E., WONG, K. H., FEELEY, J. C. and FORLINES, M. E. (1972). *Inf. Immun.* **6**, 739.
- MINDEN, P., McLATCHY, J. K. and FARR, R. S. (1972). *Inf. Immun.* **6**, 574.
- MIYAKI, K., IWAHARA, S., SATO, K., FUSIMOTO, S. and AIBARA, K. (1967). *Bull. Wld Hlth Org.* **37**, 773.
- MOSLEY, W. H. and AHMED, A. (1969). *J. Bact.* **100**, 547.
- MOSLEY, W. H., BART, K. J. and SOMMER, A. (1972a). *Inst. of Epidem.* **1**, 5.
- MOSLEY, W. H., AZIZ, K. M. A., MIZANUR RAHMAN, A. S. M., ALAUDIN CHOWDHURY, A. K. M., AHMED, A. G., and FAHIMUDDIN, M. (1972b). *Bull. Wld Hlth Org.* **47**, 299.
- MUNKERJEE, S. (1963). *Bull. Wld Hlth Org.* **29**, 753.
- MUNKERJEE, S. (1970). *Wld Hlth Org. Publ. Hlth Pap. No.* **40** (see also Basu, S. and Mukerjee, S. (1970). *Bull. Wld Hlth Org.* **43**, 509. Bystryi, N. F. et al (1971). *J. Hyg. Epidem. (Praha)* **15**, 286).
- NEW SCIENTIST (1973). *New Sci.* **60**, 530.
- NORRIS, H. T., SCHULTZ, S. G., CURRAN, P. F. and FINKELSTEIN, R. A. (1967). *J. infect. Dis.* **117**, 193.
- NORRIS, H. T., CURRAN, P. F. and SCHULTZ, S. G. (1969). *J. infect. Dis.* **119**, 117.
- NORTHROP, R. S. and CHISARI, F. V. (1972). *J. infect. Dis.* **125**, 471.
- PAL, S. C., PANDIT, C. G. and RAGHAVAN, N. G. S. (1968). *Indian J. med. Res.* **56**, 1482.
- PETERSON, J. W., LOSPALLUTO, J. J. and FINKELSTEIN, R. A. (1972). *J. infect. Dis.* **126**, 612.
- PHILLIPS, R. A. (1963). *Bull. Wld Hlth Org.* **28**, 297.
- PEIRCE, W. E. (1973). *J. exp. Med.* **137**, 1009.

- PEIRCE, N. F., GREENOUGH, W. B. III and CARPENTER, C. C. J. (1971a and b). *Bact. Rev.* **35**, 1.
- PEIRCE, N. E., GRAYBILL, J. R., KAPLAN, M. M. and BOUMANN, B. L. (1972a and b). *J. Lab. clin. Med.* **79**, 145.
- PIERCE, N. F., KANIECH, Evelyn A. and NORTHRUP, R. S. (1972c). *J. infect. Dis.* **126**, 606.
- POLLITZER, R. (1959). *Wld Hlth Org. Monogr. Ser. No. 43*. (a) p. 17. (b) p. 266. (c) p. 326.
- POWELL, C. J. Jr. and FINKELSTEIN, R. A. (1964). *J. Bact.* **87**, 1249.
- RAY CHAUDHURI, C. and CHATTERJEE, S. N. (1969). *Bull. Calcutta Sch. trop. Med.* **17**, 5.
- ROBISON, G. A., DUTCH, R. W. and SUTHERLAND, E. W. (1968). *Ann. Rev. Biochem.* **37**, 149.
- SACK, R. B. and MILLER, C. E. (1969). *J. Bact.* **99**, 688.
- SACK, R. B., CARPENTER, C. C. J. Jr., STEEBER, R. W. and PIERCE, N. F. (1966). *Lancet* **ii**, 206.
- SAKAZAKI, R. and TAMURA, K. (1971a and b). *Jap. J. med. Sci. Biol.* **24**, 93.
- SAKAZAKI, R., TAMURA, K., GOMEX, C. Z. and SEN, R. (1970). *Jap. J. med. Sci. Biol.* **23**, 13.
- SANYAL, S. C. and MUKERJEE, S. (1969). *Bull. Wld Hlth Org.* **40**, 503.
- SCHAEFFER, B. T., SUN, S. C. and WALKER, R. I. (1972). *Chin. J. Microbiol.* **5**, 27.
- SEREBRO, H. A., IBER, F. G., YARDLEY, J. H. and HENDRIX, T. R. (1969). *Gastroenterology* **56**, 506.
- SHARP, G. W. G., HYNIE, S., EBEL, H., PARKINSON, D. K. and WIKTUR Patricia (1973). *Biochem. biophys. Acta (Amst.)* **309**, 359.
- SHARP, G. W. G. (1973). *Ann. Rev. Med.* **24**, 19 (quoting Leitch, G. J., Burrows, W. and Stolle, L. C. (1963). *J. infect. Dis.* **117**, 197. Love, A. H. G., Phillips, R. A., Rohde, J. E. and Veall, N. (1972). *Lancet* **ii**, 151. Iber, F. G. et al (1969). *Amer. J. med. Sci.* **258**, 240. Love, A. H. G. (1965). *Proc. Chol. Res. Symp (NH)*, 144.
- SINHA, H. (1969). *Lancet* **i**, 1162.
- SINHA, V. B. and BHASKARAN, K. (1972). *Curr. Sci.* **41**, 311.
- SNOW, J. (1855). *On the Mode of Communication of Cholera*. 2nd ed. John Churchill. London. Reprinted 1965. Hafner Publishing Co. New York.
- SOMMER, A. and WOODWARD, W. E. (1972). *Lancet* **ii**, 985.
- SOMMER, A. and MOSELY, W. H. (1973). *Lancet* **i**, 1232.
- SPAUN, J., FEELEY, J. C., SMITH, H. C. Jr. and THOMAS, A. K. (1971). *Bull. Wld Hlth Org.* **42**, 818.
- SPIRA, W. M. and GOEFFERT, J. M. (1972). *Appl. Microbiol.* **24**, 341.
- STROMBECK, D. R. (1973). *Life Sci.* **12**, 211.
- SULTZER, B. M. and CRAIG, J. F. (1973). *Nature New Biol.* **244**, 178.
- SWALLOW, J. H., CODE, C. F. and FRETER, R. (1965). The Effect of Cholera Toxin on Water and Ion Fluxes in the Canine Bowel. *Proc. Cholera Res. Symposium*. Jan 24-29. Honolulu. p.30. United States Publ. Hlth Serv. Publication No. 1328.
- VAN HEYNINGEN, W. E., CARPENTER, C. C. J., PIERCE, W. E. and GREENHOUGH, W. B. III (1971). *J. infect. Dis.* **124**, 415.
- VAUGHAN, M., PIERCE, N. F. and GREENHOUGH, W. O. (1970). *Nature (Lond.)* **266**, 658.
- VELLA, E. E. (1970). *Brit. med. J.* **i**, 818.
- VELLA, W. (1971). *Brit. med. J.* **i**, 49.
- VELLA, E. E. (1972a). *Brit. J. Hosp. Med.* **7**, 255.
- VELLA, E. E. (1972b). *Lancet*, **i**, 796.
- WATANABE, Y. and VERWEY, W. F. (1970). *Wld. Hlth Org. Publ. Hlth Pap. No. 40*.
- WORLD HEALTH ORGANISATION (1952). *Wld Hlth Org. techn. Rep. Ser.* **52**, 6.
- WORLD HEALTH ORGANISATION (1969a). *Wld Hlth Org. techn. Rep. Ser.* **413**, 31 and 44.
- WORLD HEALTH ORGANISATION (1969b). *Wld Hlth Org. techn. Rep. Ser.* **414**, 17.
- WORLD HEALTH ORGANISATION (1971). *Off. Rec. Wld Hlth Org.* **190**, App. 12.
- WORLD HEALTH ORGANISATION (1972). *Wld. Hlth Org. techn. Rep. Ser.* **500**. (a) p.10 (b) p. 5 (c) p. 27.
- WORLD HEALTH ORGANISATION (1972d). *Wkly epidem. Rec.* **47**, 1.
- WORLD HEALTH ORGANISATION (1973). (a) *Wkly epidem. Rec.* **48**, 413 (b) *Wkly epidem. Rec.* **48**, 298 (c) *Off. Rec. Wld Hlth Org.* **205**, 29.
- WILLIAMS, E. M. A. and DOHADWALLA, A. N. (1969). *J. infect. Dis.* **120**, 652.
- WINKLE, S., REFAI, M. and RHODE, R. (1972). *Ann. Inst. Pasteur* **123**, 775.
- YEFIMTSEVA, E. P., YERMOLYEVA, Z. V., ZYKIN, L. F. and GUEREVITCH, E. I. (1972). *J. Hyg. Epidem. (Praha)* **16**, 398.
- ZIEVE, R. D., PIERCE, N. F. and GREENHOUGH, W. B. (1970). *Clin. Res.* **18**, 690.