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VECTOR-BORNE PARASITIC DISEASES IN DEVELOPING COUNTRIES

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It is a great honour to be invited to address the Asian Congress of Parasitology tonight. It is also a little daunting for a medical entomologist to address such a company of experienced parasitologists of so many sub-disciplines. When invited by the Secretary-General to speak on parasitic diseases in developing countries, I hastily requested that the field be narrowed in order that I could restrict myself to vector-borne infections. And in this age of specialization, even this field is much too wide for my competence. However, all developing countries are facing difficulties in controlling one or more of such infections, and tonight I think it appropriate that we should ask ourselves how many of these difficulties are real, how many are imagined, how many are at present insoluble, for how many others are the solutions already at hand, and why, in such cases, are they not applied.

I think we can start by looking at the progress and the lack of progress made over the past century with vector-borne diseases. We might classify our infections into 3 groups: first, those which to a large extent have now been controlled, and which, though still potentially dangerous, are not of current, widespread public health concern. One might include plague,

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typhus and probably relapsing fever and yellow fever. Then there are those which may have been brought under control in some areas at some times but which are still widespread, may occur in epidemic form, and which appear in some circumstances uncontrollable. Malaria is the major example, and I think perhaps it would be right to include also leishmaniasis. The third group includes those infections on whose distribution and prevalence we have as yet made no significant impact; and, indeed, in some cases the infections may be increasing. Bancroftian filariasis has first place in this group, and is accompanied by some of the mosquito-borne viral infections, notably dengue haemorrhagic fever. Perhaps onchocerciasis belongs here also, for although there is a massive control programme now operating in seven countries of West Africa it is too early to judge whether the outcome will be as successful as is hoped. Since this is an Asian Congress, I shall say no more about onchocerciasis.

There is a complementary approach we might take for reviewing our infections, and that is to look at their distribution in time and in space, and to identify if we can those factors which have led to the disappearance of some infections and those which have contributed to the appearance and spread of "new" infections.

When we look at the history of the infections which have been brought under control, we see that much has been achieved—but not as a result of planned campaigns. There is no doubt that the decline of plague as a public health problem has been primarily related not to the activities of health workers but to the general improvements in the living conditions and hygiene of the people in previously affected areas. Plague was on the decline in Europe long before Yersin's discovery in 1894 of the causative organism and the incrimination a few years latter of the flea vector.

Here in India the yearly number of deaths from plague 60 to 70 years ago was a massive 500,000. But plague was largely overcome by social improvements. And the same conclusion can be drawn for the decline and near elimination of louse-borne typhus and relapsing fever.

Similarly, yellow fever was disappearing from North America during its developing period long before *A. aegypti* was recognised as the vector. The last outbreak of yellow fever in New York was in 1870, and apart from an outbreak in 1905 in New Orleans the infection was virtually eliminated from North America by 1880, that is, 20 years before the vector was recognised. The reasons were simply the improved living conditions and, in particular, the improved water supplies which made water storage in domestic containers unnecessary, thereby depriving *A. aegypti* of larval habitats.

At the present time, plague, typhus and yellow fever, which separately have caused massive mortality in the past, are controlled in all populations centres. They must never be ignored; and surveillance systems must take account of them; but with present-day health standards and

technology, none of them should ever present insuperable problems.

Our first conclusion is, therefore, that social improvements in living standards of the people form a most powerful ally of the health worker. But we must note one point of similarity among the infections which have declined; namely, the vectors live on the person or in the homes of people, in other words where improvements in personal hygiene and social conditions would be expected to have the greatest and quickest effect. *A. aegypti* deserves a further mention, and we shall return to it in due course.

In the second group of infections, with which we have had some success in the past, we might pass over leishmaniasis briefly and comment that successful control has often been a side-effect of malaria operations, in particular house-spraying of residual insecticides, and that a large part of the success has been because the vectors transmitting the infection to man have been domestic in habits. As you know, there have been resurgences of leishmaniasis in areas where anti-malaria operations have been interrupted. This has been true recently in the Middle-East and most particularly in the few years up to 1977 in the State of Bihar where there has been a major epidemic of *Kala-azar*. However, leishmaniasis can be contained, as workers in Bihar have so recently demonstrated.

But what of malaria? The situation with this infection is much more complex and serious. Although there has been a steady decline during the past century in malaria in most of Europe, much of it attributable to improvements in the environment, to drainage and to better housing, in the developing countries of Africa and Asia and South America malaria is undoubtedly the chief vector-borne infection. And more than half the world's population at risk

from malaria, about 900 million, live in South-East Asia. The reasons for the decline in the outlook for malaria control are known to you all: multiple resistance of the vectors to insecticides, coupled in some areas with drug resistance in the parasites, administrative and financial shortcomings which have hindered or halted control programmes, and a general fall in confidence among public health workers that malaria can be defeated in the foreseeable future. There is certainly no reason to be optimistic that a new insecticide will be found which will solve our current problems with insecticide resistance; even when we had effective insecticides we were unsuccessful in eliminating either vector or parasite. Similarly, even if a new drug is made available our problems will be relieved but not solve. And, if and when a vaccine against malaria is ever produced, there would still be no guarantee of interrupting transmission.

There would seem to be sufficient grounds for pessimism, but surely the vast amount of information we have gathered on malaria vectors should have some relevance to a solution or a partial solution, or could be utilised in some areas at some times in order to prevent transmission.

I have always been struck by the work of Malcolm Watson, one of the pioneers of malaria control. He arrived at the beginning of 1901 in Malaya as a young Government surgeon with medical responsibility for the hospital in the coastal town of Klang and for the surrounding district. Within a few months he was faced with an epidemic of malaria—not large by standards in India or Sri Lanka, but nevertheless of primary importance to the new medical officer. Watson was not content simply to treat his patients; he decided that he had to try to prevent the population from becoming infected. He knew nothing

about the mosquitoes of the area—nor did anyone else—but he planned and started a programme of clearing the forest around the town, and draining the valleys and swamps. By the following year the benefits were clear and the incidence of malaria had fallen. Watson did not know which mosquitoes transmitted malaria in his district and it was fortunate, as we now know, that the vectors were of the *umbrosus* group, which required shade and standing water, so that forest clearing and drainage were the ideal methods to prevent breeding. Further inland, in the foothills of the main range of hills clearing and surface drainage of water give contrary results; since the important vector, *An. maculatus*, required sunlight and moving water in its larval habitats. It was some years later before *maculatus* malaria was prevented by the introduction of subsoil drainage, a method which had been used earlier in Panama.

Watson was also successful in controlling malaria in Port Swettenham, a few miles from Klang, where, as we now know, the main vector was *An. sundaicus*. The port was constructed in a low-lying mangrove swamp, and when the work was completed in 1901 officials and labourers were transferred to the new port. However, very soon malaria struck and by the end of the year two-thirds of the labourers were infected. Ships crews were being infected, there was a shortage of labour for unloading, and the port was soon on the point of collapse. Watson had made proposals for the improvement of the situation, and the Commission of Enquiry endorsed his recommendations to build sea-walls or bunds to stop the salt water flooding the mangrove area, to drain the area around the port, to fell the patches of forest and to oil larval habitats. These measures were dramatically successful, and the incidence of malaria fell during the next year or two to a low level.

I have dealt at some length with Watson and his work because I think they illustrate three points which are relevant today. First, Watson's aim throughout was the prevention of malaria; not its control, nor its eradication. There is some overlap in meaning when we use one or other of these three terms, but when we think of prevention, as Watson did, then our philosophy of vector control changes. We stop asking which insecticide should be used for the control of a particular vector; we ask instead how can breeding be prevented or reduced or how can we prevent people being bitten by the adults.

There is, of course, nothing new in this philosophy, but we need reminding of it. It is all too easy nowadays to find information on the resistance level of all the major malaria vectors to DDT or malathion or one or other of the carbamates. But how often does one see a report of someone preventing mosquito breeding, on however small a scale? We have if you like been fighting a defensive action: applying insecticides where the mosquito might come into contact with them. Watson's approach was one of attack on the mosquito habitat. Remove it or alter it.

There have often been pilot control projects against malaria vectors, but they have usually been expected to lead to a conclusion which can be applied on a national or regional scale. You may say that Watson was working in a small township in a small country. This is true, and it is this point about the scale of operations which brings me to the second lesson to be learned from Watson's work. He did not start by trying to eradicate malaria from his whole district, nor did he try to control malaria throughout the country. He started on a small-scale and established a good permanent foundation for his preventive measures. Later, when

a method was demonstrably effective it might be applied elsewhere, though sometimes this proved to be uneconomic. For example, subsoil drainage was most effective in preventing *An. maculatus* breeding, and this was the method successfully applied in 1913 to the valleys and ravines in Kuala Lumpur, but it was clear that subsoil drainage throughout rubber estates where *An. maculatus* was transmitting malaria was, for economic reasons, quite out of the question. And so, having seen the excellent results achieved in Panama from oiling still and slow-flowing water, Watson in 1914 decided to try oiling the relatively fast-moving streams in the side pockets and edges of which *An. maculatus* larvae are to be found. By using an oil with the right spreading power, he demonstrated a complete kill of the larvae, and oiling soon became the standard method for preventing *An. maculatus* malaria on estates and in the outskirts of towns where subsoil drainage could not be afforded. In other words, control measures are influenced by economic factors as well as by the mosquito ecology.

There is a third point of some general application from a consideration of Watson and his work. When he went to Malaya in 1901, he was still a young man in his twenties and most of his achievements were made during the following 10-15 years. He was young, and he was enthusiastic. And linked with his age and enthusiasm was the important point that he was given the opportunity to try out his ideas. It is not easy to envisage such a situation today where a young medical officer less than 6 months in his Government post was allowed to plan and implement major construction works without any evidence that his proposals would succeed. Nor was Watson so very confident of success! He writes: "At times a feeling

of despair came over me; indeed I was prepared for a total failure of the works, and had my answer ready prepared should I be called to account by Government ”.

Watson's despair is well understood by the workers on malaria prevention of today.

A few months ago at a meeting in London to celebrate the centenary of Manson's discovery of the mosquito as a host of *W. bancrofti*, Dr. M. T. Gillies, in reviewing the history of malaria control, divided the period from Ross's discovery of the mosquito as the intermediate host of *Plasmodium* up to the present time into four eras. The first, from 1897–1920, was a period of successes: Gorgas controlled malaria in Panama and enabled the canal to be completed; Watson was having success in coastal Malaya; and Ross was spreading optimism in his visits to Africa and elsewhere. The next era, from 1920–1945, was a period of limited progress in control, a period when the immensity of the problems was being realised, but a period when extensive studies were made in the systematics and biology of the vectors throughout the world. The third era, from 1945–1970, was the period when a new optimism swept through the world and when malaria eradication seemed attainable through the use of residual insecticides. Now we are a few years into the fourth era, one in which we have retreated to the concept of malaria control than eradication, one in which doubt and disillusion have spread through the ranks of malaria field workers. There is still hope and some degree of optimism among laboratory workers that new drugs will be developed to combat the current drug resistance in some areas, and that the development of a long-lasting vaccine may be feasible in the foreseeable future. However, in the meantime we are faced

with resurgent malaria, and it is the field workers who have the most acute problems.

One of the chief concerns must be that during the 25-year period of optimism generated by the success of insecticides, we lost a whole generation of field entomologists. Those who would have grown up to acquire an understanding of and feeling for vectors such as *An. culicifacies* and *An. stephensi* were diverted or attracted to other subjects—perhaps even parasitology—and the experience and knowledge of earlier generations may have to be acquired all over again.

This is the point where the WHO Special Programme for Research and Training in Tropical Diseases has special significance. Laboratory-based research will be encouraged of course, on immunology and chemotherapy, but to me, more importantly, this programme provides an opportunity for young workers to go into the field to study vector populations in their surroundings. It provides an opportunity for them to try out their ideas—new or old—of preventing malaria transmission.

What now of the last group of infections, on which we have made little or no impact? The most widespread is bancroftian filariasis, but let me first say a few words about another, which in South-East Asia is the cause of grave concern, and that is dengue haemorrhagic fever, which has spread so widely during the last 25 years within this region. It seems strange that so much should be known of the vector *A. aegypti*, that means for its control are established, that even without the use of insecticides populations can be reduced to a very low level, and yet during this past year there were nearly 40,000 cases of dengue haemorrhagic fever in Thailand with about 750 deaths. And in recent years in the Caribbean there have been many

thousands of cases of dengue; last September in Puerto Rico alone there were more than 4,000 cases.

I said earlier that *A. aegypti* transmitted yellow fever had disappeared from the United States largely because of improved water supplies. It will be some years yet before equivalent improvements can be achieved in the developing countries. Therefore preventive measures must be advocated now. We have effective and safe insecticides which can be used to greatly reduce *A. aegypti* populations, but because *A. aegypti* is a domestic mosquito some responsibility must be accepted by householders. Past experience has unfortunately shown that to obtain the co-operation of some householders legislation must be available to the health worker. It is quite clear that the only successful long-term *A. aegypti* control programmes have been those in which legislation could be invoked by the health authorities to ensure the participation and assistance of the public. As examples, one can quote Brazil and Singapore. In the 1930s workers in Brazil reduced *A. aegypti* to very low levels by environmental sanitation; but the public was obliged to play its part, and as a result, the threat of yellow fever was lifted from Brazilian cities. No householder in Singapore is unaware of *Aedes* mosquitoes. All of them know that a spoonful of salt in each anti-ant container at the base of food cupboards will prevent breeding. And all of them know that the water in flower vases, water jars, and the like must be changed every few days to prevent breeding. And it is equally well-known that vector control department inspectors have the right of entry into their homes to check that there are no mosquito habitats.

It seems to me fully necessary for health authorities concerned with vector-borne

diseases to be given the support of enforceable legislation. They are readily given the responsibility to prevent epidemics; they must also be given every reasonable means to fulfil this responsibility.

A. aegypti is not the only domestic mosquito for which the householder must accept some responsibility. Here in India the malaria-transmitting *An. stephensi* is another for whose control the co-operation of the householder is essential.

I was most interested to read yesterday a comment in the *Times of India* that the New Delhi Municipal Corporation has cautioned the public that they will be prosecuted if they allow water to accumulate in their premises and homes. All overhead tanks and open cisterns will have to be properly covered, according to the municipal order, and regularly cleaned.

When we move out of the house, the responsibility for preventing mosquito breeding falls squarely on the shoulders of the health authorities and on the engineers and town planners, and this is where we come back to bancroftian filariasis and its principal vector *Culex fatigans*. We cannot walk through the poorer areas of Calcutta or Rangoon or Colombo looking at blocked drains supporting thousands of *Culex* mosquitoes and blame the people living nearby for not clearing them, or for causing the blockage with refuse. The blame has to be spread much more widely. In time social improvements in housing and drainage will solve the problem. What of now?

Part of the blame no doubt falls on us. We know which insecticides could be applied to *Culex* larval habitats to reduce breeding very significantly. It is more than 10 years since workers in Rangoon and in Africa demonstrated the efficacy of a number of alternative organo-phosphorus insecticides. Why then in the past 10 years

have we made no progress in reducing filariasis? For that matter, why has so little progress been made during the past 100 years since Manson demonstrated in November of 1877 that the mosquito was the intermediate host?

Many reasons could be put forward: the most common nowadays is that the price of insecticides has escalated. This has been true of the years since 1973, and there are many illustrations of the great rise in costs of malaria control programmes. But in the case of filariasis we cannot claim that economic factors provide the sole or major reason for lack of progress.

I understand that in some cities of India, such as Bangalore and Hyderabad, *C. p. fatigans* has become widespread and prevalent only during the past 30 years. And during this period *W. bancrofti* has become a public health problem. What is the difference between the period of the 1930s and 1940s and the later period when *C. p. fatigans* and *W. bancrofti* increased in prevalence? One change has been the recent rapid rate of urbanisation both in India and other developing countries. City populations in the less well-developed countries have been growing more than twice as fast as those in the developed countries of the world—and much faster than rural populations. And, of course, unplanned growth has brought problems of water supply, water disposal and sewage disposal, all bringing benefit to *C. p. fatigans*. We can ascribe the increase in *C. p. fatigans* and *W. bancrofti* reasonably to increased urbanisation, and no-one would argue strongly against this thesis.

On the other hand, one could suggest that there may be a correlation between the increased usage of and dependence on insecticides and the increase in filariasis. It is difficult to support this argument with examples, but I feel there could well be

some truth in it. In West Africa we commonly refer to the virtual absence during the 1930s of *C. p. fatigans* from towns, and to its spread during the insecticide era. It has been argued that another mosquito of drains and polluted water, *Culex nebulosus*, was the common species in the earlier period and that owing in part to insecticide usage it was drastically reduced in numbers allowing *C. p. fatigans* to move into its niche. Now *C. p. fatigans* is very abundant. Fortunately, at present *C. p. fatigans* does not transmit filariasis in West African towns, but it seems to me likely that the parasite, which is still essentially rural in distribution (and transmitted by *Anopheles*) will become adapted to the urban vector. Whether the use of insecticides has directly affected detrimentally the control of *C. p. fatigans* may be uncertain, but I would suggest that indirectly insecticides have perhaps worked to our disadvantage. I remember a conversation a few years ago with Dr. T. Ramachandra Rao when he told me that prior to our dependence on insecticides a high standard of environmental sanitation was achieved and maintained in many towns in India by oilers and drain-cleaners, and that *C. p. fatigans* was prevented from building up high populations by simple, sensible measures, which depended, however, on good staff at all levels.

All vector control programmes depend for their success on the man at the bottom of the organisational pyramid. If he does not implement his instructions, the programme will fail. He is invariably the poorest paid in an organisation, but to be successful, his work must be consistently of the highest standards. We should be concerned about this problem of how to raise the status and efficiency of these workers.

One wonders whether nowadays we have lost the enthusiasm and conviction among workers on mosquito and filariasis prevention that was a feature of the past. Are we waiting for a new wonder insecticide which will do the dull work of environmental sanitation for us, or a new drug or a new vaccine?

I should like to end on a more optimistic note—difficult though that may seem. Let me suggest that a programme of vector control or disease prevention need not be large to be effective and that new insecticides and drugs need not necessarily be essential for success. New methods of vector control and parasite control should and will be developed, and the WHO Special Programme is designed in part to find and develop new technologies, but there is much that can be done now to prevent vector-borne infections. If we were asked to prevent *A. aegypti* breeding or *C. p. fatigans* breeding in one street in Bombay or Colombo, I think we could do

this without requiring too many resources in man-power, insecticides or transport. Equally, I think we could prevent anopheline breeding in one village and its surroundings.

Taking into account the available resources, I feel sure that we could start effective control on a small-scale in many areas. We need not concern ourselves with national or global programmes; if we look after the streets and the villages, the towns and States will look after themselves. Small-scale programmes must be administered locally, and this is an advantage. There is a great danger that, following the phases when emphasis was given successively to such methods as environmental control, chemical control, and integrated control, we have reached an era when too much weight is given to the latest method, namely disease prevention from the offices and committee rooms by remote control. I feel sure that all of us hope that resistance to this method develops rapidly.

■ ■

CHOLERA VACCINES—ROOM FOR IMPROVEMENT

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The progress in the development of an effective cholera vaccine is reviewed. The classical vaccine still in use is of low efficacy. As more insight has been gained into the mechanisms of disease and immunity in cholera rational approaches have been made in the development of more effective vaccines. The drawbacks to the use of the various prospective oral vaccines are discussed, and the development of an improved parenteral vaccine based on a combination of existing vaccine and a sub-unit toxoid is described. Some approaches to improved methods of producing somatic antigen are outlined.

Introduction

The expectation at the beginning of this century that improved standards of sanitation and hygiene would reverse the spread of cholera and limit the disease to its endemic focus on the Indian sub-continent has unfortunately not been fulfilled, and during the last two decades a pandemic has been caused by the *El Tor* biotype of *Vibrio cholerae*, spreading the disease from Indonesia to mainland Asia, Africa, the Middle-East and parts of Europe (1). Although environmental health measures undoubtedly play a significant role in cholera prevention (2, 3), the need for prophylaxis by vaccination remains, especially during population migrations (4) and after natural disasters, when sanitation facilities may be inadequate or inoperative.

Parenteral Whole Cell and Purified Somatic Antigen Vaccines

The earliest cholera vaccines used by Ferran in 1885 and Haffkine in 1894 comprised live cultures of *Vibrio cholerae* for parenteral administration, and were soon followed by the introduction of a heat killed-phenol preserved vaccine (5).

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Since that time the composition of cholera vaccines has remained virtually unchanged; and until recently the mechanism of disease and immunity against it remained obscure and methods of vaccination have remained largely empirical. Indeed, it is only since 1963 that the efficacy of the vaccine in man has been reliably established by controlled field trials (6). In no instance has a parenteral whole cell or a purified somatic lipopolysaccharide (LPS) or LPS-protein vaccine induced solid immunity against cholera, the degree of protection being 40-80 % over the first few months with rapid decline thereafter. Such limited protection led to the conclusion that the use of such vaccines could not be regarded as a primary means of cholera prevention and so the search for a more effective vaccine gained impetus.

Throughout the past two decades, as more insight has been gained into the mechanisms of pathogenicity and immunity in cholera, a more rational approach has been adopted towards devising improved vaccines. Interest has centred mainly around the development of effective oral vaccines and a suitable toxoid, although consideration has also been given

to improved parenteral vaccines based on live organisms (6), cell-free somatic antigens (7, 8, 9), ribosomal antigens (10) and flagellar antigens (9).

Oral Vaccines

Before the pathogenesis of cholera was fully understood, it seemed rational to suppose that effective immunisation against such an organism with an oral route of infection would best be produced by oral vaccines (11), which could be expected to induce local immunity most effectively. With our present insight into the pathogenesis of cholera the supposition seems no less likely, since as the vibrios are primarily restricted to the lumen and surface of the small bowel and the enterotoxin acts at the surface of the intestinal epithelium, the immune mechanism probably operates exclusively within this arena (12, 13).

Killed whole cell oral vaccines induce in man a predominantly coproantibody response (14), obviously exclusively antibacterial in nature. Such a response does constitute a significant protective mechanism against cholera, although the mechanism is unclear (6, 12, 13, 14). Coproantibody production locally has the disadvantage of short duration, although the response can be maintained by frequent revaccination (14) which is itself made more feasible by the ease of administration of this type of vaccine. The possibility of using an L-form lysate oral vaccine has also been considered (15), although the procedures governing the application of non-living oral vaccines to cholera immunoprophylaxis have yet to be established, and their practicability has been questioned (12).

Live oral vaccines have the theoretical advantage that the effective immunising dose is amplified after administration, and they may be expected to simulate the

natural disease more closely than killed vaccines, especially with regard to the induction of antitoxic coproantibody, for which a protective role in man has been proposed (12, 13, 14, 16). However, live oral vaccines derived from such an organism with a faecal-oral route of spread must obviously conform to strict requirements (6).

Several types of live vaccines have been considered (6, 12), which have developed along lines similar to oral vaccines against bacillary dysentery. However, the theoretical advantages of live vaccines outlined above are not met by the candidate vaccine strains; the streptomycin-dependent mutant would fail to induce antitoxic immunity (12), non-toxigenic mutants (17, 18) suffer the same defect, and apathogenic strains invoke a poor local immune response because of rapid elimination without colonisation of the bowel (19, 20) and perhaps weak antigenicity (20). The use of strains in which toxigenesis is depressed by certain plasmids has also been considered (21), although such organisms may also suffer from rapid elimination after administration. To overcome these problems the use has been proposed of apathogenic mutants with bowel colonising ability and which produce a structurally altered toxin molecule that is devoid of choleraogenic activity but retains its antigenic integrity (12, 22). In this way antibacterial and antitoxic immunity could be safely induced. The use of chemical mutagenesis and other genetic engineering techniques is being applied to the development of such mutants (12, 22), although it remains to be seen whether a suitable organism can be isolated.

The possibility of eliciting effective immunity by the oral administration of toxoids has been considered (12, 16), although, as with the killed oral vaccines,

the short-lived nature of the coproantibody response so stimulated would necessitate frequent revaccination (16). This disadvantage could be overcome if the gene for the production of a structurally altered non-cholera toxin could be transferred to a member of the normal intestinal flora (22).

It remains to be seen whether oral vaccination against cholera poses a worthwhile proposition; certainly until bowel-colonising mutants are selected an effective oral vaccine would need to comprise both cell and toxoid components and revaccination would need to be frequent. As has been pointed out (23, 24), it would be remarkable if solid immunity against cholera could be achieved by oral vaccination when the disease itself, which induces local and systemic antibacterial and antitoxic responses, does not induce solid immunity, even though, under controlled conditions, experimentally induced infections may confer a somewhat greater degree of protection (25, 26).

Improved Combined Parenteral Vaccines

With the failure to develop an effective oral vaccine, consideration has recently been given to the development of improved parenteral vaccines based on a combination of somatic and toxoid antigens. The rationale is that parenterally administered somatic antigens do offer a degree of protection, albeit limited, in the field, and they can induce in man a coproantibody response almost comparable to that of prospective oral vaccines (14, 20). Evidence that parenterally administered toxoid can offer significant protection in humans is less conclusive, since in the only field trial conducted the degree of protection was low (27), as was the immunogenicity of the glutaraldehyde toxoid used. Furthermore, the stimulation of serum-derived coproantibody would have the advantage of

a response of relatively long duration and strong anamnesis (14); and as the combined vaccine should induce immunity against two separate components of the pathogenic process there was good reason to predict that the effect of combination would be synergistic rather than additive (12). The synergistic protective effect between cholera LPS and purified toxin or natural toxoid (cholera toxinogenoid) was subsequently demonstrated (28), the combination of antigens inducing more than one hundredfold greater degree of protection against experimental intestinal challenge of the rabbit with live vibrios than did the single antigens.

It was suggested (13) that the limitations in the currently used formaldehyde and glutaraldehyde toxoiding treatments (reversion to toxicity and destruction of antigenicity respectively) could be overcome by chemical modification *in situ* of the toxin molecule by amino acid specific reagents, or by the development of a sub-unit toxoid. The latter proposal was based on the recent demonstration of the structure and function of the sub-units of the cholera toxin molecule, in which one type of sub-unit (L or B) binds the toxin molecule to the cell membrane ganglioside receptors, and the other type (H or A) subsequently mediates the disease by the stimulation of adenyl cyclase activity (13). L sub-units appeared to be superior immunogens and gave rise to sera with higher toxin neutralising activity compared to H sub-units (13, 29); thus, after dissociation of the toxin molecule and removal of the H sub-units, reaggregated L sub-units could be expected to form an ideal toxoid, since there would be no possibility of reversion to toxicity.

The preparation of a purified L sub-unit toxoid has been described (29). After separation the L sub-units reaggregated to form a complex similar in size to the

natural toxin and parenteral administration induced better protection against either live vibrio or toxin challenge in the rabbit ligated ileal loop than did the glutaraldehyde toxoid used in the field trial referred to above. When combined with conventional or purified LPS vaccine a synergistic protective effect against intestinal challenge of the rabbit with live vibrios was demonstrated, and the duration of protection was greater than that of the conventional vaccine alone. On this basis it was suggested that the inclusion of the sub-unit toxoid into existing cholera vaccine should result in more effective and longer lasting immunity against cholera. The verification of this suggestion in controlled field trials of the combined vaccine is eagerly awaited.

Improved Methods of Somatic Antigen Production

The motivations for manufacturers who produce vaccines on a large-scale to develop more efficient means of production are various (30). In the production of cholera vaccines the organism is most commonly grown on solid medium, although the use of large-scale cultivation vessels for batchwise or continuous cultivation in liquid medium would be a more efficient method (30, 31). However, with vaccine production the immunogenic quality of the product is of higher priority than the rate of output (32), and unfortunately the growth of cholera vibrios batchwise in complex liquid medium results in an inferior vaccine compared to growth on solid medium, as measured both in the Calcutta field trials

(6) and by the active mouse protection test (Collier and Richer—personal communication).

At the Lister Institute, in the search for a method of producing from liquid cultures organisms of equal or superior immunogenicity to those grown on solid medium, continuous culture in the chemostat has been used to investigate the effects of nutritional environment and growth rate on the immunogenicity of *Vibrio cholerae*. The basis for this study was the evidence that in Gram-negative organisms the chemical composition and biological properties—including antigenicity—of the cell envelope can be phenotypically variable (33, 34, 35, 36, 37). Comprehensive descriptions have been given of the theory of the chemostat (38, 39) and its design and construction (40, 41). Its ability to produce a homogeneous product is recognised (42) and its application to the production of vaccines of controlled antigenicity has been advocated (30, 42, 43, 44), although examples of this are few (45, 46). It is hoped to use the method to determine the conditions producing a cholera phenotype of acceptable potency, so that a scaled-up continuous system (40) or an equivalent fed-batch culture system (39) can be used for routine production. With the demonstration that antibacterial immunity in cholera is exclusively mediated by antibody directed towards LPS (13), a combined LPS-toxoid vaccine may become accepted in the future as the least reactogenic product. If so, such systems could be used to produce LPS of maximum immunogenicity and to optimise the LPS : cell ratio as has been done, for example, with nucleic acids (47).

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CULTURAL AND SEROLOGICAL DIAGNOSIS OF GONORRHOEA

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In this study of gonococcal infection, cultural and serological tests were done in males and females. In culture by using a selective medium of chocolate agar containing various drugs, *N. gonorrhoea* could be isolated in 85 % males and 58 % females. In females, maximum isolation occurred from endocervical region when a single swab was used for collection of clinical material. When two swabs were employed a combination of endocervical and rectal swabs gave the best result. In serology, hemagglutination, microflocculation, microprecipitation and radio-immunoprecipitation tests were employed. The latter gave the highest percentage of sero-positive reactions which were as high 90-95 % and 85 % in culture positive and culture negative cases, respectively. In these tests wherever lipopolysaccharides were used as the antigen, false positive reaction occurred in 20-25 % of normal sera. On the other hand, in microprecipitation and flocculation tests, there was less false positive reaction.

Introduction

Recent statistics (1) show an alarming increase in the number of cases of gonorrhoea in all five continents. The highly contagious nature of this sexually transmitted disease and complications caused by it makes it essential that a rapid, accurate and sensible diagnosis can be made with the least delay. Laboratory diagnosis of the disease is possible by examination of smears, cultural and serological studies. Smears and cultures of more than one infected sites give a higher percentage of positive results and at least three cultures are necessary before negative results can be stated with certainty. But such procedures present difficulties in many countries including India. It is therefore desirable to develop simple and reliable serological tests for diagnosis of the disease.

During 1975-76, Clinical Pathology Department of Haffkine Institute, Bombay in collaboration with V. D. Clinic, Bellasis Road, Bombay tried a number of cultural and serological procedures for the diagnosis of this disease. These are being presented in this article.

Materials and Methods

(A) *Smears and Cultures*—for these, sterile cotton swabs treated with phosphate buffer and charcoal (2) were used to collect materials from-urethral discharge, seminal fluid after prostatic massage (males), endocervix, vagina and rectum (females). One swab was used for making smears for Gram staining and the rest were put on two transport media—(a) modified Stuart (3) and (b) Transgrow (4) and inoculated on two culture media within 4 hr. The media were—(c) clarified chocolate (5) and (d) (c) modified to contain cycloheximide (5 µg), lincomycin (0.5 µg), colistin (7.5 µg), nystatin (12.5 µg) and

trimethoprim (3 µg) per ml of the media. These media after inoculation were put in candle jars (5-10 % CO₂) for 24-48 hr. and *N. gonorrhoea* identified on the basis of oxidase and sugar fermentation reactions.

(B) *Serological Tests*—4 tests were employed as below :

(1) *Hemagglutination*—LPS of 10 freshly isolated smooth strains of *N. gonorrhoea* was extracted (6) and freeze-dried. To 0.5 mg of the LPS 1 ml of 0.02 N NaOH was added, kept at 37°C for 24 hr; dialysed in cellophane bag in phosphate buffer (pH 7.2) for 3-4 days. For H.T., 15 µg LPS/ml in phosphate buffer was sensitized with 2.5% sheep r.b.c. for 37°C for 1 hr. and the test carried as per method of Meland (7). A hemagglutination titre of 1 in 64 or above indicated a positive serological reaction.

(2) *Microflocculation (M.F.T.)*—This was done by technique of Reising (8) 24 hr. growth on solid media washed in distilled water; sonicated; centrifuged at 43500 × g for 1 hr. at 5°C; supernant freeze-dried; dissolved in 1 ml of buffer (pH 7.0, phosphate) and sensitized with 0.25 ml of lecithin and cholesterol mixture (4 mg of the former in 10 ml of 0.9% of the latter in ethanol) at room temperature for 1 hr; 0.025 ml of this antigen and serum tested as in V.D.R.L. test.

(3) *Microprecipitation (M.P.T.)*—Antigen for the test was prepared as per method of Chacko and Nair (9). In the test equal amount of antigen (2 units/ml) was allowed to act with serum in a capillary tube (15-18 × 2 mm) for 1 hr. at 37°C for precipitation ring to form at the zone of contact.

(4) *Radioimmunoprecipitation (R.I. P.T.)*—LPS of *N. gonorrhoea* extracted as before (6) was labelled with ⁵¹Cr(10). In labelling, 150 µg of LPS, 150-200 mCi of ⁵¹CrCL₃ (Sp. activity 50-60 mCi/mg of Cr) and 10 ml of ethanol were mixed and extracted in a 50 ml flask with a reflux system at 80°C for 1 hr. in water bath; the extract dried on a hot plate at 70-80°C; 5 ml of distilled water and 0.2 ml of 5 % EDTA added; elute the extract through Sephadex G-50 column to get two fractions, LPS & Cr and EDTA & free Cr which showed two peaks of radioactivity in a medical spectrometer. The former's radioactivity indicated that about 50-55% of the isotope was incorporated in the antigen.

In the radioimmunoprecipitation test (11), 0.1 ml of antigen in 3% Na-deoxycholate borate buffer saline, 0.2 ml of serum and 0.1 ml of the buffer mixed and allowed to react at 4-5°C for 15 min; equal amount of 40% polyethylene glycol (PEG, m.w. 6000) added to act at room temperature overnight to precipitate 3 types of immunoglobulins (12); add 1 ml of 20% PEG to each sample of serum; centrifuge at 1000 × g for 30 min; determine radioactivity of the precipitate in a medical spectrometer. The percentage of antigen bound to immunoglobulins precipitated from each sample determined.

$$\frac{\text{Count per min. of the precipitate}}{\text{Count per min of the antigen}} \times 100$$

A retention of more than 20% radioactivity indicated a positive reaction.

In all the serological tests, a negative, positive sera and antigens, were used as controls.

Results

(A) *Smears and Cultures*—These are shown in Table I.

TABLE I

Smears and Cultures

Samples		Smears post		Culture post	
Nos.	Sources	Nos.	%	Nos.	%
860	Male	473	55	732	85
900	Female	297	33	522	58

TABLE II

Culture from Various Sites (Females)

Samples	Positive		Sources	Nos.	%
	Nos.	%			
150	88	59	C & R	88	100
			C & U	83	94
			C & V	83	94
			R & U	70	80
			R & U	73	88
			U & V	72	82
			C	83	94
			U	65	74
			R	38	48
			V	68	77

In females, maximum isolation occurred (56 %) from endocervix. In 150 females, two swabs were taken for culture and results compared with a single swab.

C, R, U, V means cervix, rectum, urethra and vagina.

The above results indicated that for a single sample and two samples, endocervix and endocervix and rectum, respectively, gave the best results.

The results of transport and culture media are shown in Table III.

The Table IV shows results of the 4 serological tests.

In Table V evaluation of H. T. and M.P. T. have been done during different stages of infection.

TABLE III

Evaluation of Different Media

Samples	Transport Post Medium				Samples	Culture Post Media			
	(a)		(b)			(c)		(d)	
	Nos.	%	Nos.	%		Nos.	%	Nos.	%
50	28	56	28	56	55 (Male)	42	76	47	85
					60 (Females)	24	40	36	60

TABLE IV

Serological Tests

Samples	Sources Nos.	Culture Post Nos.	%	% of Sero-positives in Tests								
				H. T.		M. F. T.		M. P. T.		R.I.P.T.		
				Nos.	%	Nos.	%	Nos.	%	Nos.	%	
Males ..	..	550	450	82	229	51	337	75	373	83	405	90
			100	18	40	40	55	55	68	68	85	85
		(Neg.)										
Females	..	700	400	79	284	71	320	80	352	88	380	95
			300	21	152	51	185	61	224	81	255	85
		(Neg.)										
Normal Sera	..	100				20		10		10		25

TABLE V

H. T. and M. P. T. in Culture Positive Cases

Duration of discharge in days	Nos.	H. T.		M.P.T.	
		Nos.	%	Nos.	%
3-4	200	54	27	153	77
5-7	115	85	73	92	80
7-30	135	90	67	128	96

Discussion

In gonococcal infection before 7-10 days when antibody formation does not occur (13), smear and culture examinations are important for the laboratory diagnosis of the disease. The superiority of the latter as compared to the former as a diagnostic aid, is well-known. During 1975 (14), smears and cultures for *N. gonorrhoea* were positive in 52% and 58% males and 31% and 38% females, respectively. During 1976 (15) smear results remained the same but isolation rate of the organism improved by 20-21% by using a selective medium containing various drugs of which cycloheximide particularly proved useful in elimination of commonly contaminating filamentous fungi belonging to the *Aspergillus* group. This study also showed that

in case of females, a single endocervical or combination of endocervical and rectal swabs gave a higher rate of isolation of *N. gonorrhoea*.

Serology of *N. gonorrhoea* is complex, involving superficial thermolabile and deeper thermostable antigens which produce antibodies in serum in 7-10 days and 2 weeks (13), respectively. The latter, LPS in nature contains several sugars which are reported to be common among *Neisseria* (16) and *Enterobacteriaceae* (17). This creates a serological diagnostic problem as could be seen that 20-25% non-specific seropositive reaction with normal sera in hemoagglutination and radioimmunoprecipitation tests (Table IV). Similar reaction varying from 20-40% has been reported (17) in radioimmunoassay with internally labelled antigen of *N. gonorrhoea*.

In this study, M. F. T. and M. P. T. appeared more specific and gave a higher percentage of sero-positive reaction with culture-positive gonococcal cases as compared to indirect fluorescent antibody test (14, 18). The latter test (M.P.T.) which could be repeated more than once along with H. T. showed a higher percentage of

sero-positive reaction in early and late gonococcal infected cases (Table V). These results suggest that besides LPS (9) the antigen of M.P.T. might be containing some superficial antigens which were preserved during chorio-allantoic passages and gave a sero-positive reaction in early infected cases. This test inspite of giving 10% non-specific reaction, appears to be suitable for the laboratory diagnosis of gonorrhoea cases.

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SYNTHESIS AND ANTHELMINTIC ACTIVITY OF 2-(N⁴-SUBSTITUTED-N¹-PIPERAZINYL) METHYL 5-(or 6)-SUBSTITUTED BENZIMIDAZOLES

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A series of substituted piperazinomethyl benzimidazoles have been prepared and tested for *in vivo* anthelmintic activity. The majority of the compounds showed activity above 80% at 500 mg/kg dose as compared to Yomesan showing 100% activity at 500 mg/kg dose. The results confirm the importance of nitro group in benzimidazole ring for anthelmintic activity.

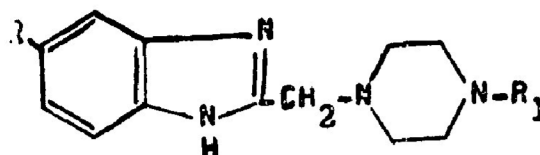
Introduction

Many benzimidazole derivatives possess antiparasitic (1), antibacterial (2) and antiviral activities (3). Of these thiabendazole, parbendazole, mebendazole etc. exhibit significant broad spectrum anthelmintic activity against intestinal parasites.

A significant advance in helminth chemotherapy was the introduction of piperazine and its derivatives in the treatment of ascariasis and filariasis. Among the benzimidazole derivatives it was observed that the introduction of a nitro group in heterocyclic ring potentiated the antiparasitic activity as in metronidazole, niridazole, etc.

Synthesis of a series of compounds of the general formula (I) was, therefore, undertaken in an effort to develop improved drug activity and further

delineate the structural parameters leading to activity within this class.

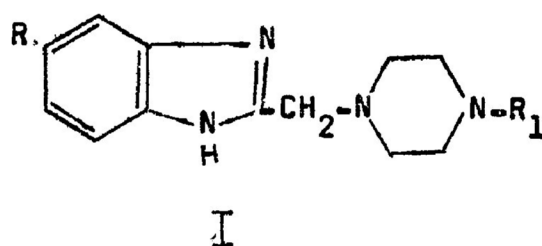


Where R=H or NO₂

R₁=Methyl, β -hydroxyethyl; benzyl, Phenyl, *p*-Chlorophenyl; *o*-tolyl; *o*-methoxyphenyl; 2-pyridyl, 2-thiazolyl.

The desired piperazinyl benzimidazoles were obtained by reacting 2-chloromethyl-5 (or 6)-substituted benzimidazoles with appropriate N-substituted piperazine. The required 2-chloromethyl-5-(or 6)-substituted benzimidazoles and N¹-substituted piperazines were prepared as per the standard procedures 4(a)–(d).

TABLE

2-(*N*⁴-Substituted-*N*¹-Piperazinyl) methyl-5(or 6)-Substituted Benzimidazoles

Serial No.	R	R ₁	Molecular Formula*	M.P°C†	Dose mg/kg	% Efficacy‡ against <i>H. nana</i>
1	H	Methyl ..	.. C ₁₃ H ₁₈ N ₄	.. 179–181	500	Not effective
2	H	β-Hydroxyethyl ..	.. C ₁₄ H ₂₀ N ₄ O·3HCl	.. 208–210		Not tested
3	H	Benzyl ..	.. C ₁₉ H ₂₂ N ₄	.. 200–201	500	92
4	H	Phenyl ..	.. C ₁₈ H ₂₀ N ₄	.. 246–248	500	50
5	H	<i>p</i> -Chlorophenyl ..	.. C ₁₈ H ₁₉ ClN ₄	.. 222–223	500	Not effective
6	H	<i>o</i> -Methoxyphenyl ..	.. C ₁₉ H ₂₂ N ₄ O	.. 214–215	500	65
7	H	<i>o</i> -Tolyl ..	.. C ₁₉ H ₂₂ N ₄ ·2HCl	.. 172–174	500	40
8	H	2-Pyridyl ..	.. C ₁₇ H ₁₈ N ₅	.. 213–215	500	Not effective
9	H	2-Thiazolyl ..	.. C ₁₅ H ₁₇ N ₅ S	.. 218–219	500	Not effective
10	NO ₂	Methyl ..	.. C ₁₃ H ₁₇ N ₅ O ₂ ·2HCl	.. 240–241	500	97
11	NO ₂	Benzyl ..	.. C ₁₉ H ₂₁ N ₅ O ₂	.. 176–178	500	95
12	NO ₂	Phenyl ..	.. C ₁₈ H ₁₉ N ₅ O ₂	.. 192–194	500	Not effective
13	NO ₂	<i>p</i> -Chlorophenyl ..	.. C ₁₈ H ₁₈ ClN ₅ O ₂	.. 225–227	500	94
14	NO ₂	<i>o</i> -Methoxyphenyl ..	.. C ₁₉ H ₂₁ N ₅ O ₃	.. 128–130	500	80
15	NO ₂	<i>o</i> -Tolyl ..	.. C ₁₉ H ₂₁ N ₅ O ₂	.. 198–200	500	95
16	NO ₂	2-Thiazolyl ..	.. C ₁₅ H ₁₆ N ₆ O ₂ S 2HCl·H ₂ O	.. 254–255	500	Not effective

*Analysed for C, H & N

†All melting points are capillary melting points and are uncorrected.

‡Reference drug-Yomesan (100 % Efficacy at a dose of 500 mg/kg).

Experimental

2-Chloromethylbenzimidazole and its 5-(or 6)-nitroderivatives were prepared from *o*-phenylenediamine or 4-nitro-1, 2-phenylenediamine and chloroacetic acid in 4 N HCl in 80 % yield. N-aryl-piperazines were prepared according to Pollard *et al.* 4(b) by heating the mixture of diethanolamine hydrochloride and substituted aniline at 170° C and then at 240° C for 7 hr.

2-(N⁴-*p*-chlorophenyl-N¹-piperazinyl)-methyl-5-(or 6)-nitro-benzimidazole—A mixture of 2-chloromethyl-5-(or 6)-nitro-benzimidazole (1.05 g, 0.005 mole) dissolved in ethanol (25 ml), N-*p*-chlorophenyl piperazine (1.08 g, 0.0055 mole) dissolved in chloroform (30 ml) and sodium carbonate (1.06 g, 0.01 mole) was refluxed for 8 hr. on steam bath filtered hot and the solvent removed by distillation under reduced pressure. The residue after washing with hexane was purified by column chromatography using alumina (neutral 15 gm) and dry benzene as eluent. The compound obtained was recrystallized from a mixture of ethanol

and hexane (1:2). Yield 29.6 % (1.1 g), m.p. 225–227° C.

Biological Results and Discussion

Piperazinobenzimidazoles were tested (5) *in vivo* against *Hymenolepis nana* at 500 mg/kg dose and 250 mg/kg dose. The results are tabulated in Table I.

Of the fifteen compounds tested, nine showed definite activity. Of these nine active compounds five showed efficacy above 90 % at 500 mg/kg dose as compared to Yomesan showing 100 % efficacy at 500 mg/kg dose. They all contained a nitro group at 5-(or 6)-position and thus confirm the importance of nitro group in the benzimidazole ring for anthelmintic activity. The most active was 2-(N⁴-methyl-N¹-piperazinyl) methyl-5-(or 6)-nitrobenzimidazole, which showed 97 % efficacy at 500 mg/kg dose.

Acknowledgements

The authors thank Mr. M. T. Jaokar and his associates for elemental microanalyses. They also thank Dr. B. B. Gaitondé Director, Haffkine Institute, Bombay for facilities to carry out this work.

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SYNTHESIS AND STUDY OF 2-(4-AMINOPHENYL) THIAZOLES FOR IN VITRO ANTITUBERCULOSIS ACTIVITY AND AS USEFUL CHEMOTHERAPEUTIC INTERMEDIATES

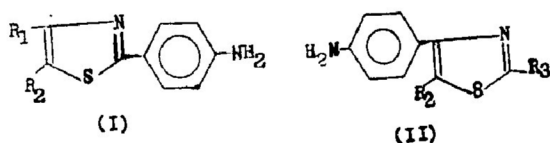
C. B. NERURKAR, D. T. CHAUDHARI, MADHUBEN R. PATEL AND S. S. SABNIS

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2-(4-Aminophenyl) thiazoles have been synthesised from various haloketones and 4-aminothiobenzamide as useful chemotherapeutic intermediates and have been screened for *in vitro* antituberculosis activity. Many thiazoles have shown significant antituberculosis activity and two of these have shown activity comparable with that of PAS (*p*-aminosalicylic acid) and Ethionamide under identical conditions of *in vitro* testing.

Introduction

We have already reported a novel method for the preparation of 2-(4-aminophenyl) thiazoles (I) with various substituents at positions 4 and 5 of the thiazole ring (1).



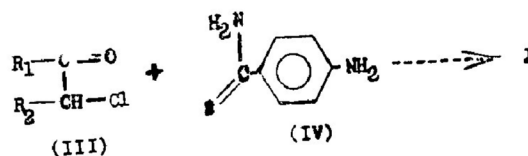
Following the same method we have prepared a few new thiazoles and considering the excellent *in vitro* antituberculosis activity shown by 4-(4-aminophenyl) thiazoles (II)(2) and their derivatives (3), we have screened these thiazoles, along with those reported earlier (1), for *in vitro* antituberculosis activity.

Chemistry

The synthesis of 2-(4-aminophenyl) thiazoles (I) was effected by condensing equimolar quantities of 4-aminothiobenzamide (IV) with various haloketones (III), in boiling ethanol, to afford the thiazoles

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as hydrochlorides in good yields and in a sufficiently pure state as reported earlier (1).



The intermediates were also prepared as reported earlier (1).

Experimental

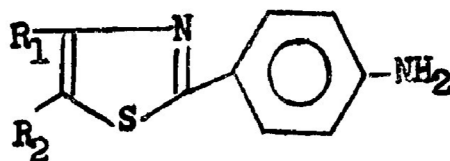
A mixture of requisite haloketone (0.01 mole) 4-aminothiobenzamide (0.01 mole) in ethanol was refluxed to clear solution. The solid thrown out in due course was filtered, washed with boiling ethanol (10 ml) and ether, and dried to get the thiazole-hydrochloride in analytically pure condition as reported earlier (1).

Biological Results

In vitro antituberculosis testing was carried out against the human virulent strain of *Mycobacterium tuberculosis* (H₃₇Rv) cultivated in Youmans' medium containing

TABLE I

2-(4-Aminophenyl) Thiazoles*



Serial No.	R ₁	R ₂	M.p. °C†	Molecular Formula‡	Antituberculosis activity µg/ml§
1	CH ₃	H	300(dec)	C ₁₀ H ₁₀ N ₂ S.HBr	2
2	C ₆ H ₅	H	280-83	C ₁₅ H ₁₂ N ₂ S.HBr	40
3	C ₆ H ₅	CH ₃	270	C ₁₆ H ₁₄ N ₂ S.HBr	10
4	4-CH ₃ O C ₆ H ₄	H	242-44	C ₁₆ H ₁₄ N ₂ OS.HCl	5
5	4-CH ₃ O C ₆ H ₄	CH ₃	258-61	C ₁₇ H ₁₆ N ₂ OS.HCl	
6	4-C ₂ H ₅ OC ₆ H ₄	H	235-37	C ₁₇ H ₁₈ N ₂ OS.HCl	5
7	4-C ₂ H ₅ OC ₆ H ₄	CH ₃	261-62	C ₁₈ H ₁₈ N ₂ OS.HCl	2
8	4-CH ₃ C ₆ H ₄	H	252-55	C ₁₆ H ₁₄ N ₂ S.HCl	5
9	4-CH ₃ C ₆ H ₄	CH ₃	258-61	C ₁₇ H ₁₆ N ₂ S.HCl	
10	4-ClC ₆ H ₄	H	272-75	C ₁₆ H ₁₁ ClN ₂ S.HCl	20
11	4-ClC ₆ H ₄	CH ₃	275-78	C ₁₆ H ₁₃ ClN ₂ S.HCl	10
12	4-BrC ₆ H ₄	H	263-66	C ₁₆ H ₁₁ BrN ₂ S.HCl	10
13	4-BrC ₆ H ₄	CH ₃	263-66	C ₁₆ H ₁₃ BrN ₂ S.HCl	Inactive
14	4-CH ₃ CONHC ₆ H ₄	H	280-83	C ₁₇ H ₁₅ N ₃ OS.HCl	
15	4-CH ₃ CONHC ₆ H ₄	CH ₃	248-50	C ₁₈ H ₁₇ N ₃ OS.HCl	5
16	4-NH ₂ C ₆ H ₄	CH ₃	280(dec)	C ₁₆ H ₁₅ N ₃ S.HCl	
17	4-CH ₃ O C ₆ H ₄	C ₆ H ₅	151-54	C ₂₂ H ₁₈ N ₂ OS	
18	H	CH ₃	275-76	C ₁₀ H ₁₀ N ₂ S.HCl	0.5
19	COOC ₂ H ₅	H	215-17	C ₁₂ H ₁₂ N ₂ O ₂ S.HBr	20
20	CH ₃	COOCH ₃	218-20	C ₁₂ H ₁₂ N ₂ O ₂ S.HCl	
21	4-FC ₆ H ₄	H	251-54	C ₁₆ H ₁₁ FN ₂ S.HCl	0.5
22	4-NO ₂ C ₆ H ₄	H	212-13	C ₁₆ H ₁₁ N ₃ O ₂	Inactive
23	2-OHC ₆ H ₄	H	265-67	C ₁₆ H ₁₃ N ₂ OS.HCl	200
24	3-CH ₃ CONH-4-CH ₃ OC ₆ H ₃	H	270-71	C ₁₈ H ₁₇ N ₃ O ₂ S.HCl	2
25	4-C ₂ H ₅ COONHC ₆ H ₄	H	284-85 (dec)	C ₁₈ H ₁₇ N ₃ O ₂ S.HCl	10
26	4-C ₆ H ₅ C ₆ H ₄	H	255-57	C ₂₁ H ₁₆ N ₂ S.HBr	Inactive
27	4-C ₆ H ₅ C ₆ H ₄	H	276-77	C ₂₁ H ₁₆ N ₂ S.HCl	Inactive
28	4-CH ₂ COOHC ₆ H ₄	H	295	C ₁₇ H ₁₄ N ₂ O ₂ S.HCl	40
29	4-OCH ₂ COOHC ₆ H ₄	H	305	C ₁₇ H ₁₄ N ₂ O ₃ S.HCl	20
30	4-OCH ₂ COOCH ₃ C ₆ H ₄	H	285	C ₁₈ H ₁₆ N ₂ O ₃ S.HCl	20
31	4-C ₂ H ₅ CONHC ₆ H ₄	H	285	C ₁₈ H ₁₇ N ₃ OS.HCl	2

*The chemistry of Thiazoles (1-17) have been reported earlier except their biological activity (1).

†Melting points are capillary melting points and are uncorrected.

‡Analysed correctly for C, H and N.

§Minimum inhibitory concentration *in vitro* antituberculosis activity µg/ml (for ethionamide 0.5 µg/ml; for PAS 0.4 µg/ml and for thioacetazone 2.0 µg/ml.).

10 % horse serum by a modified method of Doub and Youmans (4).

Out of thirty-one thiazoles, twenty-five members were tested for *in vitro* activity and eight thiazoles were active below 5 µg/ml. Two thiazoles (18, 21) were active at 0.5 µg/ml, this activity being comparable with that of PAS and ethionamide and superior to that of thiacetazone under identical conditions of *in vitro* testing.

Acknowledgement

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■ ■

DEVELOPMENT OF *ANCYLOSTOMA CEYLANICUM* IN LABORATORY HOSTS

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Ancylostoma ceylanicum could develop in infant mice, rats and guinea-pigs when infected per os. The larvae reached the adult stage but failed to attain sexual maturity. All animals including both infant and young rabbits did not harbour worms at autopsy done at various intervals during 30 days post-exposure period.

Introduction

Ancylostoma ceylanicum is a natural parasite of dogs and cats but it infects man as well (1-3). In an experimental study, Yoshida *et al.* (4) recovered many adult worms from the gut of the human volunteers following oral infection. This parasite develops directly without undergoing any lung journey in its natural hosts when infected orally (5-7). Ray *et al.* (8) reported a complete development of this parasite in hamsters and subsequently they established this strain through serial transfer in these animals. They further reported the complete development of *A. ceylanicum* in Swiss albino mice under the influence of corticosteroid (9).

This present communication deals with the development of *A. ceylanicum* in neonatal and young mice, rats, guinea-pigs and rabbits.

Materials and Methods

Ancylostoma ceylanicum strain (Hamster strain) was obtained from the Parasitology Laboratory, CIBA-GEIGY Research Centre, Bombay and the infection was maintained in hamsters as per methods

described by Ray *et al.* (8). A dose of 0.05 ml of saline suspension containing approximately 300 infective larvae was given per os to each of suckling (7 days old) and young (20 days old) mice and rats. Similarly, a dose of 500 larvae was given to each guinea-pig and rabbit. These infant and young animals following oral infection, were kept with their respective mothers. The worm load in small intestine and lungs of animals was determined by doing necropsy done every week following infection for a period of 30 days.

Results

Three infant and three young rats when examined on day 7 were found to harbour larvae which had moulted to fourth stage. Morphologically, they were distinguished from the third stage as these showed the evidence of well developed buccal capsule and prominent oesophagus. Sexes at this stage were clearly differentiated. The tail of the male had a swelling indicating developing bursal rudiments. The L₄S larvae increased in their average size from the third stage onwards from 0.420 mm to 1.098 mm during this period. Three rudimentary teeth were barely visible in the buccal capsule. On day 14, the larval recovery from infant rats, was 0.33 %

and 2.60 % and these larvae were in the initial fifth or immature adult stage (L_5S); the male measuring 2.143 mm while the female 2.217 mm in length. The hook like teeth were fully formed and protruded from the buccal capsule. The muscular structure of the oesophagus was clear. The bursal apparatus was formed but still was enclosed within the cuticle. Most of the larvae left the gut during the second and third

week period after infection. They were rarely seen in rats from which about 0.33 % larvae were recovered. These larvae showed remarkable development (2.650 mm) in size (Table I, Figures, 1-4). By the day 30, infant rats had already expelled all worms from the gut, while none of the young rats (20 days old) showed any larvae during this period of observation.

Figures 1-5—Developmental Stages of *Ancylostoma ceylanicum* in Infant Rats recovered from the Small Intestine.

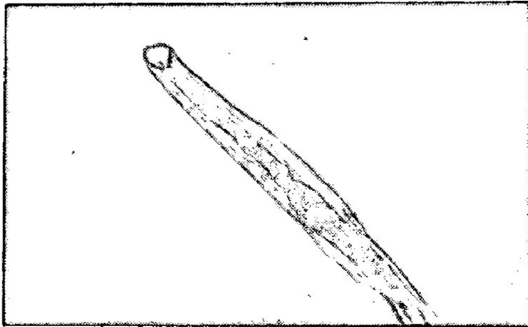


Figure 1—Head of the fourth stage larva on the 7th day.

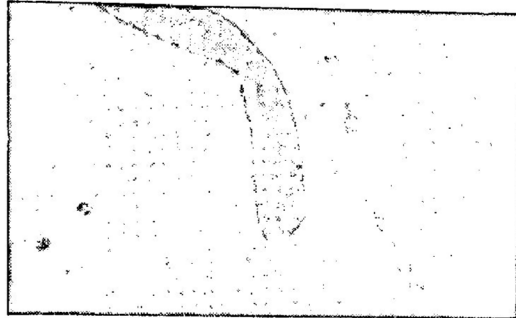


Figure 2—Tail part of the fourth stage larva on the 7th day.



Figure 3—Head of the immature adult on the 14th day.

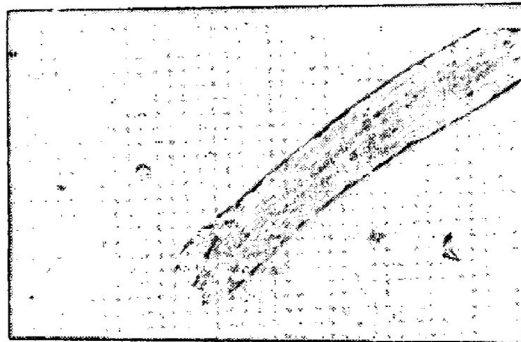


Figure 4—Bursa copulatrix of an immature adult on the 14th day.

On the examination of ten infant and ten young mice, only one infant mice yielded single adult female worm on day 14 during the period of observation (Table I, Figure 5). It showed marked growth in size

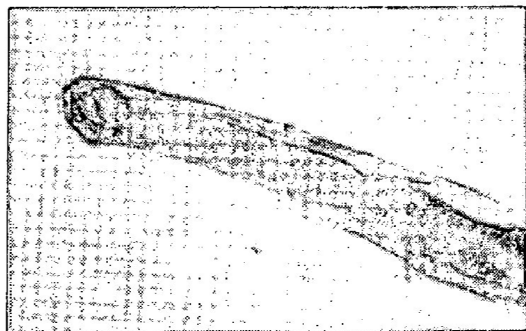


Figure 5—Head of an adult worm on the 21st day recovered from the intestine of infant mice.

(4.90 mm) with prominent buccal capsule possessing one pair of teeth, oesophageal bulb and generative organs but no fertile or infertile eggs became visible in the uterus of this female worm. Similar observation was made in guinea-pigs. The single worm recovered from the small intestine of an infant guinea-pig on the 7th day after infection, was an immature adult female (3.00 mm) (Table I). However, no other infant or young guinea-pigs indicated the presence of any larva or adult worm in the gut. It was surprising to note that none of the infant or young rabbits showed any parasite during the observation period of 30 days when examined at different intervals.

No migration of larvae into the lungs was noticed in any of the infant or young animals during daily examination carried out for a period of 7 days. No mortality occurred in any of the infected animals.

Discussion

The present study showed the incomplete development of *A. ceylanicum* in the laboratory hosts, viz; rats, mice and guinea-pigs. After oral inoculation, the development of the third stage larvae of *A. ceylanicum* in the intestinal mucosa in these animals is similar to that of development in hamsters as reported by Ray *et al.* (8). They observed that *A. ceylanicum* completed its life cycle entirely in the lumen of the gastrointestinal tract. In our investigation, the larvae could develop only up to the immature adult stage in these immunological immature host. The failure of the development and establishment of the larvae in the young hosts of 20 days old is due to perhaps to the manifestation of a higher degree of host's resistance against the larvae for undergoing further development. This could very well be understood on the basis of explanations offered by Kassai and Aitken (10), Jarret and Urguhart (11), in their study on *Nippostrongylus brasiliensis* infection in baby mice.

The time necessary for the development of larvae may vary in different hosts. According to Yoshida *et al.* (12) and Ray *et al.* (8), fourth and fifth stage larvae of *A. ceylanicum* in pups and hamsters following oral infections, first appeared on the 2nd and 5th day. This development was, however, delayed in rats, mice and guinea-pigs, used in the present study. During the course of investigation, the rabbit was found to be an unsuitable host for the development of this hookworm species. However, this is in contrast to that of *Necator americanus* which grows well and reaches complete development in this animal as reported earlier by Bhopale *et al.* (13). This phenomenon clearly differentiates the behaviour of these two hookworms in the same developing medium. Our findings indicate that *A. ceylanicum* does not undertake any lung

journey and undergo direct development in the small intestine. This observation agrees well with that of others in hamsters (8) and in dogs (12).

Acknowledgements

Grateful acknowledgement is expressed to Dr. B. B. Gaitondé, Director, Haffkine Institute, for his encouragement and advice.

TABLE I

Developmental Stages of Ancylostoma ceylanicum in Different Hosts at Various Intervals after Exposures

—	Third stage larvae before infection	Fourth stage (Final) infant rats	Immature	Adult stage		Adult stage
			infant guinea-pigs	infant rats	infant rats	infant mice
Number of positive animals		3	1	4	2	1
Days after infection		7	7	14	21	14
Number of worms recovered		17, 70, 3	1	1, 2, 8, 1	1, 1	1
Worm size :						
Total length	.. 0.420	1.098	(3.00)	2.143 (2.217)	(2.650)	(4.90)
Width	.. 0.002	0.043	(0.074)	0.063 (0.075)	(0.130)	(0.20)
Oesophagus length	.. 0.120	0.181	(0.420)	0.350	(0.480) (0.362)	(0.840)

1. Average dimensions of worms in mm. Values in parenthesis apply to females.

2. Total infant animals used—mice 10 : rats 10 : guinea-pigs 6 : rabbits 7.

Total Young animals used—mice 10 : rats 10 : guinea-pigs 5 : rabbits 8.

3. Animals sacrificed on every week but only positive results are shown in the Table.

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