IMMUNOPATHOLOGY OF AMOEBIASIS IN IBADAN

BY

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SUMMARY

Amoebiasis exemplifies a disease of protean manifestations which presents many perplexing problems. In this thesis an attempt is made to define a number of the wide variations which have been observed in the pathological manifestations of the disease.

The work consists of both retrospective and prospective Studies. The retrospective study involved a review of the 7922 protocols of the autopsies at the University College Hospital, Ibadan, during the ten year period 1958 to 1967. 135 cases in which lesions of amoebiasis were described, were selected for special study using 276 cases of other diarrhoeal diseases as controls. The results of this review defined the pathology and complications of amoebiasis seen at the UCH, during the period covered by this study.

A prospective study which included field surveys, laboratory studies on patients and controls; and in-vitro studies of the parasite, was also carried out. Parasitological, biochemical and immunological techniques were applied in the prospective study of 1291 subjects in a field survey. In addition some 200 hospital materials were included in the prospective study. The results obtained provided the basis for the suggestions made concerning the different methods that can be applied to the future study of the prevalence of amoebiasis in any given population exposed to the risk of infection by Entamoeba histolytica.

Local (Ibadan) strains of Entamoeba histolytica have been successfully cultured and the in-vitro characteristics studied. From the materials provided by the in-vitro cultures of the organism, investigations have been made on E. histolytica antigens and the corresponding antibodies produced in man. The results of these investigations have been utilized in immunological studies, designed to define the mechanism of production of some of the problems posed by amoebic infections. The severity and high fatality of the disease in pregnancy and puerperium, was studied in detail.

The observation that amoebiasis tends to be more severe and to have a higher mortality in pregnant and puerperal women was made only recently. At the time the present studies were planned, the association of pregnancy with severe amoebiasis had not been well established, and the mechanism for this association was unknown. It was, however, well known that pregnant women were more liable to severe forms of certain other infectious diseases and it was considered possible that a similar mechanism might be operating in the case of amoebiasis.

The present work confirms these clinical observations. Thus from the statistical analyses of the results of both the autopsy and prevalence studies, it became evident that the high fatality of amoebiasis during pregnancy and the early puerperium was not fortuitous but real. A fulminating type of lesion affecting the whole length of the large bowel is commoner in pregnant women and in those in the early puerperium dying of amoebiasis, than in any other cases of amoebiasis seen at autopsy. Furthermore, the biochemical

and immunological studies help to throw some light on the mechanism of the selectivity of infection by E. histolytica. These same results, also provide some explanation for the severity of amoebiasis during pregnancy and the allied states.

The conclusions, support the hypothesis of lowered resistance to infectious diseases during pregnancy. Speculative submissions are, therefore, made on the defective immune mechanism occurring during pregnancy and the early puerperium. Thus, the inability of pregnant women to produce 'sufficient antibodies' to amoebic infections, demonstrated by the reactivity of the immunoglobulins in amoebiasis, confirms the suspicion of the existence of a derangement of host-defence mechanisms during pregnancy. This raises the hope and encourages future search for the specific serum agent(s) which may account for the deranged immune mechanism. On similar basis, the characterization of amoebic antigens together with that of the corresponding antibodies, offers a new field in the study of the immunopathology of amoebiasis.

In conclusion, from the combined autopsy, prevalence, in-vitro experimental, biochemical, and immunological studies, it is submitted:

1. That the association of pregnancy with the severe form of amoebiasis at least, in this part of the world, is one of the perplexing problems posed by the disease.

2. That chronic amoebic infection is associated with the development of growth-inhibiting factors in the serum, for example, in patients with amoebic liver abscess.
3. That, on the contrary, growth promoting factors were demonstrated in the sera of pregnant/puerperal women with or without acute amoebic dysentery.
4. It is suggested that the severity of the disease in pregnant/puerperal women is a reflection of the derangement of immune response during pregnancy, with particular reference to the production of serum immunoglobulins.

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CHAPTER I

1.1 Introduction:

The amoebae that have been identified as causes of human disease fall into two categories, free-living and parasitic. The free-living amoebae are oridinarily found free in nature. They may however occur in the animal body, for example, in intestinal contents. Organisms of the free-living genera, Hartmanella (Acanthamoeba) and Naeglaria have recently been recognized as occasional pathogens, causing primary meningoencephalitis (Fowler and Carter, 1965; Symmers, 1969). Several other amoebic species have been identified as causing disease. The infection by these others are so rarely recognized that they rate little more than curiosity value.

The parasitic amoebae multiply only within the animal body, where they live as commensals or occasionally as pathogens. The species which are parasitic in man include Entamoeba histolytica, Entamoeba coli and Entamoeba gingivalis, Endolimax nana, Iodamoeba butschlii and Dientamoeba fragilis. Entamoeba histolytica is the only one that ranks as a major human pathogen. Entamoeba coli can cause disease, but usually does so as an "opportunist" - that is, when the patient's resistance to its invasion is lowered by other disease or by side effects of treatment. The latter mechanism occurs most frequently when immunosuppressive agents such as steroids or cytotoxic agents are involved.

More rarely the other parasitic amoebae mentioned above have been known to cause "opportunistic" infections. 'Amoebiasis', however, in the context of this thesis concerns the presence of Entamoeba histolytica in man, either as commensals or as symptomatic invaders. It is generally believed that the invading amoebae produce tissue damage. The orgamism is found as a harmless commensal in the vast majority of human beings. Little is known about what causes the parasite to assume an invasive character.

Although infestation of the human colon by Entamoeba histolytica is world-wide in distribution, the tropical and subtropical countries with humid climates are reputed to have a high incidence of the invasive forms of the infection. Comparatively little information is available on the incidence of amoebiasis in Nigeria (Alele, 1966). Conflicting ideas still persist on the definition of the parameter that can be applied to the study of the prevalence of the disease in any given community. At the 7th International Congress on Tropical Medicine and Malaria, it was shown that the prevalence of infection with Entamoeba histolytica and of symptomatic amoebiasis could not be assessed from a review of the world literature (Elsdon-Dew, 1963). Differences in the criteria used for the identification of Entamoeba histolytica and the varied symptomatology attributed to the parasite made it impossible to correlate data from investigators throughout the world.

Of special interest is the local observation on the apparent severity of the disease in pregnancy and the puerperium (Edington, 1967; Lewis and Antia, 1969). This observation is similar to the more universal one on the severity of the disease in the young and debilitated children (De Silva, 1962; Macdougall, 1959; Salas, 1958; Walt, 1959; Scragg, 1960; Olatubosun, 1965 and Nnochiri, 1965). The predisposition to, and increased severity of amoebiasis in patients previously given immunosuppressive agents such as corticosteroids has also been the subject of several communications (McAllister, 1962, Lisert, Hannibal and Sanders, 1959; Kanane, Knight, 1969, and Mody, 1959).

The first and in general terms, the most obvious theory which can be advanced to explain the phenomenon of differential severity and selectivity of infection by Entamoeba histolytica, is that some species of the organism are virulent while others are not. This was the hypothesis which Brumpt favoured, and was prepared to go so far as to postulate the existence of virulent and non-virulent species. Thus Brumpt (1928) described Entamoeba dysenteriae (the form causing amoebic dysentery), Entamoeba dispar, a non-pathogenic form producing cysts morphologically identical with those of E. dysenteriae, and E. hartmanni, a species, also non-pathogenic and producing cysts of less than 10u in diameter. Maleney, Frye and Leathers (1939) demonstrated marked

differences in the pathogenicity of different strains of E. histolytica for kittens and concluded that the differences were intrinsic in the amoebae. All the foregoing evidence notwithstanding, one strongly feels that this differential severity of amoebiasis may have an immunological basis. Other factors - considered in the next chapter may also be of importance. This view is in accordance with the suggestion that the immune response of the host may exert selective pressure on 'fitness' for survival of many parasitic species (Dineen, 1963). It is possible, therefore, that the peculiar behaviour of the disease is brought about by conditions which either increase the level of commensal infection, or produce factors which convert the commensal amoeba to pathogenic organisms or do both.

Since the beginning of the twentieth century, considerable interest has been shown in the possible role of immune response in the pathogenesis of amoebiasis (Izar, 1914). However, there are few experimental studies designed to ascertain the nature of the amoebic antigens involved in the host/parasite relationship of amoebic infections. Indeed, insight into the antigenic nature of some parasitic materials has been derived by inference and not by direct isolation and characterization. It is considered that there is probably a minimum threshold level of antigenic information necessary for stimulation of an immunological response in amoebic infections.

The threshold level of antigenic information, being defined as, the sensitizing of, and consequent development by the host, of an acquired resistance to additional amoebic infections. It is possible, therefore, that the antigenicity of E histolytica can be modified by immunity. It is clear therefore, that the causes of the possible immunological derangements in pregnancy, the puerperium, and debilitated states, when amoebiasis is known to be very severe, can only be fully appreciated from the study of the character of the immune response stimulated by each stage of the causative parasite.

Unfortunately, the available data on the biological and immunological activities of amoebic antigens are limited. This is probably due to the problem of obtaining sufficient numbers of organisms. This has been a serious handicap to investigations, - metabolic, immunological or otherwise - on E. histolytica (Reeves and Ward, 1965; Swart and Warren, 1962). Indeed Kun, Bradin and Dechary (1965) stated that they were abandoning studies on the organism because of the difficulty in obtaining material. These impressions were again confirmed during the early stages of the present studies.

1.2 AIMS AND OBJECTS

Bearing in mind the limitations of the existing knowledge on the various aspects of amoebiasis, notably, the complete lack of information on the cultural characteristics of the local strains of *E. histolytica*, the study programme was designed with the following objectives:

(i) To assess the role of amoebiasis with its consequent pathological changes and complications, as a cause of death, with special reference to deaths during pregnancy and the puerperium in Ibadan. This is described in chapter 3 following a review of the literature in chapter 2.

(ii) To investigate and define all various parameters applicable to the accurate assessment of the prevalence of amoebiasis in a given sample of the Nigerian population. These investigations are described in Chapter 4.

(iii) To establish and maintain *in-vitro* cultures of the local strains of *E. histolytica* and utilize these in the study of the immunopathology of amoebiasis in Nigeria. These are included in Chapters 5 and 6.

(iv) To investigate the role of immunity in amoebic infections, with special reference to the antigenic reactivity of the local strains of *E. histolytica*, and also the nature and reactivity of human immunoglobulins. These investigations are described in Chapter 7.

CHAPTER II

GENERAL REVIEW OF LITERATURE

2.1 Historical Sketch

"The history of mankind is' the history of its disease" - (Joan Tate)

Intestinal amoebiasis, is a disease now fairly widespread in the tropics. The disease appears to have been a problem even to the ancients. Sushruta (? 600 B. C.) describes what appears to be the clinical features of amoebic dysentery (Atisara) and incriminates" germination of parasites in the intestines", drinking impure water" and " excessive drinking of liquor" as some of the causes of the disease. Amoebic liver abscess was probably known during the time of Hippocrates, since drainage of pus from this organ was said to have been successfully practised by the Master (Rajasuriya and Nagaratnam, 1962).

What is considered to be one of the earliest historical references to hepatic amoebiasis is the account of the last illness of Alexander the Great (Savill, 1956). During his campaigns in Asia he had developed dysentery as a result of drinking impure water. Life in the army had made him a votary of both Bacchus and Venus. Then at the early age of 32, the end came suddenly and without warning. Some believe that he caught malaria from the mosquitoes of the marshy plains near Babylon; others that the illness was due to abscess of the liver.

Alexander, the Great was dead by the twelfth day - 13th June, 325 B. C - of an illness associated with increasing weakness and steady mounting temperature. However, the question as to whether he died of malaria or amoebiasis, historians have not yet decided.

The first reference to amoebiasis in Europeans was in Ceylon in 1672, during the visit of a French Embassy from the Court of Louis the XIV of France to Rajasingha the II of Kandy (Puris, 1920). In more recent times medical writers have made noteworthy references to the prevalence of amoebiasis, both intestinal and hepatic. Dr. John Davy a brother of the famous Sir Humphrey Davy, who was stationed in Ceylon as Surgeon to His Majesty's Forces in 1821, commented on the increasing incidence of inflammation of the liver and liver abscess among the British troops which he ascribed to the "abuse of ardent Spirit" (Davy 1821).

James Annesley (1828), was acquainted with a form of dysentery in India, in which the clinical picture and morbid anatomical features of dysentery were accompained by liver disease. The intestinal disturbances at that time were attributed to a "morbid state of biliary secretion". With regard to the cause of the dysentery Annesley incriminated cold nights, intoxicating liquor, noxious terrestrial exhalations, fatigue, exposure to the night air and even intestinal worms, although considered the last of little importance considering their prevalence amongst those unaffected by dysentery (Annesley 1841).

However, this notion as to the pathogenesis of such acute diarrhoeal disease associated with hepatic disturbances, has long been shown to be incorrect (Losch, 1875).

The history of amoebiasis up till the second half of the 19th century was mostly speculative and suggestive. The foundation of the history of amoebiasis was properly laid with the observation of amoebae in the faeces of man.

Losch (1873) was probably the first to describe a proven case of amoebic dysentery. In the clinic of Professor Eichenwald in St. Peterburg, Losch discovered the amoebae in the stool of a young Russian peasant, named MarKoff. MarKoff was a 24 year old peasant from Archangel. He contracted dysentery in St. Peterburg, and suffered from a relapsing dysenteric illness which ended fatally. MarKoff's illness was characterised by periodic attacks of diarrhoea with blood and mucus, much loss of weight, severe prostration and a terminal pleuritis. Amoebae were repeatedly observed in this patient's stool and Losch gave an excellent description of these organisms which he named Amoebae coli. The numbers of amoebae fluctuated and increased with clinical exacerbations. The course of the disease was little influenced by current conventional treatment. There seemed to be some response to quinine administered rectally.

Losch advanced another step in establishing amoeba as the causative organism by administering faecal material orally and rectally to four dogs, one of which developed dysentery and multitudes of amoebae were observed in its stool. At autopsy there was some ulceration in the animal's large bowel.

This was not the first occasion on which amoebae had been found as parasites of man. Another Russian, G. Gros had in 1849, discovered and briefly described an amoeba parasitic in the mouth. In 1860, Lambl of Prague reported the finding of amoebae in the intestine of a child who died of enteritis, although R. Leuckert doubted the accuracy of Lambl's descriptions and was later supported in this view by C. Dobell who managed to obtain the rare original papers of Lambl (Dobell, 1919).

Timothy Lewis and D. D. Cunningham (1870) in the course of their studies on the aetiology of cholera observed amoebae in human faeces. They regarded the amoebae as non-pathogenic species, Entamoeba coli. Thus it seems that Losch was the first to observe the Entamoeba of dysentery.

Evidence continued to accumulate that amoeba might be of aetiological importance in dysentery and in liver abscess. In 1883, Koch saw amoebae both in cases of dysentery and in the wall of a liver abscess in Egypt.

Staphanos Kartulis (1886, 1887, 1889, 1891 and 1904), working in Alexandria found amoebae in stools of patients suffering from dysentery, in pus from liver abscess and in section of the large gut. Eleven of 334 cases of liver abscess were complicated by brain abscess. In two of these he saw amoebae both in brain and liver substance. He was also able to produce dysentery in cats by the rectal injection of faeces from human cases of dysentery. Hlava (1887) in Prague was able to infect cats by the same method. In the United States, Osler (1890) found amoebae in a patient suffering from dysentery complicated by liver abscess. In 1892, Kovacs infected kittens, and in 1894 Kruse and Pasquale produced dysentery in a cat by the rectal injection of liver pus containing amoebae but bacteriologically sterile.

In 1890, Councilman and Lafleur of Johns Hopkins Department of Pathology showed the parasite of amoebic dysentery to be common in Baltimore. They published the most exhaustive account of the disease. Their work is a classical contribution to the pathology of the disease. They were the first to introduce the terms "amoebic dysentery and "amoebic abscess of the liver".

In a careful clinical and pathological study of fourteen patients Councilman and Lafleur (1891) came to these conclusions: "Amoebic dysentery is clinically and aetiologically distinct. Ulcers in the gut are undermined and the products of inflammation are absent.

Liver abscess often with extension to the lung may complicate the disease. The amoeba in the liver abscess may exist without other organisms, causing liquefaction and not purulent inflammation. The disease has a worldwide distribution, and is what has been referred to as tropical dysentery." They described under the title of "broncho-pleural fistula" in one patient, a liver abscess ruptured into the lung. Amoebae were seen in the sputum and at post mortem the abscess was found to communicate with the duodenum, and the gall bladder, as well as, the lung. Perforation of the large gut and death from intestinal haemorrhage were also recorded in other patients.

An important landmark in the investigation of amoebiasis was the establishment of the modern concept of the transmission of the disease by Quincke and Roos (1893). They noted, by properly controlled experiments, all the essential characters of Entamoeba histolytica. These include its size, clear ectoplasm, active movement and ingestion of red cells, and confirmed its pathogencity for cats. By contrast, they showed that the amoebae from non-dysenteric cases were larger, sluggish in movement and never contained red cells. They were also the first to observe the encysted form of the parasite and demonstrated that these were infective by mouth for the cat. They correctly concluded that this was the mode of infection in man.

Huber (1903) described cysts with one, two, three or four nuclei but never more. He distinguished them from the cysts of Entamoeba coli, but although he told Schaudinn of these observations they were ignored. At the end of the nineteenth century, despite the weight of evidence pointing to an amoeba as an aetiological agent in dysentery and liver abscess, confusion still existed because of observations on patients in whom amoebae were found but to which no significance in the causation of the disease could be attached (Grass, 1879; Casagrandi and Barbagello, 1895; Callendruccio, 1890; Celline and Fiocca, 1894). It was Schaudin in 1903 who paved the way to a clearer understanding of this problem by differentiating between a harmless Entamoeba coli and a pathogenic Entamoeba histolytica.

Leonard Rogers in 1903 showed the presence of the disease as a separate infection in India, where its existence had previously been frequently denied. He also showed clearly its association with liver abscess.

2.2 GEOGRAPHICAL DISTRIBUTION

The extreme variety of opinion as to what constitutes the disease immediately clouds any attempt at establishing a valid geographical distribution. However, Entamoeba histolytica seem to have been found in most parts of the world and in most peoples examined, with the exception of the Eskimos (Brown et al, 1950); Laird and Meerovitch, 1961)

and of the Poles (over 90,000 examinations with nil report).

In general, however, the distribution of amoebiasis is worldwide. Surveys have shown that a significant percentage of the world's population harbours the parasite irrespective of latitude. It occurs sporadically in most subtropical and temperate countries, but it is much more frequent in tropical ones. In the tropical portions of America, Asia and Africa; and also in the West Indies, the Malay Archipellago, and the Phillipine Islands, it has usually been the prevailing form of dysentery.

As regards the suitable index applicable to the study of the disease it has been shown that available data, - either parasitological or clinical, give but little information on the distribution of amoebiasis. The most reliable data is based on the occurrence of liver abscess, though this too is clouded by differing outlooks on the condition. The communities which are familiar with such abscess, and ipso facto with amoebic dysentery, do not regard these with the same awe as do those encountering sporadic cases. In fact, cases appearing as sporadic may be the result of failure to report the real occurrences.

2.3 PATHOGENESIS AND MECHANISM OF PRODUCTION OF AMOEBIC LESIONS

2.3.1 PATHOGENESIS:

Many studies have been conducted to determine the conditions causing invasion by E. histolytica. Concomittant bacterial infection has received much attention. Westphal (1937) infected himself and carried the amoebae as a commensal for several months, then swallowed the bacterial flora associated with another case of amoebiasis, and promptly developed acute amoebic dysentery. Usually the bacterial flora associated with acute amoebic dysentery are not themselves pathogenic, although the dysentery bacilli have sometimes been found in such cases.

Deficiencies of thiamih and of ascorbic acid have been shown to favour invasion in experimental animals (McCarrison, 1919; Sadun, Bradin and Faust, 1951). Other dietary factors may be important also. Faust, Scott and Swartzwelder, (1934) showed that experimental dogs fed on a fish diet developed acute amoebic dysentery, but improved rapidly by changing to a diet of liver. General lowering of resistance, has also been claimed as a factor. The possession of the spreading factor, hyaluronidase by Entamoeba histolytica has been described by Bradin (1953) and might be a factor in invasiveness.

However, it is generally agreed that exposure and infection in human amoebiasis result from ingestion and swallowing of the viable cysts of

Entamoeba histolytica. The cysts pass uninjured and unmodified through the stomach and proximal levels of the small intestines. The encysted organism begins to exhibit protoplasmic activity only when the gastric secretions have been neutralized or slight alkalinity has been produced by the intestinal juices (Swartzwelder, 1939). This activation and thinning of the cyst wall in the medium of the intestinal juices cause a rupture of the wall and allow the amoeba to emerge as a naked protoplast (metacyst). Simultaneously or shortly thereafter the cytoplasm divides into a number of small amoebae corresponding to the number of nuclei in the metacyst. As many as four metacystic trophozoites are produced from the metacyst. These little amoebae (metacystic trophozoites) now pass down the remainder of the small intestine and are carried into the lumen of the caecum. If excystation fails to occur in transit down the remainder of the small intestine, the fate of the cysts is uncertain but since excystation probably does not take place in the large bowel, the cysts may at times pass through the entire digestive tract and be evacuated in the faeces, without colonization.

Once the little metacystic amoebae reach the caecal area, where normally there is a moderate degree of stasis in the faecal flow, they have their first opportunity to make chance contact with the cuticular surface of the mucosa, either at the tips of the digital processes extending out from the glands, partway within the glands or in the depth of the

glands (James, 1923; Meleney, 1934; Faust, 1932, 1943). Contact, even for a brief time, allows the amoebae each to digest a small cavity in the mucosal cells, in which the parasites can take residence. They ingest the lysed material, grow, then divide by binary fission and thus initiate a colony. Tissue contact may or may not occur at this level of the large bowel. If tissue contact does not occur, the amoebae then pass further down in the liquid faeces, to make contact at the lower level, particularly in the sigmoid colon or rectum, where primary colonization may then develop. On the other hand, they may re-encyst and be evacuated in the faeces without causing infection. In a majority of exposures with small numbers of cysts it is almost probable that this latter is the case and that infection fails to occur.

Although evidence preponderates in favour of the view that Entamoeba histolytica is characteristically a tissue invader, there is suggestive evidence that this amoeba, may remain temporarily as a surface-contact parasite of the mucosa producing superficial erosion, excess production of mucus, and developing extensive colonies in the crypts (Hoare, 1950). However, whenever favourable circumstances develop tissue invasion characteristically occurs. As long as the amoebae remain in the intestinal lumen it seems likely that they depend on the metabolites of certain associated enteric bacteria for growth and multiplication (Deschiens, 1937, 1938). Once they invade the intestinal mucoas the host cells appear

to provide the necessary stimulus and bacteria are no longer essential.

Most workers, agree that the primary disturbance in amoebiasis is in the large bowel and that all other forms of lesions are metastatic. The regions that are most likely to be thus affected include the liver, the brain, the lungs and skin (Fig. 2).

2.3.2 Mechanism of production of bowel lesions:

Once the amoebic process has become well established, it tends to involve the lymphatics and blood vessels of the submucosa, with thrombosis of the latter which allows the amoebae to enter the luminar of these vessels. Moreover, extensive submucosal extensions frequently occur, so that adjacent subsurface lesions coalesce by tunnels. The superficial tissues are thus deprived of their blood supply, causing a sloughing of the overlying layers. By similar mechanism, peristaltic movements of the involved bowel well squeeze out many amoebae and necrotic detritus through the necks of the ulcer into the lumen of the bowel producing partial collapse of the tissues.

In fulminating amoebiasis, and similarly in chronic infection but considerably less rapidly, the amoebae may invade the muscular coats, then break through the barrier and reach the subserosa. Meleney (1934) states that they "penetrate the muscle cell and cause oedema, hyalinization and finally necrosis of this tissue .... In the subserous tissue the amoebae produce oedema followed by the formation of fibrin on the serous surface, which leads to adhesions of apposing peritoneal surfaces. If the necrotic process penetrates through the muscle layers, the serosa gives way and perforation occurs.

2.3.3 Spread of Intestinal Lesions:

Original sites of tissue invasion and colonization by Entamoeba histolytica provide opportunities for development of amoebic processes at other levels of the bowel.

As already noted earlier on, it has been suggested that the first opportunity for development of the primary amoebic lesions is in the caecal area, since this is the first level of the bowel where any appreciable amount of intestinal stasis occurs. Amoebic trophozoites extruded from these primary foci serve as the source for tissue invasion at lower levels of the bowel, and particularly in the sigmoid colon and rectum. Moreover, additional ulcerative invasion of the caecum is likely to take place as a result of direct contact of the extruded trophozoites with undamaged caecal mucosa. By similar mechanism, regurgitation of these trophozoites into the posterior segment of the ileum allows tissue invasion of this part of the intestine. Primarily implanatation apparently never occurs in the wall of the ileum, nor do the amoebae enter the mucosa at higher levels of the small intestine.

The development of a preponderance of primary lesions in the caecal area is a well recognized fact. The pathologic process of amoebic colitis is characteristically a progressive one, with the earliest sites of tissue damage at the caecal level and gradual or rapid involvement at lower levels of the large bowel.

However, in cases of fulminating amoebiasis there may be invasion throughout the entire large intestine (Fig.4). Also in a relatively small per cent of cases the primary invasion of the lower colon and rectum occurs concomittantly with that of the caecum or exclusively in the lower segment of the large bowel.

The cumulative lesions as observed in autopsy findings (Clark, 1925; Faust, 1943), indicate that amoebic ulceration occurs most frequently in the caecal area (caecum, appendix, ascending colon) and next in the lower portion of the sigmoid colon and rectum, while the intermediate levels of the colon are less liable (Fig. 3).

2.4 EXTRA - INTESTINAL AMOEBIASIS

Amoebiasis outside the intestinal tract is usually secondary to amoebic invasion of the large bowel. The anatomical locations where extra - intestinal amoebic lesions have been demonstrated include practically all soft organs and tissues, of which the liver, lungs, brain and skin are most commonly involved.

2.4.1 Hepatic Amoebiasis

Metastatic lesions may occur when the amoebae in the course of their burrowing penetrate a small vein and get carried off to the liver.

Hepatic amoebiasis may take one of three forms. These include (1) hepatitis associated with colonic amoebiasis without evidence that amoebic invasion of the liver has occurred; (2) acute amoebic hepatitis and (3) amoebic liver abscess. (1) Loeber and D'Antoni)1947) first called attention to an enlarged tender liver in children suffering from colonic amoebiasis without any of the usual clinical signs of hepatic involvement in amoebiasis such as leukocytosis, fever and X-ray evidence of diaphragmatic impairment. Sodeman (1950a) refers to this condition as a "lower grade hepatic process" (that occurs) "before the stage of acute hepatitis .... "with enlargement or tenderness of the liver but very little in the way of symptomatic response". The histopathologic picture in this type of hepatitis has not been reported.

2.4.2 Acute Amoebic Hepatitis:

Acute amoebic hepatitis is the stage of amoebic invasion and colonization of the liver preceding development of one or more abscesses.

The pathogenesis of acute amoebic hepatitis is better explained by referring to the amoebic lesions in the intestine, particularly those in the caecal area. Lytic necrosis in the submucosal layer involves not only the interstitial cells but the mesenteric blood vessels as well. Frequently the walls of the mesenteric blood venules are eroded to such an extent that amoebae gain entrance to these vessels and get carried into the intra-hepatic portal vessels.

This obviously is the origin and mechanism of the metastatic amoebic lesions of the liver already mentioned above. However, only a small proportion (not over 5 per cent) of persons with confirmed amoebic colitis have evidence of coincident or subsequent amoebic hepatitis. This, therefore, raises the question of the possible existence of immuno-pathological basis for this "hepato-colonic complex" in amoebiasis.

It is generally accepted by pathologists and clinicians that Entamoeba histolytica enters the liver via the portal blood stream. The study of Carrera (1950) on hepatic amoebiasis following experimental development of amoebic colitis in kittens confirms the findings of Rogers (1922) and Palmer (1938) on human material. This deals with the fact that the earliest colonization of the amoebae is associated with thrombosis in branches of the portal vein, and particularly in the small interlobular veins.

In this regard, however, it is a very significant fact that, in the case of guinea pigs with intestinal amoebiasis, the amoeba goes on to the liver without being able to establish itself there or to produce lesions (Bock and Mundrow-Reichenow, 1955; Rees, Taylor and Reardon, 1954). Maegraith and Harinasuta (1954) demonstrated that amoebae introduced into the mesenteric vein did not succeed in establishing themselves in liver. All these strengthen the possible existence of immunological basis for "hepato-colonic complex in amoebiasis."

2.5. Pleuropulmonary Amoebiasis

The pleurae and lungs constitute the second most common and most important extra-intestinal locations of amoebiasis. There are several demonstrated ways whereby these organs and tissues may become involved. These include the following sources:- (Ochsner and De Bakey, 1936).

(1) From the intestine via the bloodstream or mesenteric lymphatics and inferior vena cava, without hepatic involvement, (14.3%)

(2) by concomittant but independent involvement of liver and lungs (10.4%)

(3) with pulmonary extension of an hepatic abscess (37.2%)

(4) due to a bronchohepatic fistula with pulmonary involvement (19.6%)

(5) by empyema extension from an hepatic abscess (15.6%)

From the above quoted figures it is evident that in most cases pleuropulmonary involvement is an extension of hepatic amoebiasis. However, amoebiasis of the pleural cavity is only about 5 to 10 per cent as frequent as hepatic involvement.

2.5.1. Mechanism of Production:

In the pleuropulmonary extension of hepatic lesion, the typical mechanisms involve:

(i) the doming up of the diaphragm above a sub-diaphragmatic abscess of the right lobe of the liver.

(ii) Adhesion of the lower lobe of the right lung to the diaphragm,

(iii) lytic penetration through the tissues of the diaphragm and

(iv) erosion by the amoebic process directly into the lung.

2.6. IMMUNOLOGY OF AMOEBIASIS

Any immunological concept in a disease entity must involve the question of humoral, as well as, cellular mechanisms. The humoral aspects should include details such as antigen-antibody reaction and its adaptability to reproducible techniques such as immobilization test, complement fixation test, haemagglutination test, immunoflourescent techniques, gel diffusion precipitin test and general reactivity of immunoglobulins. There is sufficient evidence to show that these various immunological techniques have at one time or the other been adapted for use with Entamoeba histolytica antigens.

2.6.1. Complement Fixation Test

The first postulation or suggestion of immunological concept in amoebiasis was the attempt of Iza (1914) to devise a complement fixation test for infection with Entamoeba histolytica. He claimed to have obtained positive results with such test in five individuals and three kittens infected with the parasite. His work was repeated by Von Hage (1920), who failed to confirm it, but in (1921), Scalas reported that he obtained positive results with such a test in cases of amoebic dysentery.

Apparently little attention was paid to his communication and no other publication appeared upon this subject until Craig in 1927, and 1928 demonstrated that complement fixation occurred in the blood serum of individuals infected with Entamoeba histolytica in the presence of suitable antigen.

The demonstration by Craig that complement fixing substances occur in the blood serum of individuals infected with Entamoeba histolytica has since been confirmed by numerous investigators, notably Spector, (1932); Menendez (1932); Sherwood, and Heathman, (1932); Tsuchuja, (1932); Weiss and Arnold, (1934 and 1937); Meleney and Frye, (1935-1937); Shiragawa, (1935); Stone, (1935); Paulson and Andrew, (1936); Rees, Bazievich, Reardon and Jones, (1942); Ghosh and associates, (1948); Terry and Bozievich, (1948);; Hussey and Brown, (1950); Dolkart, (1951); Medearman, and Dunham, (1952); Kun Yun Kon, (1958). In recent years there has been a re-awakening of interest in this serological technique. Fairly more recent work includes that of Kessel and co-workers (1965) and Kashwal and co-workers (1966).

2.6.2 Immobilization Test:

When amoebae are incubated in the presence of antiserum, they are affected to various degrees and in various ways according to the type of observation carried out. Thus they may be temporarily immobilized,

their ability to ingest red blood cells may be changed and finally the growth rate may be affected. The inhibition of growth of Entamoeba histolytica in culture did not reveal any differences between strains (Nakamura, 1959; Swart and Warren, 1962). However, red blood cell phagocytosis did show strain differences (Shaffer and Ansfield, 1956).

The immobilization reaction has been studied by several workers (Biagi and Buentello, 1961) as a method of serological diagnosis of amoebiasis in man. No differences between strains of Entamoeba histolytica was noticed during these investigations.

In contrast with the other studies utilizing immobilizing reaction, Zaman (1960) carried out an extensive comparative study with several species and observed the degree of immobilization of the homologous and heterologous species. Entamoeba coli was found to be unsuitable for immobilization studies owing to its normal restricted activity. Some cross-reactions to Entamoeba histolytica were observed, since Entamoeba histolytica amoebae were partly immobilized by anti Entamoeba coli serum.

2.6.3 Indirect Haemagglutination Test:

This technique has been used by very few workers, notably Kessel and associates (1961); Maddison, Powell and Eldson-Dew (1965); and Milgram, Healy and Kagan (1968). Other studies include that of Wells and Goldman (1961) on the evaluation of haemagglutination test for amoebiasis.

2.6.4 Flourescent Antibody Test:

The basis of the flourescent antibody technique is that the antibody-antigen reaction is made visible by coupling a flourescent dye to the antibody. It is then possible to observe flourescence in the case of a positive reaction. Until recently the antigen used has been amoebae obtained from culture, but Zaman (1965) has shown that the flourescent antibody test can be applied to cysts. The disadvantage of this test is that, in general, cross-reactions are seen between zoologically related protozoa. However, differences in intensity are detectable, and a technique for measuring the intensity has been devised by Goldman (1960). Goldman and his colleagues have made detailed study of relationship of Entamoeba species with this technique.

The relationship between Entamoeba coli and Entamoeba histolytica were first studied by Goldman, (1953, 1954). Antisera to both species were prepared and although some cross-reaction was observed the heterologous flourescence was removed by absorption with the appropriate species of amoeba. The use of these antisera enabled Goldman (1954) to successfully indentify unknown amoebae as either Entamoeba histolytica or Entamoeba coli. Other species and genera of parasitic amoebae were also studied (Goldman, 1945) with this method. Amoebae of Dientamoeba fragilis and Endolimax nana showed virtually negligible flourescence, while Entamoeba invadens reacted less than either

Entamoeba histolytica or Entamoeba coli, but more than Dientamoeba fragilis or Endolima nana. Entamoeba moshkovskii did not significantly stain with an anti-Entamoeba histolytica serum (Goldman et al (1960). Since Goldman's (1953) demonstration of specific antibody in rabbits immunized with E. histolytica antibody by similar means in human subjects has now been reported (Goldman, 1966; Jean, 1966) and also by Boonpucknavig and Nairn (1957).

2.6.5 Gel Diffusion Precipitin Test:

A precipitate often forms when an antigen and its antiserum are mixed. If a gel is the supporting medium and the reactants diffuse towards each other through the gel, the precipitate will be formed as one or more bands. This technique allows the analysis of antigenic structure of an organism.

In view of the controversy as to the potency and stability of amoebic antigen(s), it is envisaged that the antigen required for the investigation of the problem under review be purified and characterised. The precipitin test has therefore been chosen for this exercise. The most compelling reason for this preference lies in the sensitivity and simplicity of the test. It also has the advantage of revealing the non-specific systems which may appear when antigens are as heterogeneous as are those prepared from parasitic animals (Maddison and Eldson-Dew, 1961).

The number of precipitation lines reported for Entamoeba histolytica vary from three to about ten (Nakamura and Baker, 1956; Maddison, 1965; Siddiqui, 1961; Sen et al., 1961a, b., and Atchley et al., 1963). The results obtained seem to depend upon the potency of the antiserum and antigen. Nakamura (1961) observed that better results with human sera were found if antigen was prepared from newly isolated strains of Entamoeba histolytica. The presence of bacterial factors further complicates the analysis, and while these variables can be investigated in an experimental system, it is said to be more difficult in the case of human serum (Neal, 1966). Maddison and Eldson-Dew (1966) showed that one of the precipitin lines with human serum was not due to Entamoeba histolytica but to accompanying Clostridium welchii antigen-antibody reaction.

While antigenic structure of Entamoeba histolytica has not been fully elucidated, some observations on cross-reaction with other species have been made. Sen et al (1961b) prepared anti - Entamoeba histolytica serum and found no reaction with Entamoeba moshkovskii antigen or antigen from the free living amoebae Naeglaria gruberi and Schizopyrenus ruselli. Talls et al., (1962) showed that there were no common antigens between Entamoeba histolytica and Entamoeba invadens. Siddiqui and Balamuth (1963) reported that no reaction was found when heterologous antigens were tested against anti-Entamoeba histolytica and anti-Entamoeba invadens sera. The antigens were prepared from Entamoeba histolytica,

Entamoeba invadens, Entamoeba moshkovskii, Hartmanalla rhysodes and Mayorella palestinenis.

2.6.6 Immunoglobulins in Amoebiasis:

This so far is the least investigated of the immunological aspects of amoebiasis. The studies so far collected from the literature include that of Shirley Maddison and her co-workers (1968) on the Reactivity of human immunoglobulins in amoebiasis and that of Ali Khan and Meerovitch (1968) on serological characterization of gamma M (19S) and gamma G (7S) rabbit antibodies in response to Entamoeba histolytica antigens. The first group of workers mentioned above, carried out their studies, on sera from only two amoebiasis patients and consequently their results cannot be subjected to any serious statistical analysis. They finally suggested that investigations of sera from young infants and adults presenting short clinical histories of amoebiasis and follow-up on these patients would clarify the role of different classes of immunoblogulins in amoebiasis.

2.6.7 Cellular Immunity in Amoebiasis:

This aspect has so far received little or no attention especially in human amoebiasis. Investigations have most frequently been carried out on immunized animals, and the results obtained have been equivocal. Heathman (1932) and Menendez (1932) showed a delayed type of hypersensitivity to Entamoeba histolytica in immunized animals. In addition,

Heathman (1932) was able to transfer passively the skin-sensitizing antibodies in sera from immunized animals. The investigations by Maegraith and Harinasuta (1954) suggest that in the guinea pig, prolonged intestinal infection maintained experimentally may induce a state of hypersensitivity with possible increase susceptibility to invasion of liver by the amoeba.

However, the reported work on this aspect as regards human infection includes that of Leal (1923), Kun Yun Kon (1958) and Shirley Maddison and associates (1968). Delayed and very rarely immediate, hypersensitivity was detected by Leal in human as a result of infection with Entamoeba histolytica. Shirley Maddison and her co-workers state that a high proportion of amoebic diagnostic sera gave positive PCA (Passive Cutaneous Anaphylaxis). Skabes also investigated skin reaction using boiled extract of the intestinal mucosa and of material scraped from the intestines of persons suffering from amoebic dysentery. However, it was not confirmed his, method was of any value in the study of cellular immunity in amoebiasis.

2.7 THE PRESENT STUDY

The above review shows that considerable work has been done on what may collectively be termed the immunopathology of amoebiasis, all over the world except of course in West Africa in general and in Nigeria

in particular. So far there is no record of any detailed studies on the nature of the antigen and antibodies involved in the host parasite relationship in human amoebiasis. So also has no effort been made to apply such studies to the elucidation of a particular clinico-pathological problem such as the severity, and selectivity of infection by Entamoeba histolytica in pregnancy/puerperium which has reasonably been observed by various workers as stated earlier on.

2.7.1 Amoebic Antigen:

The biochemical contents of the whole amoebic antigen(s) and fractions have not been identified nor the fractions evaluated clinically or experimentally. It has been suggested, however, that different amoebic antigenic components may separately be involved in different serological techniques with particular reference to haemagglutinins and precipitins (Maddison et al., 1965).

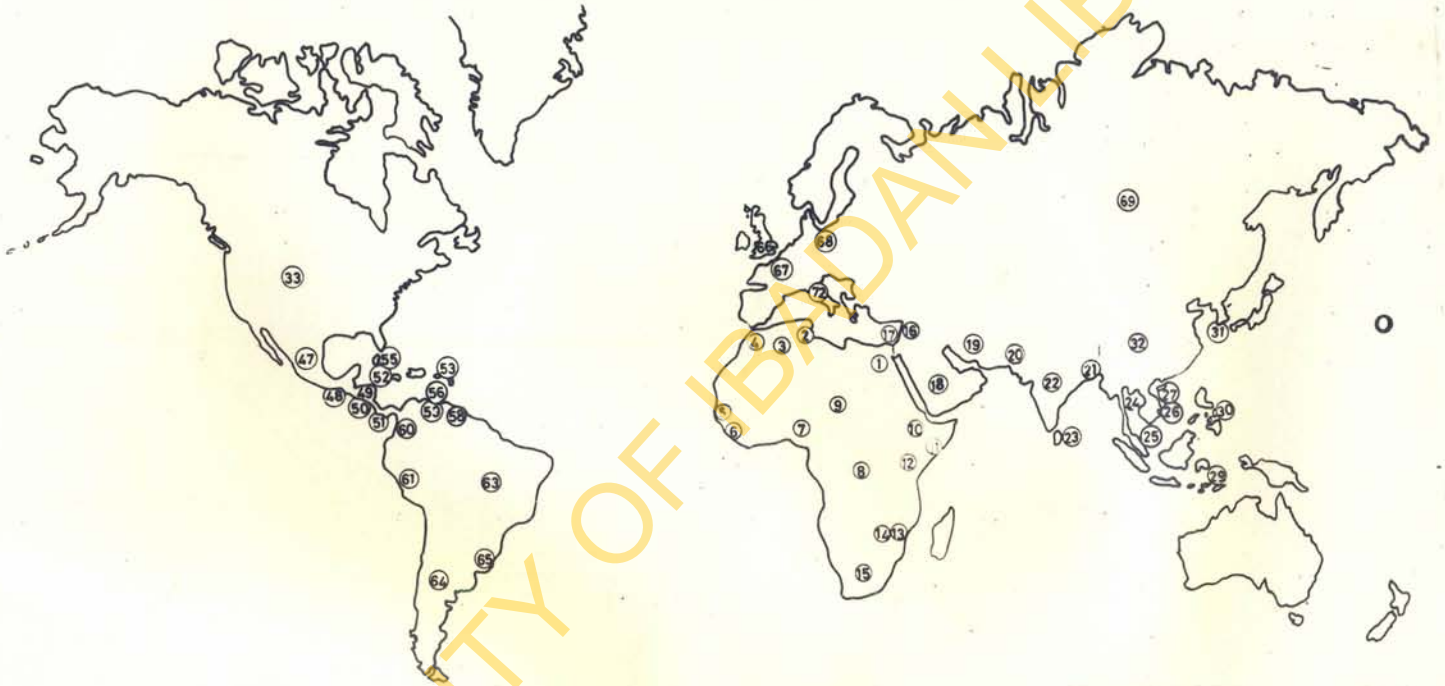
There is wide variation in the observations of various workers applying the above-mentioned immunological techniques to the study of immunology of amoebiasis. Thus taking amoebic gel diffusion for an example, Maddison (1965) has shown that this test can detect antibodies against specific components in a high proportion of patients with invasive amoebiasis, in a smaller proportion of cyst-passers and much lower percentage of patients without symptoms attributable to Entamoeba

histolytica. On the other hand Kun Yun Kon (1953) and other workers found more positive results in patients with chronic diarrhoea compared with invasive amoebiasis. In view of the variability in the results of various workers it is conceivable that the inconsistency may lie in the quality of the antigen(s) employed in the studies. Furthermore an elucidation of the concepts of amoebiasis as a disease in which derangement of immunological mechanisms may account for its peculiar infectivity and defferential severity; the biochemical and biophysical properties as well as, the stability of amoebic antigen(s), appear to be a significant factor.

It is hoped that this study will, establish immunological studies in amoebiasis in Nigeria, as well as fill in the gap which has existed in the whole of the study of the antigenicity of Entamoeba histolytica.

Finally, the application of the locally prepared antigen to the study of human amoebiasis demands a reasonable purification and characterisation of the antigen(s). Therefore, emphasis has been laid on this aspect of antigenic reactivity in the present study.

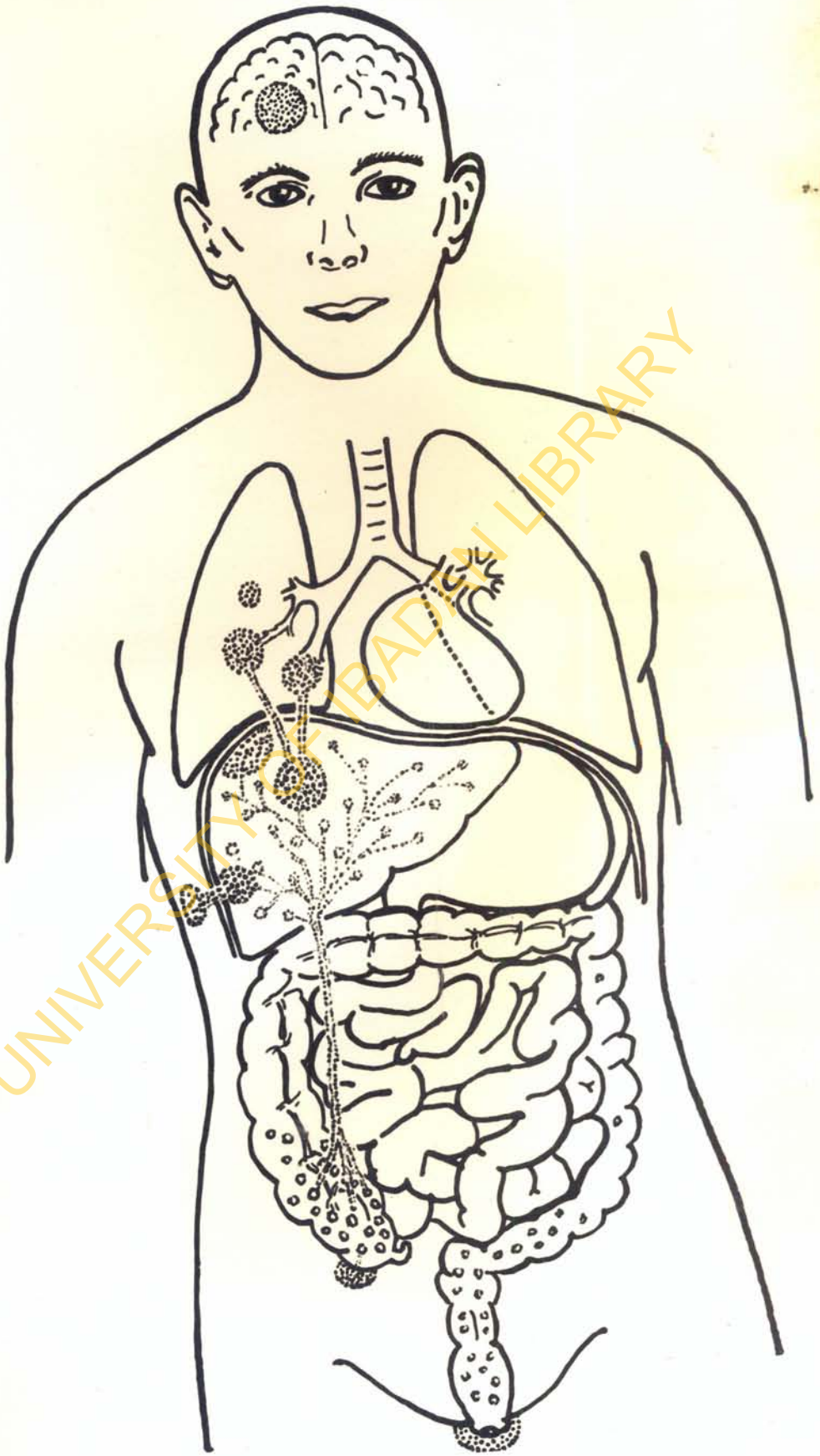
GEOGRAPHICAL DISTRIBUTION OF AMOEBIC LIVER ABSCESS (FROM CASE REPORT)



AFRICA		ASIA		NORTH AMERICA				SOUTH AMERICA		EUROPE	
1 Egypt	33	16 Syria	2	33 United States	141	49 Honduras	1	57 Cayenne	6	66 Britan	15
2 Tunisia	14	17 Isreal	99	34 Pennsylvania	1	50 Costa Rica	13	58 British Guiana	1	67 France	9
3 Algeria	4	18 Saudi Arabia	1	35 Missouri	2	51 Panama	50	59 Venezuela	4	68 Germany	2
4 Morocco	48	19 Iraq – Bagdad	1	36 Indiana	3	52 Jamaica	4	60 Colombia	47	69 Russia	1
5 Senegal	90	20 West Pakistan		37 Carlifornia	6	53 Puerto Rico	2	61 Peru	1	70 Rotterdam	1
6 Sierra-Leone	17	21 East Pakistan		38 Delaware	1	54 Martinique	1	62 Chile	89	71 China	1
7 Nigeria	106	22 India	1000+	39 Virginia	18	55 Cuba	27	63 Brazil	6	72 Italy	33
8 Republic of the Congo	130	23 Ceylon	257	40 Tennesse	10	56 Curacao	11	64 Argentina	3		
9 Chad	3	24 Thailand	93	41 Kentucky	2			65 Uruguay	36	OCEANIA	
10 Ethiopia	10	25 Malay States	14	42 North Carolina	14					73 Hawaii	3
11 Somalia	1	26 Indo-China	502	43 Alabama	16						
12 Kenya	23	27 South Vietnam		44 Georgia	24						
13 Mozambique	10	28 North Vietnam		45 Louisiana	2183						
14 Rhodesia	56	29 Indonesia	79	46 Texas	35						
15 South Africa- Durban	528+	30 Philippines	101	47 Mexico	682						
Johannesburg	12	31 Korea	110	48 Guatemala	8						
		32 China	354								

(To Face Page 35)

Fig. 2. Diagram showing (1) important primary foci of amoebic lesions in the large intestine and (2) the more common secondary extra-intestinal sites (After Faust, 1954).



2.

(To Face Page 36)

Fig. 3 Diagram showing the relative frequency of amoebic lesions at different levels of the large bowel (After Faust, 1943).

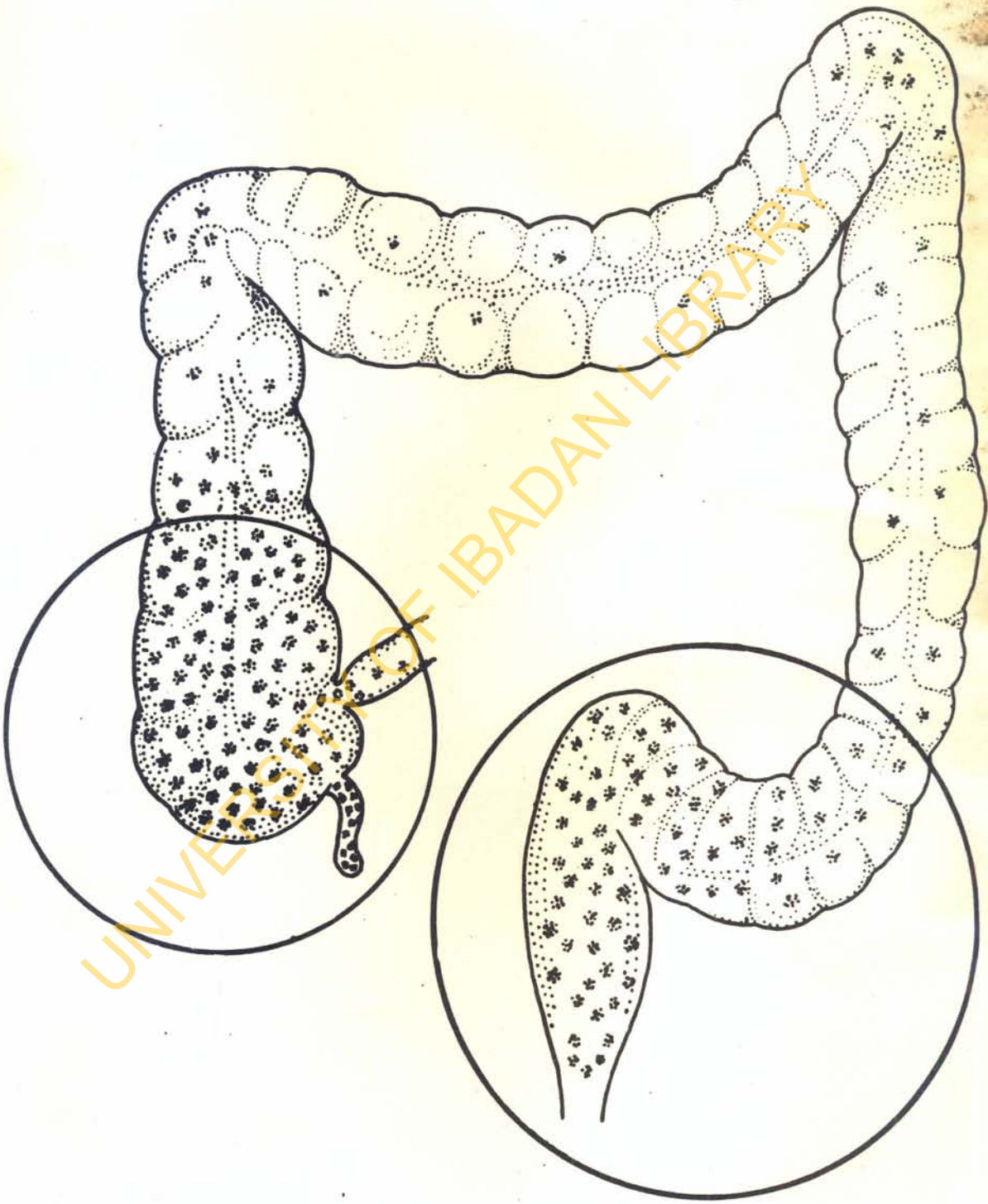
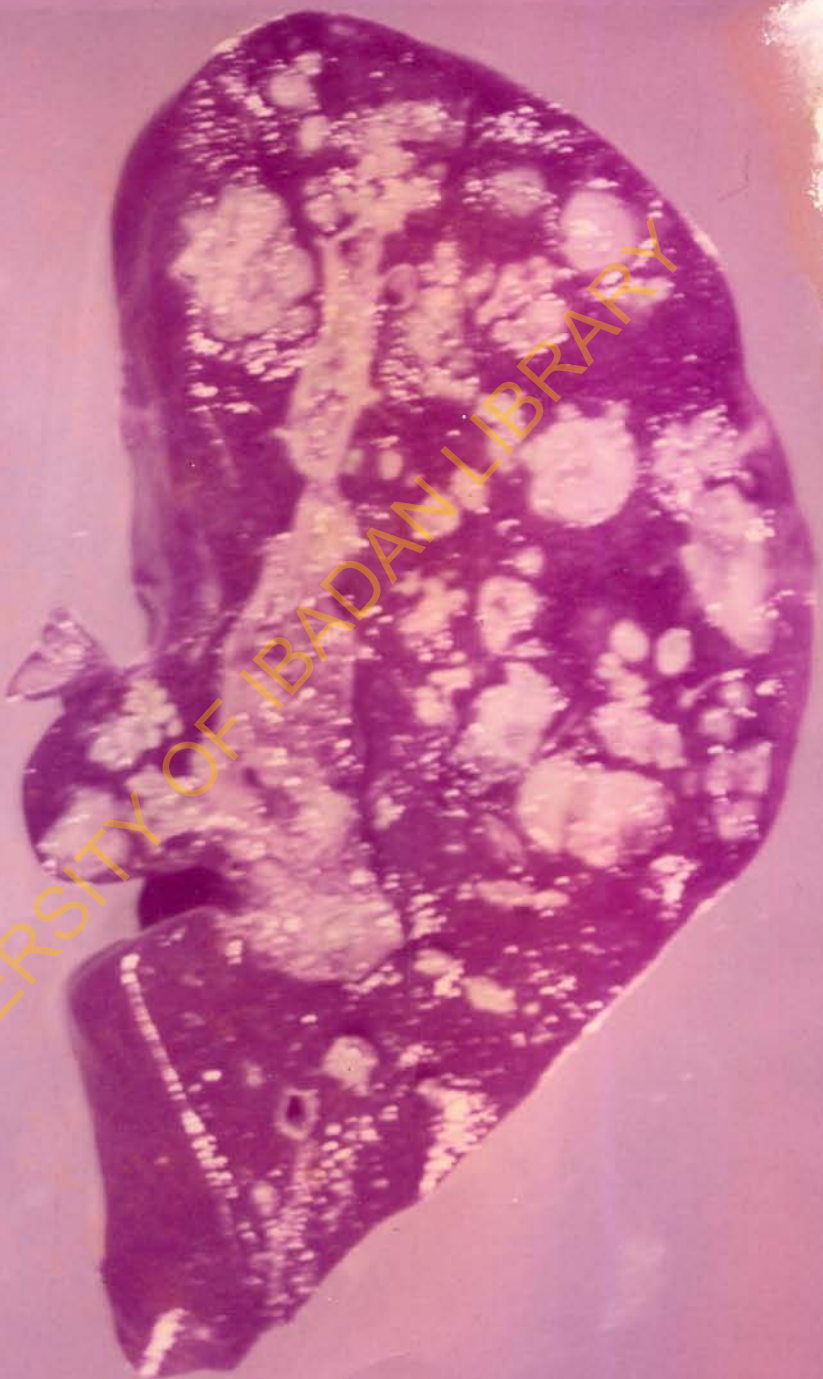


Fig. 3.

(To Face Page 37).

Post-Mortem Appearance of Pyogenic Liver Abscess.

(Note the diffused Nature of the lesions).



CHAPTER III

PATHOLOGICAL CHANGES AND COMMON COMPLICATIONS OF AMOEBIASIS SEEN AT U. C. H. IBADAN

3.1 Introduction:

In an attempt to define the frequency and pathological changes seen in amoebiasis in the U. C. H. Ibadan, a retrospective study covering all post mortem materials available from the period 1958-1967 was undertaken and is described below.

In any retrospective post-mortem study, however, of ulcerative intestinal lesions, post mortem autolysis frequently renders the interpretation of histopathological findings difficult. This is especially so in the case of amoebiasis as the histological identification of *E. histolytica* in such intestinal lesion may be extremely difficult. Accordingly in order to obtain the best possible material for histopathological examination a prospective study was additionally undertaken.

3.2 Materials and Methods:

3.3 Prospective study:

Both the macroscopic and microscopic appearances of 15 cases of intestinal amoebiasis were examined in this process.

The method of preservation used in this study was similar to that of Loehry and Creamer (1966) with some modifications. At necropsy the abdominal cavity was opened without disturbing its contents. The greater

omentum and transverse colon were laid back over the stomach to expose the small intestine. The duodeno-jejunal flexure was then identified, transfixed and ligated leaving 2-3cm of jejunum proximal to the ligature free for division. The hepatic flexure was similarly ligated. At this stage the intestines were touched only at the points of ligature.

A T-shaped cannula was then tied into the most superficial presenting loop of small intestine, great care being taken not to handle more intestines than absolutely essential. Through this cannula fixative was run in, and the intestines allowed to distend to a pressure of about 40 cm of water. Forty per cent formaldehyde was used as fixative to ensure rapid fixation with good penetration.

The intestine was then sealed by ties on either side of the cannula to prevent leakage, and left undisturbed for at least one hour. When it became necessary to remove the gut, the intestines were divided beyond the ligatures. These two points were then 'joined' by cutting along the dorsal attachment of the mesentery, and then lifted out with the mesentery as a handle. Immersion in 4% formaldehyde-saline for a further 24 hours completed fixation.

Tissues for histological examinations were stained routinely with Haematoxylin and Eosin. When indicated Bauer and Hotchkiss-McManus periodic acid-Schiff; modified Gomori's methanemine silver nitrate stain

(for glycogen), Best's Carmine (for polysaccharides), and Gidley's stain for amoebae were also utilized.

3.4 Results:

The epithelium of the small intestine was well preserved after fixation by the method described. The large intestine treated by the same method did not differ greatly from the untreated part. It also failed on section to show any appreciable difference in the microscopic appearances of the unaffected part of the bowel in cases of amoebic colitis. This result was probably due to good refrigeration of the bodies. It was therefore decided to abandon the special method of fixation and the proposed retrospective studies were carried out on the routinely preserved material in the department.

3.5 Retrospective studies:

3.5.1 Materials and Methods

7922 post mortem protocols which represent the total number of autopsies performed in the University College Hospital, Ibadan, during the ten-year period, 1958 to 1967 constitute the materials available for this study.

The post mortem protocols together with the corresponding clinical notes were studied. The causes of death were classified according to age, sex, affected body system and complications. The number of deaths

attributable to the diseases of the gastro-intestinal (Digestive) tract was further classified into diarrhoeal and non-diarrhoeal causes. The diarrhoeal diseases were again separated into chronic and acute stages. The final analysis deals mainly with the acute diarrhoeal diseases which for the purpose of the present study were grouped into causes due to amoebic colitis, typhoid enterocolitis and miscellaneous enterocolitis.

The criteria for attributing deaths to any form of acute diarrhoeal diseases in general include detailed clinical evidence of the specific causative agent; clinical/and or post mortem exclusion of any other antecedent relevant disease, and the autopsy findings, as well as, histological confirmation. In addition to these, for deaths to be attributable to amoebiasis, parasitological identification of amoebic products (cysts or trophyzoites) in stools and/or histological demonstration of tissue invasion by E. histolytica is absolutely necessary. This affords the writer an opportunity to assess various histochemical stains suitable for tissue diagnosis of amoebiasis.

The autopsy findings in amoebiasis were classified into intestinal lesions with their complications and extra-intestinal amoebiasis with special reference to amoebic liver abscess. Finally in order to assess the role of amoebiasis as a cause of death in the University College Hospital, Ibadan, in general and particularly in pregnancy/puerperium, the various data obtained from studies of post mortem materials were subjected to statistical

analysis. Such data include age and sex distribution; male: famale ratio; frequency of the disease in pregnant and non-pregnant women as compared to frequencies of other forms of acute diarrhoeal diseases in similar 'states'. This latter exercise was included in search for the confirmation or otherwise of the previous clinical observations on the apparent severity of amoebiasis in pregnancy and the puerperium.

3.5.2 Results:

In all a total number of 7922 post mortem protocols constitute the records of all autopsies performed in the department of pathology, University College Hospital, Ibadan during the period under review. Autopsies of Stillbirths, neonatal and perinatal deaths accounted for 1743 of all the autopsies. For the purpose of the various analyses in this section, the latter figure was excluded from the total number of autopsies which then brings the corrected value of "ALL" autopsies performed to 6179. Of this figure, diseases of the gastro-intestinal tract in general, were recorded as specific causes of death in 816 cases (13.20%) of corrected value of all autopsies. Of this number, 530 histology slides of the small and large intestines were included in the tissue specimens taken at autopsy during the period under review. This included 65 sections which were recut from the posterity pots both for missing sections and faded existing ones. Analysis of the protocol entries and histological confirmation of the available slides showed that acute diarrhoeal diseases accounted for 411

(6.65%) of all deaths (i.e. corrected autopsies). These were found to be nearly evenly distributed into 135 (2.19% c.a.) cases of amoebiasis; 140(2.27% c.a.) typhoid entero-colitis and 136 (2.20% c.a.) of miscellaneous (acute-entero-colitis). (Table I)

One hundred and thirty-two of the remaining 169 sections showed no lesions in the intestines, the remaining 37 slides showed non-specific as well as, changes that failed to satisfy the criteria for classifying a lesion in this context as acute diarrhoeal disease.

Of the 135 cases of amoebiasis, histological confirmation was made in 65 cases already shown to have tissue invasion by Entamoeba histolytica. 13 of these recorded as doubtful (? large round bodies present") in the protocols, were confirmed by applying special stains mentioned above. 22 of the 56 stained but previously unexamined slides of the intestine contained Entamoeba histolytica in the intestinal tissue, 8 unsuspected cases which were previously diagnosed as "necrotizing" colitis were also found to have typical amoebic ulcers and also to contain few degenerating amoebae within the necrotic debris and at the edge of the ulcers. Further 12 cases were included in this series purely on the strength of the typical amoebic ulceration of the gut, seen at autopsy. Finally, there were 28 cases of amoebic liver abscess, 10 (35.71%) of these had concurrent intestinal amoebiasis. Entamoeba histolytica trophozoites were demonstrated in four cases of amoebic liver abscess.

3.6 Common Complications of Intestinal Amoebiasis in Ibadan

An analysis of the common complications of intestinal amoebiasis found at autopsy in this series revealed that liver abscess occurred in 28 (20.74%); peritonitis 28 (20.74%); intestinal perforation in 17 (12.59%); broncho-pneumonia in 14 (10.37%); pericarditis in 5 (3.7%); lung abscess in 5 (3.70%) and brain abscess in 2 (1.48%), of 135 cases of intestinal amoebiasis (Table 2).

Considering the overall occurrences of these various complications in cases when (816) cases of gastro-intestinal tract diseases were recorded as causes of death, the number of percentage occurrences were 36 (4.41%); 105 (12.86%); 82 (10.05%); 66 (8.09%); 8 (0.98%) 17 (2.08%) and 4 (0.49%) respectively. Applying the Chi Square (χ^2) test of significance and using Yates correction where necessary statistical analyses showed that there was significant association between these complications and death due to amoebiasis. Thus amoebic liver abscess complicating intestinal amoebiasis at death was found to be significant at 0.01 per cent level ($\chi^2 = 95.28$, $p < .0001$); peritonitis at 1% level ($\chi^2 = 9.58$, $p < .01$), intestinal perforation at 1 per cent level ($\chi^2 = 7.95$, $p < .01$); pericarditis at 0.1 per cent level ($\chi^2 = 11.20$, $p < 0.001$) and lung abscess at 5 per cent level ($\chi^2 = 4.53$, $p < .05$). It was found that the association between pneumonia and death due to amoebiasis is not significant ($\chi^2 = 1.07$, $p < 0.2$) while brain abscess occurred in only 4 cases of gastro-intestinal diseases and two cases

of amoebiasis, these figures being too low for statistical analysis (Table 2).

In view of the fact that most of the complications listed above were direct consequences of intestinal leakage resulting mainly from intestinal perforation or seepage, it was decided to assess the significance of intestinal perforation as a cause of death in other gastro-intestinal affections in general and in amoebiasis in particular. As shown in Table 3, there is significant association between intestinal perforation and deaths due to various forms of acute diarrhoeal diseases, the per cent level of significance being as follows; amoebiasis 1 ($P < .01$); typhoid .01 ($\chi^2 = 27.54$; $P < .001$); duodenal ulcer .001 ($\chi^2 = 38.47$, $P < .001$). There was also significant association in cases of gastric ulcer ($\chi^2 = 30.91$, $P < .001$) and acute "appendicitis ($\chi^2 = 10.59$, $P < .01$) but the number in these cases appeared too low for serious statistical evaluation. When these values were compared with the value obtained for abdominal tuberculosis which may be regarded for the purpose of this study as a form of chronic diarrhoeal disease, the test showed no significant association of intestinal perforation in the latter condition ($\chi^2 = 0.13$, $P > .7$) (Table 3)

3.6.1 Some Observations on the Complications of Intestinal Amoebiasis

Apart from extra-intestinal amoebiasis, the complications of intestinal amoebiasis are notably, appendicitis, perforation/peritonitis of the intestinal wall, massive haemorrhage, amoebic granuloma, and post-dysenteric Colitis.

3.6.1.1 Appendicitis:

This complication occurred in none of the 135 cases of proven amoebiasis encountered in this series. Anatomically, the lesions may be in the wall of appendix, caecum, adjacent segment of the ascending colon or the margins of the ileo-caecal valve. Pathologically, they do not differ fundamentally from the chronic type of ulceration produced by E. histolytica in other sites of the large bowel.

The relative absence of appendicitis as a complication of amoebiasis in this series is also worthy of note. This complication was reported in 19% of autopsied cases in Mexico (Acevedo, Biagi, Cerecedo and Santoyo, 1960); in 20.5% of cases in Chile (Badinez and Siques, 1952), in 40% of cases in Panama (Clark, 1925), and in 31% of cases in Venezuela (Fuenmayor et al 1964). The variations in the reported prevalence of amoebic appendicitis in autopsies probably reflect the care taken in the examination of the appendix more than actual regional differences. The relative absence in this series appears to be a true reflection of the low incidence of the condition in the Nigerian population. Thus Joly and Thomas (1954) recorded 14.9 per cent for appendicitis in their surgical cases. In the same series amoebae were found in only 2 (7.77%) cases of a total number of the 26 cases in which reliable and repeated stool and other reports were available. Odunjo (1969) recorded what might be regarded as amoebic eppendicitis in 3.7 per cent of his 135 autopsied cases of

intestinal amoebiasis in the Lagos University Teaching Hospital. All these findings further confirm the notion that the part played by intestinal parasites in the aetiology of appendicitis in the tropics cannot be great (Edington and Gilles, 1969).

3.6.1.2 Perforation/Peritonitis

In tables 2 and 4, of the 135 cases of amoebiasis in this series, 28 (20.74%) cases showed peritonitis at autopsy whilst only 17 (12.59%) cases had accompanying intestinal perforation. The rates of the occurrence of peritonitis in other gastro-intestinal diseases in which perforation occurred in this series compared reasonably well with the corresponding rates of intestinal perforation in the respective conditions. The findings in cases of amoebic colitis complicated by peritonitis confirm the view that low seepage of intestinal contents from thin and porous bowel is a more usual cause of peritonitis than perforation of a deep penetrating ulcer (Powell and Wilmot, 1966).

Statistical analysis of the results in this series shows peritonitis/perforation of the gut to be a significant cause of death in amoebic colitis ($P < .01$). Perforation of the intestinal wall may occur in relatively quiescent cases of intestinal amoebiasis in which the amoebic lesion progresses much more extensively beneath the surface of the mucosa than superficial appearance of the wall would suggest. In the acute dysenteric

type the colon as seen at autopsy may be paper-thin with numerous perforations, or thickened with granulomatous perforations (Armstrong; Wilmot, 1949). In many instances perforation with release of enteric bacteria into the peritoneal cavity proceeds to peritonitis, although mesenteric adhesion may develop and prevent generalised peritonitis.

3.6.1.3 Massive Haemorrhage

Only two of the cases studied in this series had massive haemorrhage as a complication. Incidentally both cases also had superimposed typhoid entero-colitis.

This complication usually results from the erosion through the walls of mesenteric blood vessels over relatively extensive areas of deep amoebic ulceration of colon (Strong, 1901; Graig, 1944).

3.6.1.4 Amoebic Granuloma

Amoebic granuloma or amoeboma is not a common complication in this series. Only two cases were encountered, one in an outstation surgical biopsy, and another gave rise to the only cutaneous amoebiasis seen during the period of this study. The pathological findings in these cases conform to the classical description below.

Most of the recent clinical investigations refer to this complication as "Amoeboma". Macroscopically, the lesion is a relatively firm and nodular tumour mass, with a fibrous outer wall and one or more internal "abscesses" which contain necrotic tissue elements and amoebic

trophozoites. Although amoebomas may develop at any site along the length of the large intestine, Nino (1942) found that they have predilection for the caecum (34%), the transverse colon (17%) and sigmoid colon (14%).

On cut surface there is an outer covering of oedematous ulcerated mucosa, infiltrated with fibrous tissue which frequently fixes the mass to adjacent portion of the intestinal wall.

Microscopically there is a zone of granulation tissue immediately within an oedematous ulcerated mucosa. The granulation tissue is characterized by inflammatory cellular infiltrates made up of lymphocytes, few polymorphs and many eosinophils (not observed in uncomplicated amoebic colitis). There is also lymphoid hyperplasia, few fibroblasts and the core of the tumour may contain necrotic tissue cells and amoebic trophozoites and possibly giant cells.

Amoeboma may superficially resemble tuberculous abscesses, carcinoma, actinomycosis, and other granulomatous growth in the intestinal wall, the histological picture is usually distinctive.

3.6.1.5 Ulcerative post dysenteric colitis

The paucity of both clinical and post mortem diagnosis of this condition in the material studied was responsible for the delibrate omission of this complication as an entity in this work. Lewis and Antia (1969) in their clinical review of 295 cases of amoebiasis in the University

College Hospital, Ibadan, included seven cases as being possibly complicated by "pipe-stem ulcerative colitis". They also state that few of their cases with fairly long history of intermittent diarrhoea may have had this condition, but no single case coming to autopsy (UCH) was diagnosed as a case of "post-dysenteric" ulcerative colitis during the period under review. However, during the prospective study it was possible by the aid of amoebic gel diffusion test to make confident clinical diagnosis of amoebic post-dysenteric ulcerative colitis in 3 cases with chronic intermittent diarrhoea with no cysts or trophozoites of Entamoeba histolytica in their stools

Furthermore, it was possible to return the same post-mortem diagnosis in two cases diagnosed clinically as chronic diarrhoea. This was made following positive gel diffusion reaction using the blood collected at autopsy. Also in retrospect, the histopathological appearances of cases recorded in the protocol as "ulcerative colitis" show mainly disruption of mucosal surface, endarteritis of few vessels, destruction of muscle layer and mild inflammatory infiltrate of plasma cells and lymphocytes. None of these autopsied cases has the features of acute non-specific ulcerative colitis. The histological criteria for the latter having been based on: (1) oedematous thickening of the lamina propria; (2) increased cellularity of the lamina propria consisting mainly of plasma cells; (3) diminished mucus content of crypt epithelium and (4) focal degeneration

of the crypt epithelium. It may however, be mentioned that the first three criteria occur in other acute diarrhoeal diseases where rectal biopsies were available but only one (Fig. 6a) showed in addition mild evidence of focal degeneration of the crypt epithelium. Incidentally, the serum of this patient was negative for amoebic antibodies on gel diffusion, but unfortunately, the patient was lost to follow-up.

From the foregoing, whilst the existence of non-specific ulcerative colitis in Nigerians, cannot be firmly disputed at this stage, it is the view of the writer that cases previously diagnosed at autopsy as "non-specific ulcerative colitis" in this series may in fact be cases of "amoebic post dysenteric colitis". This view is in keeping with that of Steward (1950) who found that post dysenteric colitis was more commonly a sequel to acute amoebic dysentery. It is, therefore, being suggested that this diagnosis be given prominence in the (UCH) autopsied cases of chronic diarrhoea especially where a retrospective review of such case notes suggests previous acute colitis with 'active Entamoeba histolytica trophoziites' in the stool during the 'recent past'. Also that confirmation or otherwise, of the existence of non-specific ulcerative colitis, awaits a more detailed combined clinical and histopathological studies of biopsy materials, as well as, serological studies of such suspected clinical cases.

3.7. AMOEBIASIS AND ASSOCIATED CONDITIONS.

3.7.1 Amoebiasis in Pregnancy:

As shown in table 3, a comparative age and sex distribution of acute diarrhoeal diseases in the necropsy studies in this series show that of a total of 135 proven cases of amoebiasis, 58 (42.95%) occurred in females at all ages. On the other hand, of a total number of 140 cases of typhoid enterocolitis, 59 (42.14%) occurred in females, while the corresponding figure of 136 cases of miscellaneous colitis 71(52.20%) occurred in females at all ages.

Taking all forms of acute colitis occurring in adults into consideration, a single highest occurrence was in females with amoebiasis in the age group (25-34) which accounted for 14.07 per cent of all cases of amoebiasis occurring in both sexes. When cases of entero-colitis occurring in females between the ages 15-34 were analysed into female pregnant (FP) and female non-pregnant (FNP); it was found that 68% of cases of amoebiasis occurred in pregnant women as compared to 23.57% and 12.50% occurring in similarly pregnant women with typhoid and miscellaneous colitis respectively. Even when the child-bearing age is extended to 44 years, the corresponding percentage of deaths due to amoebiasis (54.30%) in pregnancy is still higher when compared to typhoid and miscellaneous colitis; where the corresponding values of deaths in pregnancy due to these diseases are 16.70% and 9.60% respectively (Table 5).

Statistical analysis showed significant association ($\chi^2 = 12.34$; $p < .001$) between amoebiasis and death in pregnancy and the puerperium, whilst similar statistical analysis of cases of typhoid enterocolitis occurring in similar states showed no significant association ($\chi^2 = 0.62$; $p > 0.3$) (Table 3). Age specific incidence for pregnancy was not available for the period covered by this review.

Admittedly the figures quoted above reflect the increased prevalence o of amoebiasis in males, and do not necessarily indicate that the disease is apt to be more severe in males. In fact, Gomez (1960) found that although males made up 64% of his clinical cases only 53% of the fatalities occurred in this same group of patients. The present series shows that amoebiasis has a fatal outcome in pregnancy and the puerperium as already confirmed above.

3.7.2 Hepatic Cirrhosis and Amoebiasis:

In the present study amoebiasis was found to be associated with several other conditions. Notable amongst these was pregnancy and puerperium, conspicuous for their frequent occurrences. These have been noted earlier on and will be considered further in the following chapters.

Malignant conditions were occasionally found in association with amoebiasis in the present series. Thus there were cases of choriocarcinoma, carcinoma of the stomach, and three cases of Hodgkin's lymphoma. The present findings agree with the view that the isolated reported cases of

amoebiasis and malignancy must be regarded as fortuitous and merely coincidental (Edington and Gilles, 1969). However, an altered immunological state which may accompany some malignant conditions, such as anergy to antigens as in malignant lymphoma, notably, Hodgkin's disease, may be a factor to be reckoned with in this 'amoeba-malignancy' association.

However, one interesting and rare association was the finding of a case of liver abscess in a cirrhotic liver during the prospective part of this work. The rarity of this association has been reflected in various surveys by its conspicuous absence. However, Biagi and his co-workers (1962) state that hepatic abscesses are very rare in cirrhotic patients. Among the possible explanations put forth to account for this rare association is the fact that in hepatic cirrhosis, the liver contains only a small quantity of cholesterol (Ralli, Rubbin and Ringer, 1941). Another possible explanation is the possibility that a cirrhotic liver may be so resistant, as a result of its tremendous fibrosis to both the entry and subsequent spread of the organisms. Indeed portal hypertension which is an invariable complication of cirrhosis (Edington and Gilles, 1969) may be another important factor. It is known that this condition causes congestion of the alimentary canal and thereby prevents the carriage of the causative organisms into the liver.

Since the suggestion as regards the level of cholesterol is more easily examined experimentally, the role of cholesterol in amoebiasis is therefore, examined in some details in chapter six of this thesis.

3.7.3 COMMENTS AND GENERAL ANALYSIS OF DATA:

The relative paucity of deaths due to amoebiasis in this series (2.19%) of all autopsies highlights the fact that none representative data cannot be used to give an indication of the frequency of events in a community. To conclude on the basis of this autopsy studies alone, that the incidence of amoebiasis in Nigeria is 2.19% will be dangerous especially when one considers the fact that almost the same figure, and some cases much higher figures have been recorded in situations where the incidence of the disease would be expected to be at least the same as in Nigeria. Thus the percentage deaths caused primarily by amoebiasis, in all autopsies in Columbia is 3.5% (Duque, 1963); Mexico 5% (Flores, Nunez and Biagi, 1959; Alfaro, Albores and Brandt, 1963); Venezuella, 6.2% (Gomez and others, 1960). The foregoing observations notwithstanding, some interesting points emerged from the autopsy studies.

3.7.3.1 Age Distribution: The present study confirms that all age groups are susceptible to death by amoebiasis. In this series of 135 autopsy cases of fatal amoebiasis 36 (26.66%) were children under 15 years of age, and 3.9% were patients above 50 years of age. This contrasts with the age distribution in Lagos where the corresponding age distributions in children under 15 years of age (43.38%) and in adults over 50 years of age (11.3%) were respectively higher than in the present series

(Odunjo, 1969). Three patients were under one year of age, and this compares favourably with the age incidence in East Africa and Central America, where amoebic dysentery and its complications were not uncommon before the age of one year (McDougall, 1960; Scragg, 1960; and Salas, 1953). Children made up 72% of the autopsy series of Fuenmayor (1964).

Deaths due to amoebiasis was found to be 3.9% of all children' autopsies in Columbia (Duque, 1963); 4.2% in Mexico (Salas, 1953); and 6.2% in Venezuella. Also in this series, 22 cases (16.30%) occurred in the age group (0-6) and 12 cases (8.88%) in the age group (7-16). This confirms the view that amoebiasis occurs in the age group nil (although none occurred between 0-28 days of life in this series) to 6 years but failed to sustain Wilmot's (1962) contention that it does not occur between the ages (7-16) years, as those in the age group seem to enjoy a greater immunity to ill effects from E. histolytica infection than others. However, fatalities due to amoebiasis occur most commonly in this series during the third and fourth decades of life (41.48%). This is in accordance with the general impression that the greatest fatalities occur at this age group. Indeed, the major contributors in this series, at this age group are pregnant/women in the puerperium with amoebic colitis.

3.7.3.2 Sex Distribution: As to sex predilection, males are more affected than females, although as stated by Edington and Giles (1969), any difference in incidence of the disease between males and females are probably related to exposure rather than a true sex susceptibility to the infection. While the age-sex distribution in this series supports the idea of "exposure-incidence" factor at all age groups except the (15-34) age group where females predominate, it would appear that there are other factors to be considered apart from this exposure-incidence one. There were 72.4% males in the series of Florez (1966); 65% in that of Duque (1963) and 57.05% in the present series. Again in the series of Olatubosun (1965), males predominate over females in the ratio 10:4, whilst in the series of Odunjo (1969), the ratio of males to females was about 3:1 and in this series about 3:2.

PATHOLOGICAL CHANGES IN AFFECTED PARTS

3.8. Introduction:

There are certain aspects of the pathological changes in parts affected by amoebiasis which deserve some comment. The observation in this section cover mainly the retrospective and some of the prospective post-mortem studies.

3.8.1 Difficulties in Recognition of Bowel Lesions at Autopsy

As shown in this chapter during the review of slides, of the 14 cases of enterocolitis previously diagnosed at autopsy either as "non-specific"

or "necrotizing" colitis, some degenerated amoebae were detected in the colonic mucosa on further review of the slides. Also histological confirmation for tissue invasion by E. histolytica was made in 13 cases previously diagnosed as doubtful (? large round bodies). The presence of E histolytica in some of these unsuspected cases, could be inferred by the examination of the H. & E. (haematoxylin & Eosin) stained sections alone but usually not very convincingly. The usual special histo-chemical stains then became mandatory in such unconvincing sections. Some of the special stains employed for this purpose are mentioned below.

3.3.2 Histochemistry of Amoeba in Tissue

The five special stains employed in this study were all designed mainly to bring out the polysaccharides in E. histolytica. The stains are (i) Best's carmine (Meriwether, 1935); (ii) Bauer and Hotchkiss-McManus periodic acid - Schiff techniques for selective staining of amoebae (Hallman, Michaelson, Plumental and Delamater, 1955); (iii) Gridley's stain for amoebae (personal communication); (iv) Gomori's methenamine-silver nitrate reaction for glycogen and other polysaccharides (Gomori, 1946) and (v) Modified methenamine-silver nitrate technique (Mowry, 1959). The last which was methenamine-silver nitrate technique modified by the use of periodic acid oxidation, and described in detail in (Appendix 1) was used consistently in this work. With this stain, the contrast between the parasite and the surrounding tissue is greater than in the other methods and this

helped greatly in surmounting one of the difficulties of histological diagnosis of tissue invasion by Entamoeba histolytica.

3.3.3. Bowel Lesions: Macroscopic Appearances

Macroscopically, in some cases, there are discrete, minute button ulcers, probably corresponding to the sites of entry into the mucosa. These buttons are seen as very small, oedematous, reddened papillae, at times with evidence of haemorrhage, and the advancing lesions resemble the neck of a 'micro-flask'. In other cases there is usually marked undermining of the mucus membrane, giving rise to overhanging and swollen edges to the ulcer. The ulcers tend to spread along the mucosal folds, so that they are broader than they are long. In about 25% of cases the membrane between the ulcers appears normal. In the remaining 75% of the cases with suspected superimposed secondary bacterial infection, there is congestion and sloughing, with the bases of the ulcers being covered with necrotic dark-doloured material.

3.3.4 Microscopic Appearances

Histologically, in greater percentage of proven cases, there is marked lytic necrosis, and stromal oedema. The histological appearances of the lesions are remarkable for lack of infiltration of neutrophilic leukocytes or of macrophages. The ulcers show invasion of the mucosa and underlying tissues by the amoeba with necrotic degeneration and some lymphocytic

infiltration. Polymorphs appear in numbers only in cases where the macroscopic appearances are suggestive of possible superimposed bacterial infection. In many cases in which the initial tissue invasion occurs the lesions are limited to the mucosal layer and only superficial ulceration develops.

3.3.5 Chronic Modification of Bowel Lesion

Five of the cases encountered during the prospective studies, show changes which may be regarded as chronic forms of the above-mentioned appearances. Some of these changes are described below:

Macroscopic changes: On the mucosal surfaces of these cases the lesions take the form of shallow weeping ulcers as extensive as a centimetre in diameter, with a hyperaemic raised margin. Sometimes, the lesions show diffuse granulating mucosal covering over a number of invaded mucosal glands but show no evidence of ulceration. More commonly, these chronic changes take the form of raised nodules with sharply delimited edges and minute depressed, yellowish pores surrounded by reddened ring and opening into small enlarged bases filled with gelatinous material and necrotic tissue.

Microscopic changes: In these chronic cases, the edges of the ulcer consists of a matrix of fibrous tissue. The underlying cellular components are made up of dense infiltrate of neutrophil leukocytes extending into the submucous or muscular coats and sometimes providing a relative compact wall around the margins of the necrotic base.

3.3.6 Comments On Chronic Modification of Bowel Lesion:

This histologic type may not be a distinct entity but rather represents an advanced picture usually resulting from the superimposed invasion of enteric bacteria. It is conceivable that the latter may provide the stimulus for host cell infiltration because in uncomplicated cases, colonies may establish themselves in the mucosa with remarkably little reaction on the part of the tissues (Edington and Gilles, 1969).

3.9. PATHOLOGICAL CHANGES OF EXTRA-INTESTINAL AMOEBIASIS

3.9.1 Hepatic Amoebiasis ("Acute Amoebic Hepatitis")

Although in some individuals with evidence of intestinal amoebiasis, a clinical syndrome of fever, tender liver and mild leukocytosis, which responds dramatically to treatment with emetine hydrochloride occurs, the majority of observers agree that conclusive pathological prove of amoebic hepatitis as an entity is lacking. None of the cases encountered in this series shows histological lesions sufficient to warrant the diagnosis of acute amoebic hepatitis.

However, some cases of acute amoebic colitis with concurrent amoebic liver abscess, shows some focal changes in an otherwise unaffected parts of the liver free from the frank abscess formation.

Microscopically, these changes consist of thrombi in the 'free' areas of the liver, composed of fibrinous filament and leukocytes. These are usually found in close contact with the hypertrophic Kupffer cells.

In addition there may be lytic necrosis and occlusion of some of the blood vessels. Possibly from the necrotic wall of the blood vessels there may be an extension into the lobule and at times the central collecting vein may be involved in the erosive process. In some cases the lesion is usually free of inflammatory reaction while in more advanced cases of liver abscess, the liver shows extensive infiltration of large numbers of neutrophilic leukocytes. This is possibly in response to the necrotic host tissue itself as has been suggested by Carrerra (1950).

In the few cases where amoebic colitis is accompanied by diffuse hepatitis, no amoebae were found in the liver. In fact excluding the reports of Doxiades and his co-workers (1961), the pathological findings in these cases have been non-specific.

3.9.2 Hepatic Amoebiasis (Amoebic Liver Abscess)

No absolute demarcation can be made between the termination of amoebic hepatitis and the beginning of amoebic liver abscess. This is because the latter is said to be a radial extension of the lytic necrosis of hepatic cells by the amoebae in one or more sites, the centres of which become filled with a puree of necrotic debris (Meleney, 1934).

In the present studies, a total number of 23 cases were encountered during the review of the retrospective autopsied cases.
A further number of eight cases of clinically and gel diffusion proven cases were added in the course of the prospective studies. Of this total number

of 36 cases, 5 cases (11.11%) occurred in females, 3 cases in children under 5 years of age, and the rest in adult males. Two of the juvenile cases, occurred in female infants, 10 and 11 months old respectively and the third was a 3 year old boy.

Two of the cases encountered during the prospective studies exemplified some rare and unusual features. Thus one was found in association with cirrhosis of the liver. The other failed to respond to the usual amoebic treatment although there was positive serum reaction on agar gel. The liver abscess of the latter case was found at autopsy to be walled off by a very thick fibrous capsule (Fig. 7).

Apart from these rare findings the pathological changes conform to the classical appearance described below. However, in two cases the abscesses were confined to the inferior surfaces of the liver. In two other cases the abscesses involved the left lobe of the liver exclusively.

3.9.3 Macroscopic Appearances

The smallest abscesses are usually a few millimetres in diameter. They are solid and white and still resemble normal liver. In slightly large ones, the contents are gelatinous and yellow; and still larger ones contain reddish brown fluid and shreds of necrotic tissue. In cases that come to autopsy soon after the onset of the disease, it appears that the abscess has no opportunity for development of a fibrous capsule, but in chronic process there is usually a limiting wall. As mentioned above,

such wall was found to be accentuated so as to interfere with treatment in one of the cases in this series. Except for lesions occurring in children and in two adults, "multiple" abscesses were not encountered in the cases under review. The majority of the single abscesses develop in the right lobe of the liver. This usually occurs either just below the diaphragm or at the lower aspect near the surface. The solitary ones may be as large as a grapefruit, with trabeculated strands of more resistant stroma (Fig. 8).

3.9.4 Microscopic Appearances:

Microscopically, there are three recognizable zones (Fig. 9). These are:- (a) The outermost zone of relatively normal tissue which is in the state of being invaded by amoebae.

(b) A median zone in which the vital tissues have been destroyed and only the stroma remains; and

(c) the necrotic centre.

The wall of the abscess is usually composed of a shaggy fibrous lining surrounded by a fibrovascular response that is infiltrated by a few mono-nuclear leucocytes.

3.9.5 Pleuro-Pleumonary Amoebiasis

The right copular of the diaphragm is usually raised in cases of amoebic liver abscess and direct extension of the abscess to the pleural cavity or base of the right lung is not uncommon. This type of complication occurred

in three of the cases of liver abscess under review. Two other cases of lung abscess were found in connection with amoebic colitis without liver involvement.

3.9.6 Macroscopic Appearances

In case of pleuro-pulmonary amoebiasis, there is localized pneumonitis, with formation of an abscess containing a gelatinous menstrum and blood. Occasionally, there is a fistulous opening into a large bronchiole or bronchus, so that the reddish-brown contents of the pulmonary abscess, and occasionally, of the hepatic abscess are coughed up and discharged. In cases in which the amoebae have reached the lung by the bloodstream, the diaphragm is not involved and the pulmonary abscess at first lies wholly within the parenchyma of the lung.

3.9.7 Microscopic Appearances

Histologically, there is localized pneumonitis with abscess formation. There is marked vascular congestion and inflammatory cellular infiltrates made up mainly of large mononuclear cells from the walls of the alveoli. There are no amoebic trophozoites seen in the cases encountered in the course of this study, but there are few undigested elastic tissue elements.

3.10 CEREBRAL AMOEBIASIS

Only two cases of cerebral amoebiasis were encountered in the course of the present study. Both cases were in adult males and both were associated with amoebic liver abscess (Fig. 10).

Amoebiasis of the brain is very uncommon compared with hepatic and pleuropulmonary infection. Izar (1932) found 56 records of cerebral amoebiasis in the literature, of which 29 were from Egypt. Huard (1937) collected a total of 59 cases. Lambardo and his co-workers found that 8.1% of their 210 autopsied cases had brain involvement. Olatubosun (1965) reported one case in his necropsy survey of amoebiasis in Nigerian children, while Odunjo (1969) found 4 cases of a total of 2743 autopsies out of which 135 cases were due to intestinal amoebiasis.

Although inoculation is considered to be exclusively haematogenous in origin, with rare exceptions, brain involvement is associated with hepatic or pleuropulmonary amoebiasis, most frequently as a metastasis from the lungs (Ochsner and De Bakey, 1942). An exception is the case which came to autopsy in the Charity Hospital of Louisiana, New Orleans, August, 1947 (Swarzwelder and McGill, 1949). Living Entamoeba histolytica were recovered from a solitary brain abscess. In this case primary lesions were found in the colon but there was no evidence of hepatic or pleuropulmonary amoebiasis.

3.10.1 Macroscopic Appearances

The affected area of the amoebic brain abscess appears oedematous and congested. The outer wall of the abscess is thin, its inner aspect fuzzy and the contents are of a chocolate pasty material. The brain abscess is characteristically sterile.

3.10.2 Microscopic Appearances

Microscopically, there is congestion and thrombosis of the (blood) capillaries in the contiguous tissue. There is lytic necrosis and abscess formation, with degenerate tissue cells, erythrocytes, and leukocytes in the abscess cavity. In the inner portion of the wall of the abscess are usually found many lymphoid cells, degenerate nerve cells and amoebic trophozoites.

3.11 CUTANEOUS AMOEBIASIS

The only case of cutaneous amoebiasis encountered in the course of the present study falls into group (b) of Engman and Meleney's (1931) suggested sources of infection listed below.

The skin is the fourth most common anatomical location outside the large bowel for the development of E. histolytica. It has been suggested by Engman and Meleney (1931), that cutaneous amoebiasis may develop in any of the following ways:

(a) from drainage of an amoebic liver abscess following spontaneous rupture or surgical intervention.

(b) from drainage of an appendical or colonic lesion.

(c) as a perianal extension of amoebiasis of the colon; or

(d) without definite evidence of previous visceral amoebiasis.

3.11.1 Macroscopic/Microscopic Appearances

Grossly there is oedematous elevation of the involved skin, with induration of the margin of the developing ulcer. The characteristic features of this lesion include a rapidly spreading ulcerative process with irregular margins, with an overhanging edge of gangrenous epidermis. The advancing zone peripheral to the ulcer has a dusky red hue which gradually merges with the normal skin. The ulcer itself contains a blood-tinged material of foetid odour and has a dirty greyish necrotic base.

Microscopically, in the necrotic tissue is found amoebae advancing into the peripheral zone (Fig. 11).

3.12 GENITO-URINARY AMOEBIASIS

In the present study one case of genito-urinary amoebiasis was encountered in a 3 year old male infant in whom amoebic liver abscess was found at autopsy (Fig. 12).

Genito-urinary amoebiasis belongs to the group of rare anatomical type. Few cases which were believed to be direct extension from amoebiasis of the colon or liver have been reported. Indeed veneral skin infection and urethral amoebiasis following rectal intercourse has been described (Mylins and Ten Seldam, 1962; Sasea and Amin, 1962).

TABLE I

ANALYSIS OF AUTOPSIES - ACUTE DIARRHOEAL DISEASES
(U.C.H., IBADAN, 1958 - 1967)

CAUSE OF DEATH	MALES	FEMALES	TOTAL	PERCENTAGE OF ALL AUTOPSIES	PERCENTAGE OF CORRECTED AUTOPSIES	PERCENTAGE OF G.I.T.D.
1. GASTRO-INTESTINAL DISEASES	469	347	816	10.30	13.20	-
2. ACUTE DIARRHOEAL DISEASES	224	187	411	5.19	6.65	50.36
3. AMOEBIASIS	78	57	135	1.70	2.19	16.54
4. TYPHOID ENTRITIS	81	59	140	1.80	2.27	17.16
5. MISCELLANEOUS (ACUTE ENTERO-COLITIS)	65	71	136	1.72	2.20	16.66

(a) ALL AUTOPSIES PERFORMED = 7922
(b) STILBIRTHS, NEONATAL AND PERINATAL AUTOPSIES = 1742
(c) CORRECTED VALUE FOR 'ALL' AUTOPSIES (a-b) = 6179
(d) G.I.T.D. = Gastro-intestinal Tract Diseases.

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TABLE II

COMMON COMPLICATIONS OF INTESTINAL AMOEBIASIS (AUTOPSIES) U.C.H., IBADAN, 1953 - 1967

NATURE OF COMPLICATION	OCCURENCE IN 135 CASES OF AMOEBIASIS		OCCURRENCE IN OTHER G.I.T.D. (691 cases)* (Non-Amoebic)		Test of Different Proportion. P (Valued)
	No.	% of Cases of Amoebiasis	No.	% of Cases of G.I.T.D.	
LIVER ABSCESS	28	20.74	8	1.18	.001
PERITONITIS	28	20.74	77	11.31	.01
INTESTINAL PERFORATION	17	12.59	65	9.55	.01
PNEUMONIA	14	10.37	52	7.64	.30
PERICARDITIS	5	3.70	3	0.44	
LUNG ABSCESS	5	3.70	12	1.76	
BRAIN ABSCESS	2	1.48	2	0.29	

* G.I.T.D. = Gastro-Intestinal Tract Disease.

TABLE III

DISTRIBUTION OF INTESTINAL PERFORATION IN 816 GASTRO-INTESTINAL DISEASES. (AUTOPSIES - U.C.H., IBADAN, 1963-1967)

RECORDED CAUSE OF DEATH	NO. OF AUTOPSIES	No. of INT. PERFORATION	PERCENTAGE OF	
			SPECIFIC CONDITION	'ALL' PERFORATIONS
AMOEBIASIS	135	17	12.59	20.73
TYPHOID	140	31	22.15	37.81
DUODENAL ULCER	40	16	40.00	19.51
GASTRIC ULCER*	11	7*	63.63	8.54
"APPENDICITIS"	13	5	38.47	6.10
ABDOMINAL TUBERCULOSIS	83	6	7.23	7.32
OTHERS	394	-	-	-
TOTAL	816	82	-	-

* Perforations confined to STOMACH

H Appendix only.

TABLE 3A

AMOEBIASIS THROUGH AUTOPSY
U. C. H. 1958 - 1967
ASSOCIATION BETWEEN AMOEBIC COLITIS AND DEATHS IN PREGNANCY/PUERPERIUM

STATE	FO	FE	FO-FE	$(FO-FE)^2$	$\frac{(FO-FE)^2}{E}$
AMOEBIASIS					
PREGNANT	17	11	6	36	3.27
NON-PREGN	14	20	-6	36	1.80
MISCELLANEOUS					
PREGN.	2	8	-6	36	4.50
NON-PREGN	19	13	6	36	2.77

$$\chi^2 = \frac{\sum (FO-FE)^2}{E} = 12.34$$

fn = 1 p = < 0.001

$X_c^2 = 9.22$; $0.005 > p > .001$

TABLE 3B

ASSOCIATION BETWEEN TYPHOID ENTERITIS AND DEATHS IN PREGNANCY/PUERPERIUM

STATE	FO	FE	FO-FE	$(FO-FE)^2$	$(FO-FE)^2/E$
TYPHOID					
PREGN	6	5	1	1	0.20
NON-PREGN	30	31	1	1	0.32
MISCELLANEOUS					
PREGN	2	3	-1	1	0.33
NON-PREGN	19	18	1	1	0.05

$$\chi^2 = \frac{\sum (FO-FE)^2}{E} = 0.62$$

n = 1, p = 0.5

$X_c^2 = 0.3$; $p > 0.5$.

AMOEBIASIS THROUGH AUTOPSY U.C.H. 1958 - 1967

FATAL ACUTE DIARRHOEAL DISEASE IN PREGNANT AND NON-PREGNANT WOMEN (15 - 44 years)

TYPE	PREGNANT	NON-PREGN	TOTAL	% PREG.	% NON-PREG
AMOEBIASIS	17	14	31	54.8	45.2
TYPHOID	6	30	36	16.7	83.3
MISC.	2	19	21	9.6	80.4
TOTAL	25	63	88	-	-

$X^2 = 16.769$; $p < .001$

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Fig. 4. Typical lesion of Fulminating Intestinal amoebiasis.

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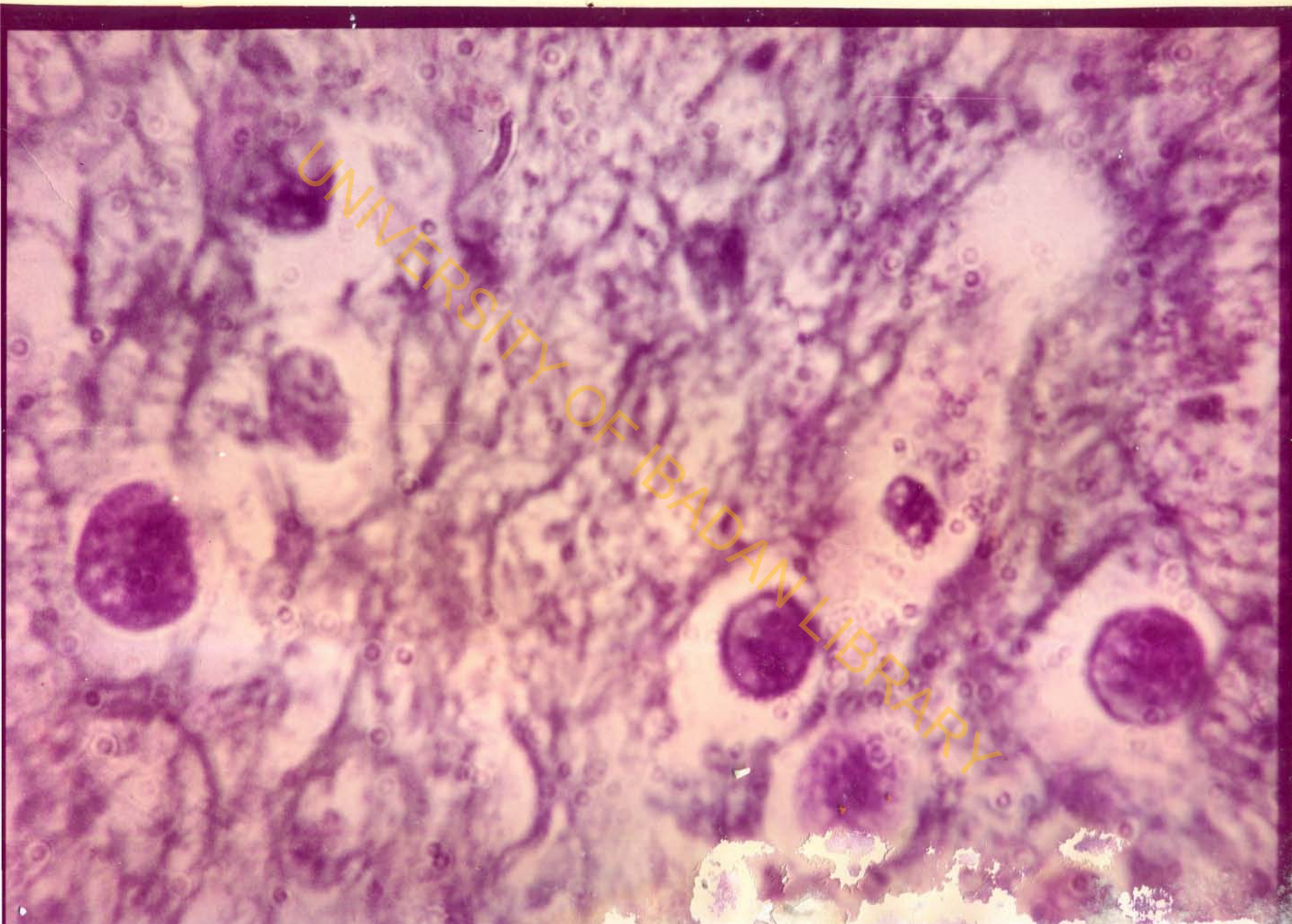
Fig. 5 Histochemistry Of Amoebae In Tissue.

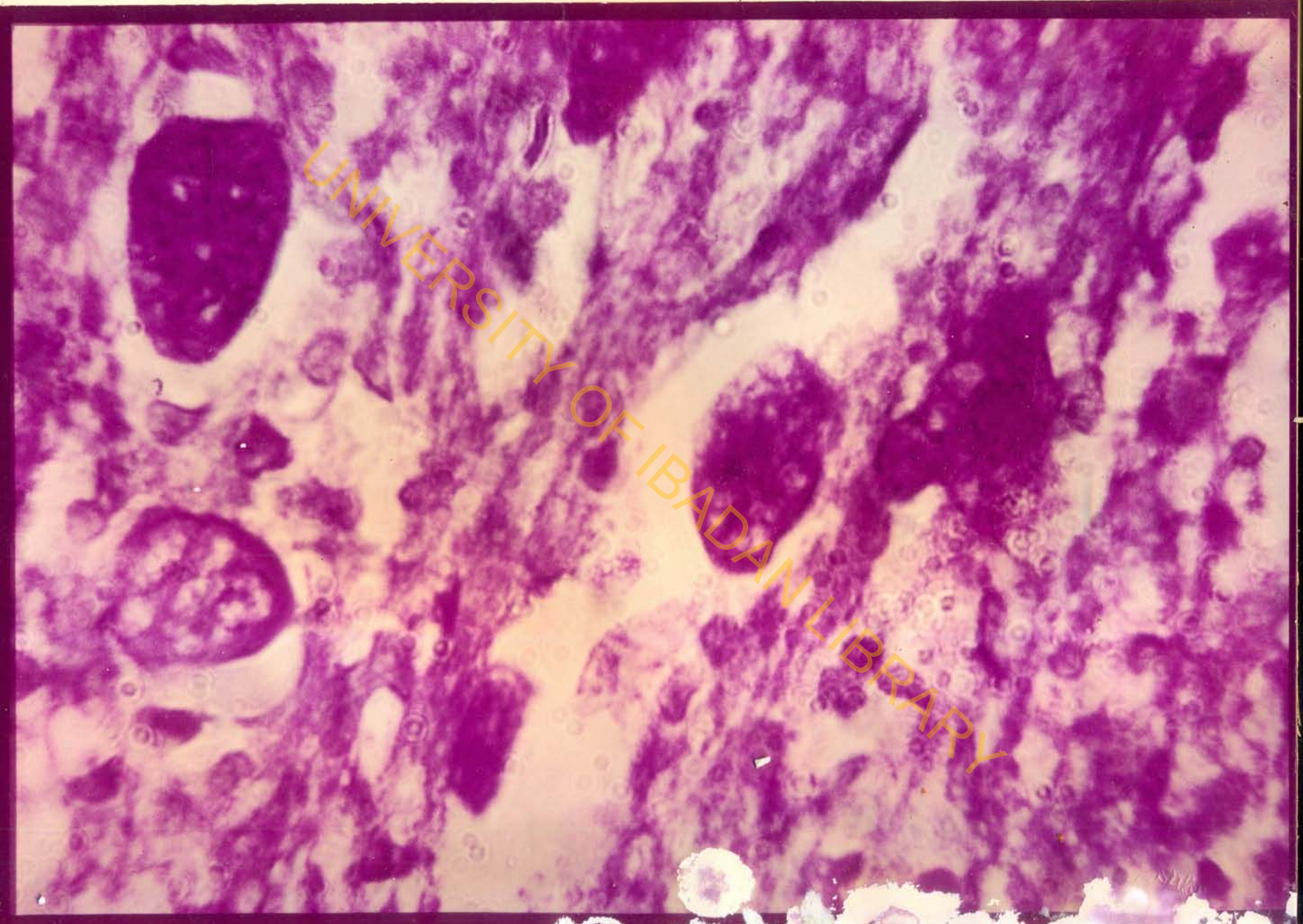
A. Modified Methanamine (PAS-Light-Green) x 900.

B. Periodic Acid Schiff's (PAS) x 900

C. Best's Carmine / Alum Haemat. x 400 &900.

D. Gridley's For Amoebae x 400.





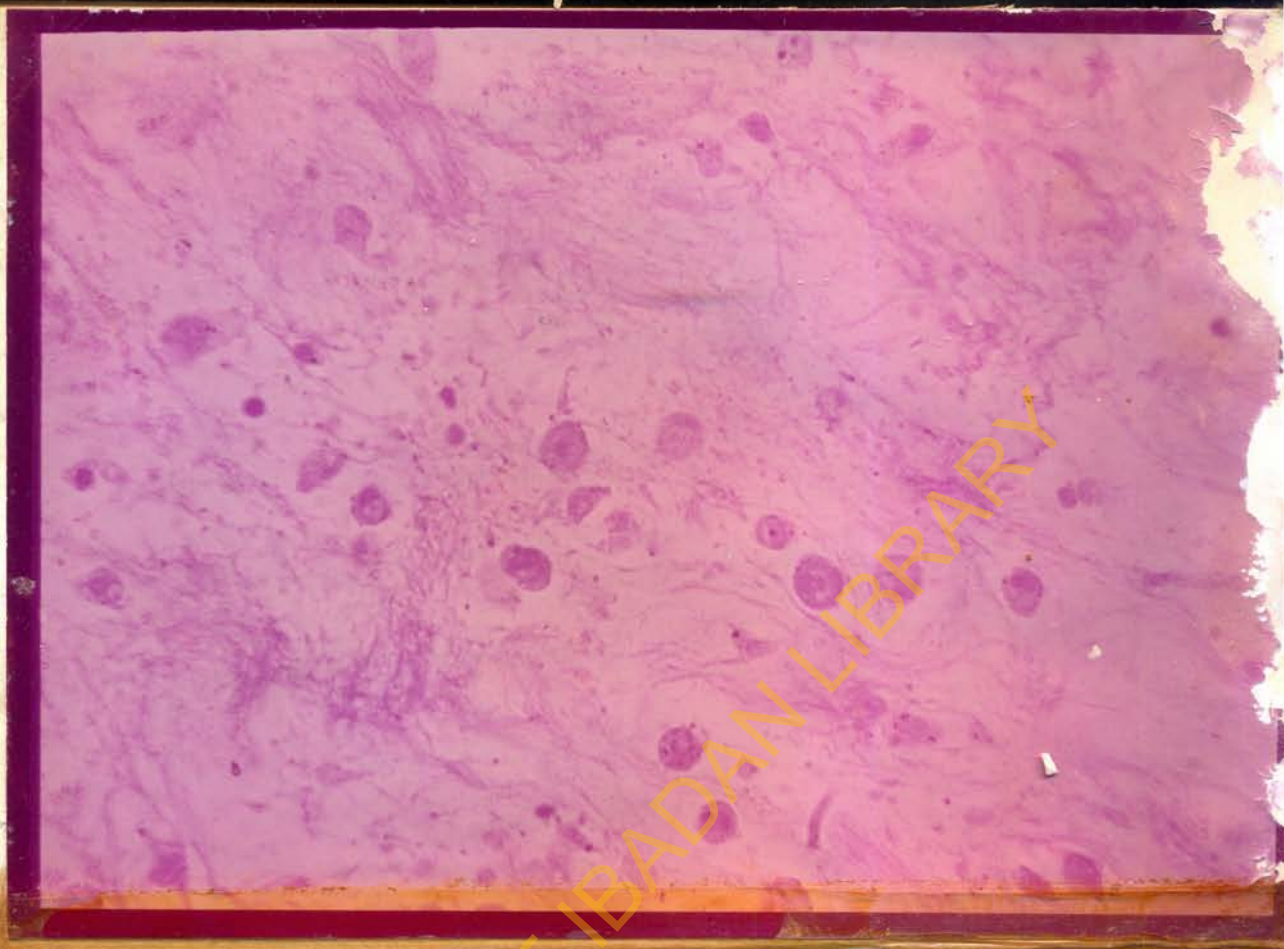


Fig. 5
C

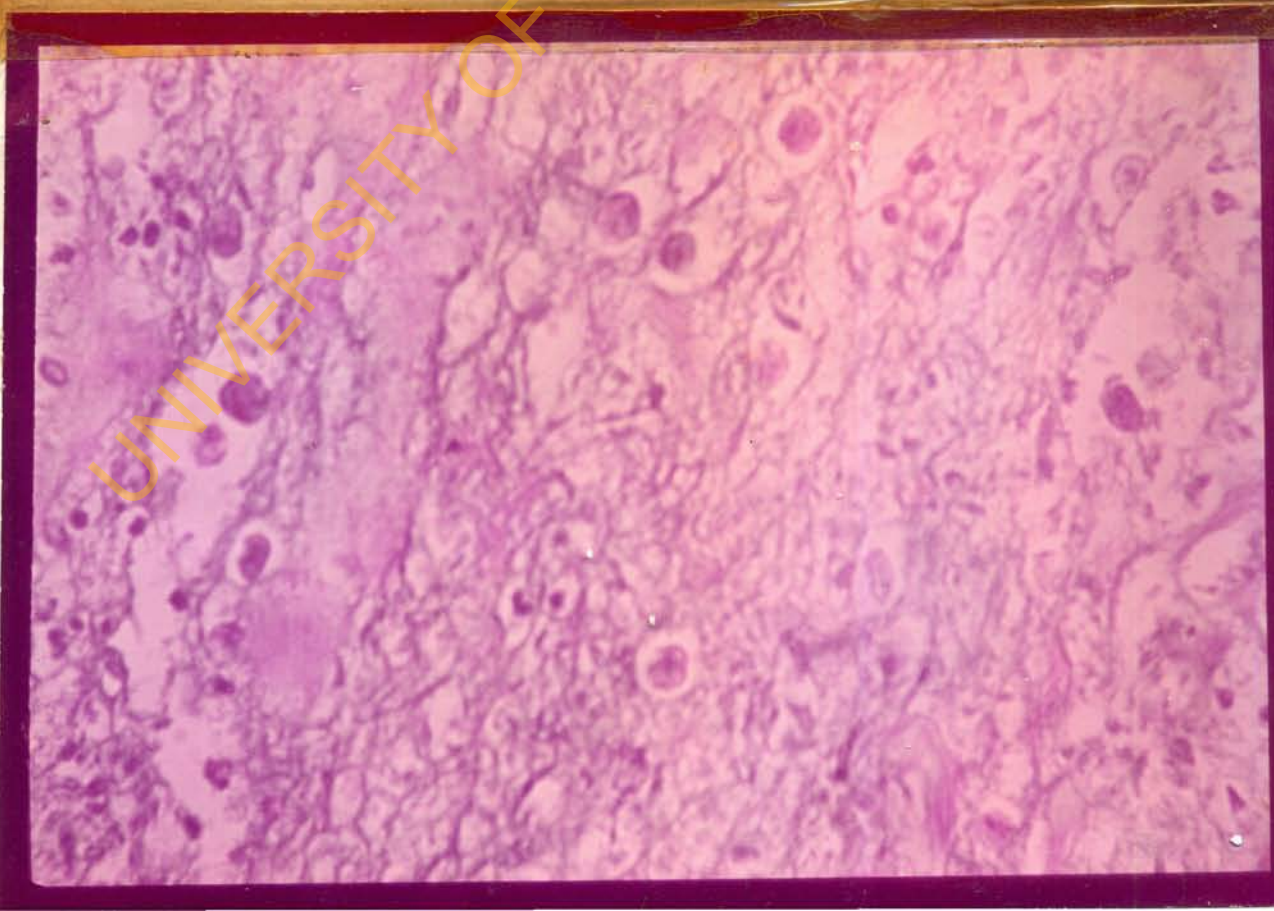


Fig. 5
D

(To Face Page 77)

Fig. 6 Histological Appearance Of An Intestinal Lesion simulating Non-Specific Ulcerative Colitis.

Fig. 6b Amoebae Invading The Submucosa And The Muscle Coat.

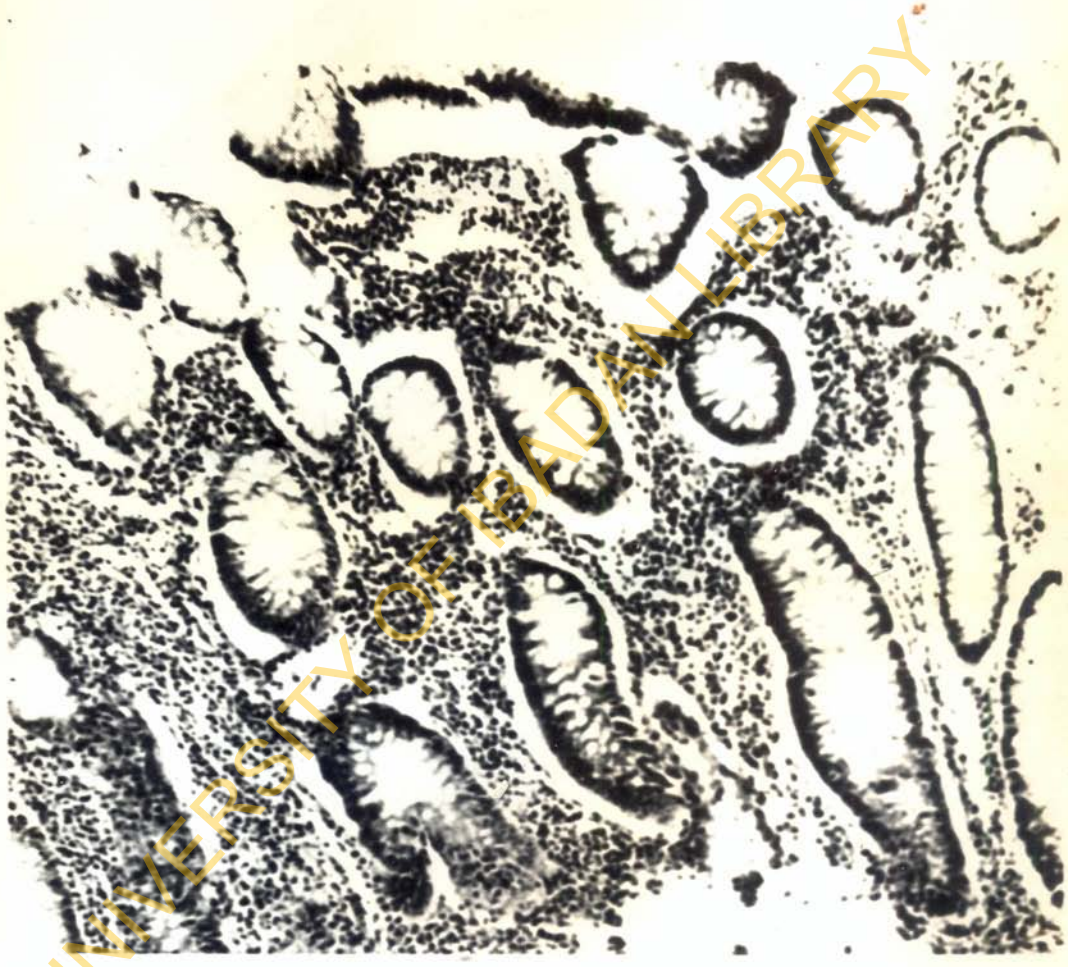


Fig. 6

Fig. 6b

TABLE 'AMOEBIASIS THROUGH AUTOPSIES'

COMPARATIVE AGE AND SEX DISTRIBUTION OF ACUTE DIARRHOEAL DISEASES - U. C. H. 1958-1967

	AMOEBIASIS							TYPHOID ENTERITIS							MISCELLANEOUS ENTRO-COLITIS						
AGE GROUP	BOTH SEXES	MALES	FEMALES	FP	FNP	PERCENTAGE M	PERCENTAGE F	BOTH SEXES	MALES	FEMALES	FP	FNP	PERCENTAGE M	PERCENTAGE F	BOTH SEXES	MALES	FEMALES	FP	FNP	PERCENTAGE M	PERCENTAGE F
0-28days	0	0	0	-	-	-	-	0	0	0	-	-	-	-	7	6	1	-	1	4.41	0.73
29dys-1yr	3	-	3	-	3	-	2.22	5	2	3	-	3	1.42	2.14	34	13	21	-	21	9.55	15.44
1 - 4 yrs	14	8	6	-	6	5.93	4.44	17	10	7	-	7	7.15	5	33	15	18	-	18	11.04	13.23
5 - 14	19	10	9	-	9	7.41	6.66	25	16	9	-	9	11.43	6.43	7	3	4	-	4	2.21	2.94
15 - 24	9	3	6	5	1	2.22	4.44	38	19	19	4	15	13.57	13.57	12	5	7	1	6	3.68	5.14
25 - 34	35	16	19	12	7	11.86	14.07	31	15	16	2	14	10.71	11.43	13	4	9	1	8	2.94	6.62
35 - 44	21	15	6	0	6	11.11	4.44	14	13	1	-	1	9.28	0.71	15	10	5	0	5	7.35	3.68
45 - 54	22	17	5	-	5	12.59	3.71	8	4	4	-	4	2.86	2.86	7	4	3	0	3	2.94	2.21
55 - 64	10	6	4	-	4	4.44	2.97	1	1	-	-	-	0.72	-	6	3	3	-	3	2.21	2.21
65 - 74	2	2	-	-	-	1.49	-	1	1	-	-	-	0.72	-	2	2	-	-	-	1.47	-
TOTALS	135	77	58	17	41	57.05	42.95	140	81	59	6	53	57.86	42.14	136	65	71	2	69	47.80	52.20
						100							100							100	

FP = FEMALE PREGNANT
FNP = FEMALE NON-PREGNANT

Table 4

78

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Fig. 8 Solitary Amoebic Liver Abscess

(Note the massive size of the abscess)

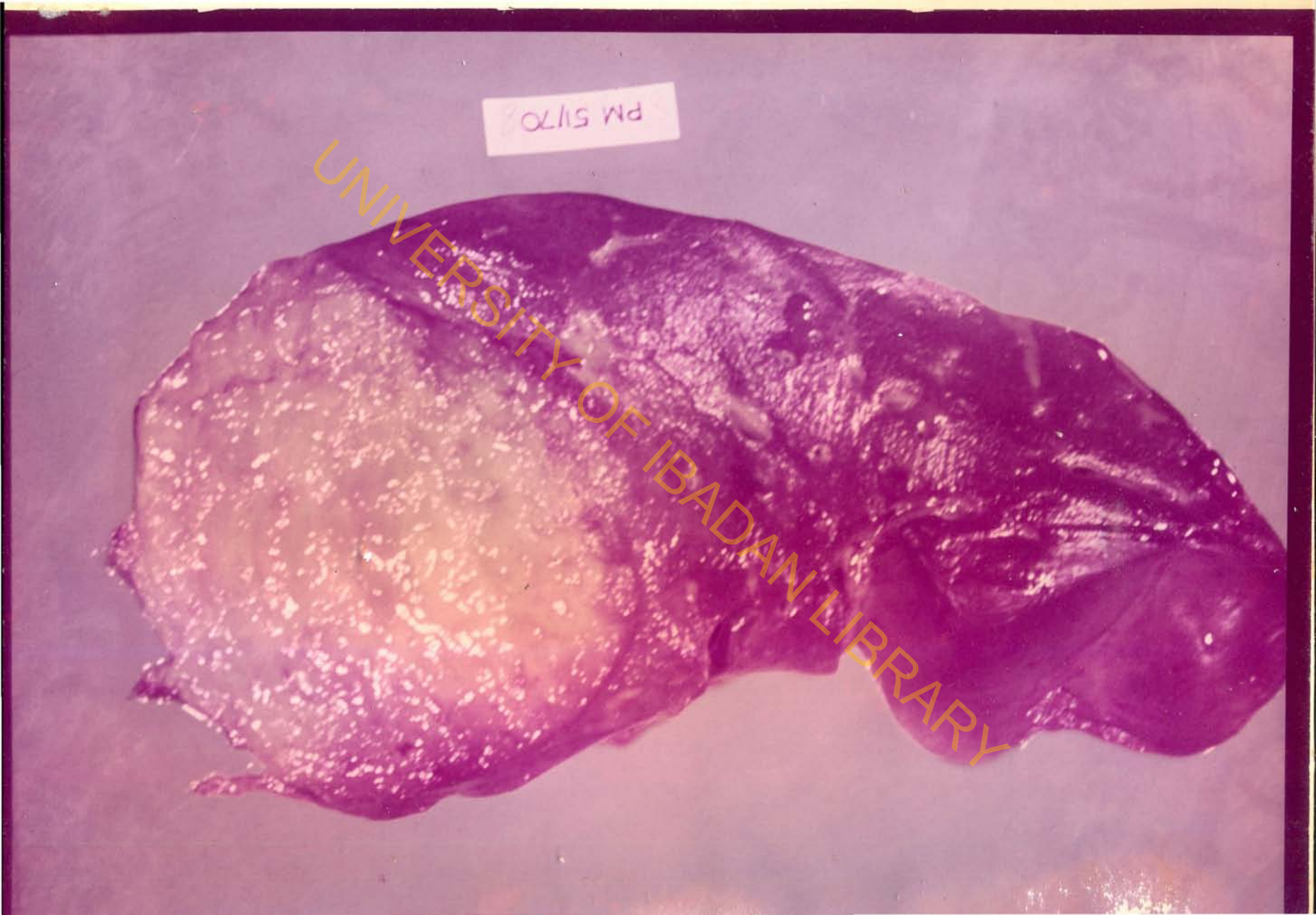

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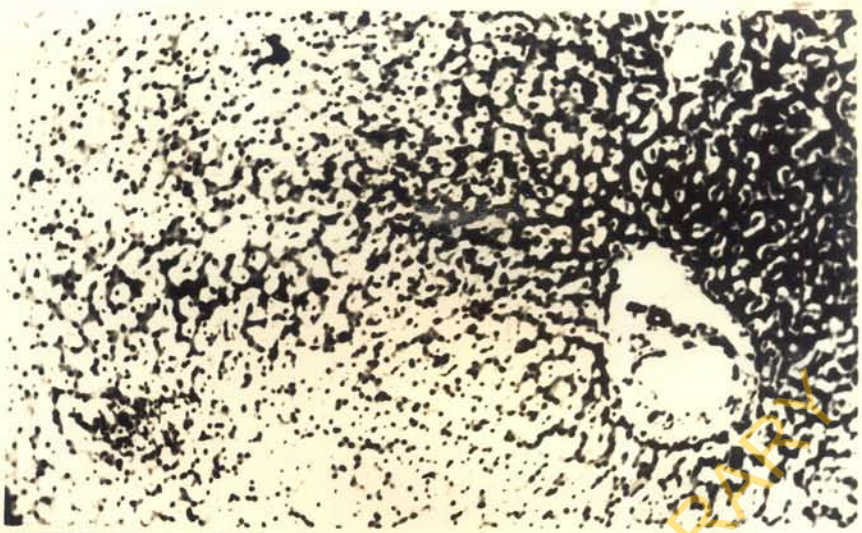
Fig. 9. Histological Appearance of Amoebic Liver abscess:

1 = 1st zone of relatively normal liver.

2 = 2nd zone showing early liver damage by E. h.

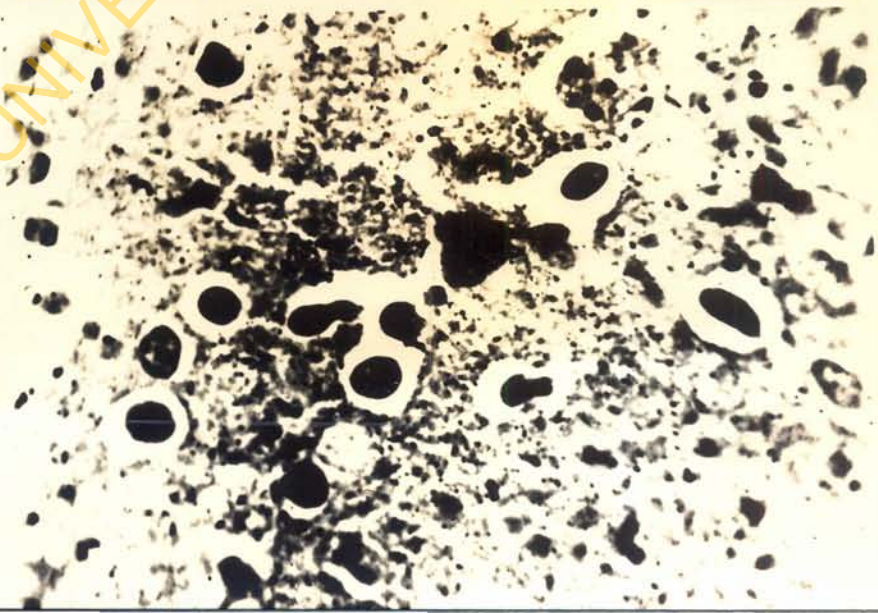
3 = 3rd zone showing amoebae in Necrotic Liver Tissue.

1



2

3



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1 & 2 Fig. 7. Amoebic Liver Abscess Walled Off By Thick Fibrous Tissue.

3. Fig. 12. Amoebic Liver Abscess in a 3-year old child with Genito-Urinary Complications.

4. Fig. 11. Amoebae In Tissue (Cutaneous Amoebiasis).

5. Fig. 10. Amoebic Brain Abscess Complicating Amoebic Liver Abscess.

1

PM 244/70

2.

Fig. 7

-3

Fig. 12

4.

Fig. 11

.5

PM 137/70
(Amoebic brain)
absceses

Fig.10

ACUTE DIARRHOEAL DISEASES AND DEATHS IN PREGNANCY / PUERPERIUM

AGE AND SEX DISTRIBUTION (U.C.H. IBADAN) 1958 – 1967

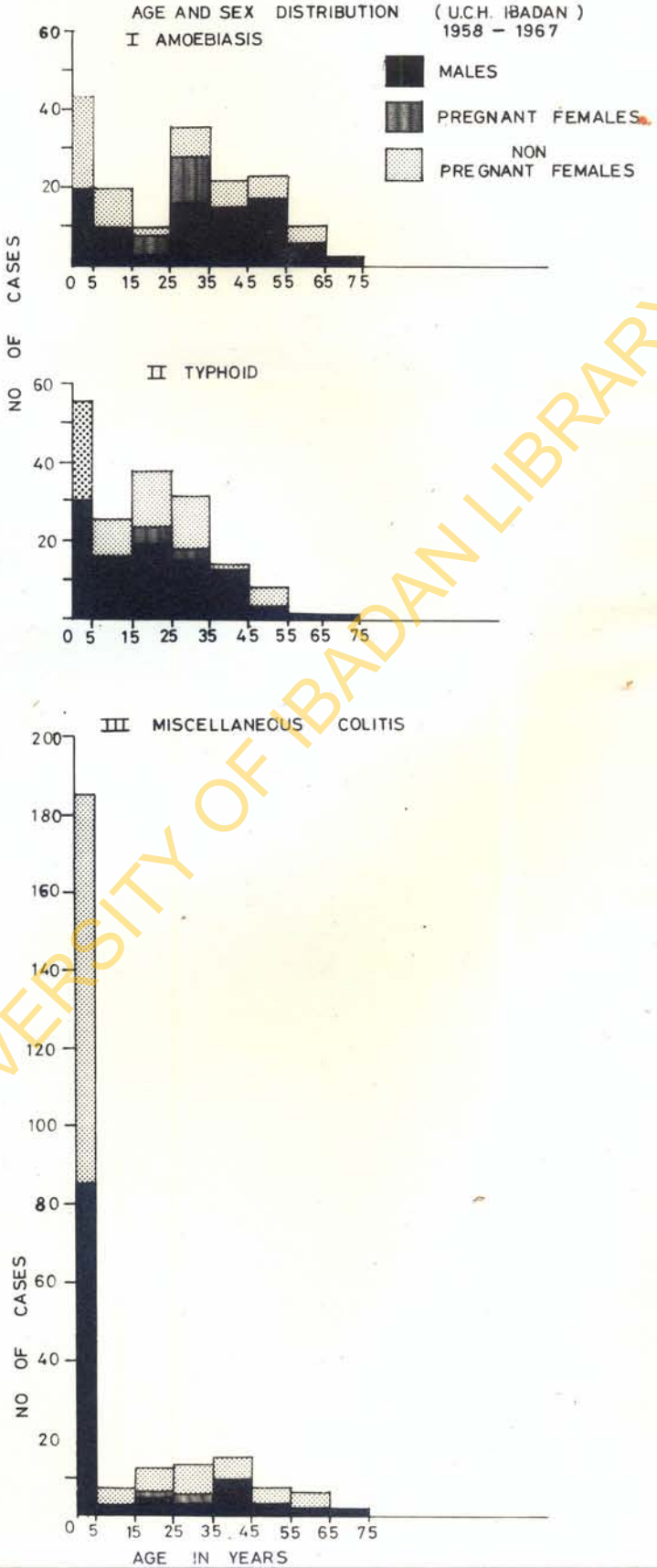

Fig. 13

FIG 14.

BIASIS THROUGH AUTOPSY (U.C.H. 1958-1967)
MALE / FEMALE RATIO

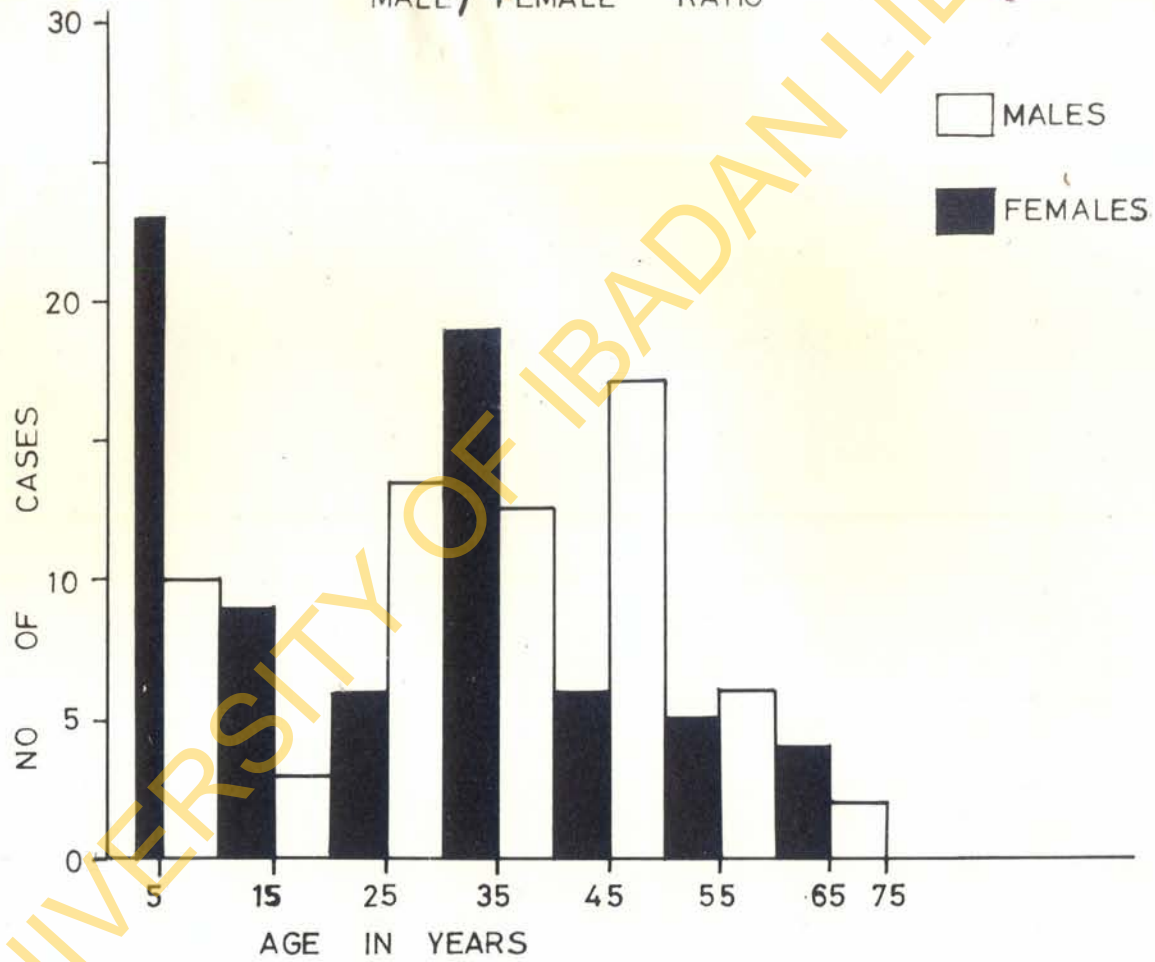

Fig. 14

CHAPTER IV

PREVALENCE AND PROBLEMS OF AMOEBIASIS IN NIGERIA

4.1 Introduction

Amoebiasis is commonly seen in clinical practice in Nigeria but the overall incidence is not yet known (Alele, 1966). There is sufficient evidence to show that amoebiasis is endemic in Nigeria (Falconer, 1958); Olatubosun, 1965; Essien, Ahimie and Laja, 1965; Nnochiri, 1965; Adi, 1966; Salako, 1967; Odunjo, 1969; and Antia and Lewis, 1969). In the Federal Territory of Lagos Okpala (1961) reported that 10.9% of Government workers harboured E. histolytica and Laukner and co-workers (1961) reported that amoebiasis accounted for 3.6% of medical admissions at the University College Hospital, Ibadan, during the calendar year January to December, 1958.

Other earlier investigations into the extent of the problem of amoebiasis in Nigeria, included the work on the incidence of some pathogens in the 'healthy' Lagos population. In that investigation it was shown that of 1816 persons examined 22 (or 1.2%) had cysts of E. histolytica in their stools (Mandal, 1964; quoted by Essien et al. 1965). Of 921 hospital autopsies performed in the Pathology Department, General Hospital, Lagos (1961-1964 March), amoebiasis was responsible for the deaths of 39 persons (4.1%) and of this number 20 (or 51.3%) were due to amoebic liver abscess. Also of 19719 stool specimens examined in the Pathology Department of the same hospital in 1961-1963, 1106 specimens (4.1%) were positive for E. histolytica (cysts and vegetative forms). Marsden (1960) using direct microscopy

found that the carrier rate of E. histolytica in a series of unselected patients in Lagos was 26.6 per cent.

At Ibadan, Cowper and Woodward (1960) found E. histolytica in twelve out of one hundred unselected employees at the Moore Plantation; Cobban (1959) working in the same city, recorded 319 cases of clinical dysentery in the out patient Department of the Teaching Hospital, in Ibadan, from April, 1957 to April, 1958. Of these 75 (9.5%) were diagnosed microscopically as amoebic. He also recorded that in a routine examination of the stools of 105 hospital servants and catering staff in 1959, E. histolytica cysts and trophozoites were found in 23 (21%) (Table 7).

From the foregoing, it appears that there is yet no definite information on the actual incidence of the disease in Nigeria. It would, however, be impossible, because of the many difficulties involved, as Maddison et al. (1965) noted, to establish an accurate assessment of the incidence of E. histolytica in populations in various parts of the world. Although it is difficult to reconcile the divergent observations made on the incidence of amoebiasis in Nigeria; differences in the sample of population studied, in the orientation of the investigators, and in the methods of study may have contributed to the discrepancies. As it would not be practicable to carry out a full random country-wide survey in the whole Nigerian population during the short period available for this study, attempts were made in this chapter to devise and apply various parameters, to the prevalence study of amoebic infection in a given sample of the Nigerian population.

In this study, attempts were made to apply at least three methods to the investigation of the prevalence of amoebiasis in Ibadan township. It was hoped that an evaluation of the parameters thus employed would provide a suitable basis for a large scale future survey of the incidence and nature of the transmission of the disease in any community.

The parameters applied are as follows:

(i) Retrospective study of post-mortem materials (Chapter 3).

(ii) Prospective coproparasitological School Survey.

(iii) Serological (immunological) Survey.

4.2 Coproparasitological School Survey

4.2.1 Materials and Methods:

STUDY POPULATION: One thousand two hundred and ninety one specimens of stools were obtained from School children, teachers, parents and food handlers drawn from fifteen Primary Schools in Ibadan township as follows:

(1) One thousand and eighty-six school children with ages ranging from five to eleven years. This group included eighty-seven children from the University Staff School, serving as control.

(2) Sixty-six adult female food handlers attending to the school children at the various schools.

(3) Ninety-one both male and female primary school teachers from the various schools examined.

(4) Forty-eight adult indigenous Nigerians, who are parents of some of the school children included in the study.

4.2.2 Collection of Stools

Going by the impression that infestation by E. histolytica is usually from drinking or eating food previously contaminated by the products of this organism, it was decided to first examine food-handlers in various schools in Ibadan. It was thought that the load of parasites in the stools of the food-handlers would give an indication as to the expected load of parasite carrier-rates among the school children served by these foodhandlers. For this reason the foodhandlers were first of all interviewed on matters relating to their general health including the history of both recent and previous acute diarrhoeal diseases. Having thus taken the history and explained the purpose of the study to these foodhandlers, stool (specimen) cartons were given to them. They were asked to transfer a small portion of their early morning stools into the carton with the warning that they should avoid the admixture of urine with the stool specimen. Early, on the following day of the distribution of cartons, the stool specimens were collected and quickly transported to the University College Hospital, Ibadan for parasitological examination. Where a foodhandler failed to defaecate early in the morning, she was advised to take simple measures such as taking copious cold water first thing in the morning.

The results of the parasitological examination of the foodhandlers' stool specimens served as a rough guide as to the selection of schools to be studied. Thus, by such preliminary study, it was possible to

include both groups of schools where the foodhandlers were cyst passers as well as, those in which the foodhandlers were non-cyst passers. It was surmized that a purely random school selection might fail to provide enough children in each type of the schools. For the same reason the parents examined included parents of children who carried E. histolytica in their stools and of those who were free from E.h. infestation at the material time (Table 8a).

Having thus decided on the schools to be examined, cartons were similarly distributed to ten pupils, comprising equal number of males and females from each class. Similar instruction as to the simple precaution, indicated above was given and the children asked to produce specimens of stool the following morning. Again these were collected first thing in the morning and quickly taken over to the hospital for necessary parasitological examination. Where a child failed to produce specimen, efforts were made to obtain one before the close of the day. In case where such efforts failed, the class teacher was asked to re-allocate the carton to another child who might be able to produce some specimen of stool without any difficulty.

On the basis of possible infestation by E. histolytica from ordinary contact resulting from closer association, the school teachers and the parents of some of the children examined were included in the study. For this purpose, the parents of the children whose stools were examined were next summoned to a meeting by the headmaster of the school concerned.

At such meetings, the purpose of the studies, was explained to the parents. They were then asked to produce and send specimens of stools through their respective children the following morning.

The specimens were treated as in the previous cases and examined for parasites especially E. histolytica. Finally specimens of stools were collected from the teachers in some of the schools involved and processed in the same manner.

4.2.3 Laboratory Examination of Stools:

In the laboratory the stools were examined both macroscopically and microscopically. Direct microscopy of faeces was made in saline, followed by concentration according to the technique of Ridley and Hawgood (1956), whence deposit was examined in a double strength Lugol's iodine. If cysts were found, the Lugol's iodine slide preparation was further examined, in a drop of Sargeaunt's (1962) solution to define chromatoids and nuclei. Observation and remarks were also made on the presence of other parasites in each specimen of stools. In some doubtful cases, the specimens of stools were cultured both for confirmation and also as part of attempts to establish long-term in-vitro cultures of E. histolytica as will be presently described.

The figures obtained from the coproparasitological school survey were classified into school children who were passing E.h. concurrently with their parents and to those who were passing E.h. but with parents who were non-passers. Similar analysis was made in respect of teachers/school

TABLE

CORPORO-PARASITOLOGIC SCHOOL SURVEY (AMOEBIASIS IN IBADAN)

SCHOOLS	GROUP OF INDIVIDUALS EXAMINED											
	SCHOOL CHILDREN			FOOD HANDLERS			TEACHERS			PARENTS		
	TOTAL	POSITIVE STOOLS	%	TOTAL	POSITIVE STOOLS	%	TOTAL	POSITIVE STOOLS	%	TOTAL	POSITIVE STOOLS	%
1. St. Peters, (Aremo)	172	33	19.19	9	1	11.11	-	-	-	18	7	38.89
2. St. Brigids, (Mokola)	56	12	21.23	3	2	66.66	-	-	-	-	-	-
3. Islamic School (Agugu)	34	12	29.41	4	1	25.00	-	-	-	-	-	-
4. Wesley Prac. School (Elekuro)	94	10	10.64	8	5	62.50	16	4	25.00	-	-	-
5. I. C. C. School (Eleta) 1	105	14	13.33	3	1	33.33	16	2	12.50	3		-
6. " (") 2	66	9	13.64	3	1	33.33						
6. St. Theresa's	106	21	19.81	3	-	-	12	5	41.25	27	3	11.11
7. I. C. C. School (Oje)	69	15	21.74	3	1	33.33	16	5	31.25	-	-	-
8. I. C. C. School (Adeoyo)	71	17	23.94	5	1	25.00	9	1	11.11	-	-	-
9. St. Stephen's (Inalende)	226	67	29.65	6	-	-	22	3	13.64	-	-	-
10. Sacred Heart (Inalende)	-	-	-	3	-	-	-	-	-	-	-	-
11. Christ's Apostolic School (Eleta)	-	-	-	7	-	-	-	-	-	-	-	-
12. St. John's (Aremo)	-	-	-	4	-	25	-	-	-	-	-	-
13. Christ The King	-	-	-	1	-	-	-	-	-	-	-	-
14. Methodist School (Bodija)	-	-	-	2	1	-	-	-	-	-	-	-
15. St. John's (Eleta)	-	-	-	2	-	-	-	-	-	-	-	-
16. Staff School (U. I.)	87	1	1.15	-	-	-	-	-	-	-	-	-
	1086	209	19.24	68	15	21.21	91	20	21.98	48	10	20.84

90A

Table 12

children; and foodhandlers/school children. The resulting figures were subjected separately to statistical analysis, with a view to confirming or otherwise, the sources of infestation for the school children (Table 9).

4.2.4 Results

Tables 8 - 10 show the results of the parasitological examinations carried out on the specimens obtained during the school survey. From these tables it would be seen that a total number of one thousand, two hundred and ninety-one (1291) specimens of stools were examined microscopically for the presence of parasites in general and E. histolytica in particular. Analysis of the various figures shows that the carrier rate for E.h. among the school children excluding those of the University Staff School, ranges between 10.64% and 35.29% of the total number examined in each school. The overall percentage 'carrier' rate among the school children examined in Ibadan township was found to be 20.82% (excluding the Staff school figure); and 19.24% when the latter figure was included. Similarly, 20 (21.98%) of 91 teachers, 14 (21.21%) of 66 foodhandlers, and 10(20.84%) of 48 parents were excreting E.h. in their stools at the time of the examination. In all, 253 (19.60%) of specimens examined contain E. histolytica, cysts or trophozoites.

It is interesting to note that in two of the schools included in the study, none of the nine foodhandlers examined showed E.h. in the stool.

On the other hand 33 of the 332 school children examined in these two schools were excreting E. h. at the time of the examination. Tables (3a, b, c and d) show that the results of the stool examination of the parents, foodhandlers and teachers show no significant correlation between the carrier state in the school children.

Table 10 showing the comparative parasite carrier rates in Ibadan gives an indication that E. histolytica tops the list of 'potential' pathogens associated with frank symptomatic disease state in the local population. This is so because the parasite (ascaris) which in actual fact tops the list is not usually associated with any symptom, except occasionally in very young children.

4. 3. Serological (Immunological) Survey:

4. 3. 1 Materials And Methods:

The material used in this aspect of the study included amoebic antigens prepared from the local strains of E. histolytica, and sera from human sources. The sera used in the study were obtained from a total number of 66 foodhandlers; 48 parents and 62 teachers. The stools of these individuals were examined parasitologically for carrier states. More specimens were obtained from other 12 parents, 29 teachers, and 89 Blood Donors attending the Blood Bank (UCH, Ibadan). The stools of these latter groups were not examined microscopically for parasites. In all a total number of 306 specimens were studied in this section.

The full description of the method of preparation of amoebic antigens used will be found in chapter seven.

Examination of sera for precipitins to antigens of E. h. was performed by micro-agar gel diffusion according to the Auernheimer and Atchley (1962) modification of Ouchterlony method. In each trial, 5ul of amoebic antigen was placed in a central well and 40ul of one of sera from six different individuals, was separately pipetted into one of six peripheral wells. Additional slides were prepared similarly using as antigens, the extracts from the bacterial associates (Esch. coli strain K12) and horse serum; since these latter were incorporated into the original culture medium for E. histolytica. All tests were incubated in humid chamber at room temperature (20° - 25°C) and examined daily for about 43 hours before transferring into 4°C for another 48 hours with frequent examination for precipitin lines.

4.3.2 Results:

Twelve (12) of the sixty-six foodhandlers drawn from the schools where examination of stool specimens was made carried antibodies to E. h. in their sera at the time of the examination. Similarly, 16 (13.57%) of the 91 primary school teachers examined; and 7 (11.67%) of the 60 parents carried antibodies to E. h. in their sera. 10 (11.24%) of the 89 Blood Donors examined gave positive gel diffusion precipitin reaction for amoebic infection. Stools of this latter group were not examined for the presence or absence of E. h.

However, only five of the 12 foodhandlers with amoebic antibodies in their sera concurrently carried E. h. in their stools. Similarly, nine of the sixteen teachers and six of the ten parents, with antibodies in their sera had E. h. in their stools at the time of examination. This series of observations give an indication that the serological technique employed in this study might be detecting antibodies to past tissue invasion by E. histolytica.

4.4. COMMENTS

4.4.1 Importance of the Application of Various Parameters to Prevalence Studies:

The results of the three methods of investigation, namely, autopsy, coproparasitological and serological, varied widely. Thus 2.19% of all autopsies studied was due to amoebiasis; 253 (19.60%) of 1291 stools examined had E. h. whilst only 45 (14.71%) of 306 sera examined gave positive gel diffusion reaction with E. h. antigens (Table 12). Many factors may be responsible for this wide variation. For instance, as shown in Table 6, showing the seasonal and annual distribution of deaths due to amoebiasis over a ten-year period in this series, it may be suggested that the term epidemic be changed to endemic when applied to the prevalence of amoebiasis. This is because the disease seems to be present in the population in which it is established, all the time and does not appear to occur in strictly epidemic form. Its occurrence appears to fluctuate from

month to month and for this reason the timing of the present survey may have some influence on the results.

The wide variation between the results of these three methods of study may also be accounted for by the fact that the parameters were measuring different categories or states of the disease. Thus whilst autopsy study suggests the mortality caused by the disease, examination of stools give essentially the carrier rate and perhaps, including few cases of recent symptomatic amoebiasis. Gel diffusion test was actually measuring occurrence of tissue invasion occurring at a reasonable period preceding the examination. Finally, the universal controversial nature and weakness of amoebic antigens, as well as, the sensitivity/limitations of the serological test employed will have to be taken into consideration in interpreting the results of the serological aspects of the study.

It is also interesting to note that of the sixty-six adults examined during the school survey, none of the total number of ten pregnant women at various stages of pregnancy encountered during the survey had E. h. in the stool. Although the number of pregnant women encountered in this survey appears too small for meaningful analysis, it is considered that this relative absence of cyst passers may be of some importance. This is in the sense that it may be possible that cases occurring during pregnancy may result from fresh contacts. It may, in fact, be possible in future studies, to postulate that contacts which may have otherwise resulted in

carrier states in a non-pregnant woman may indeed manifest as a full-blown symptomatic amoebiasis when they occur in pregnancy. Such hypothesis would then be based or used as an explanation for the undue severity and preponderance of deaths due to the disease in pregnancy and allied states as shown in the preceding chapter.

However, it is obvious that a more conclusive evidence may be forthcoming from a separate and more detailed comparative study of E. h. passers among the non-pregnant females of child-bearing age on the one hand, and the pregnant women in various stages of pregnancy on the other hand.

The dictum "association is not cause" is true but cause is often found through examination of association. In accordance with this rule, therefore, rather than jumping to any conclusion on the basis of the present studies alone, a more detailed examination of the problem of the role of amoebiasis in pregnancy is envisaged in the following chapters.

4.4.2 Possible Source of Infestation by E. h. (Or Carrier State):

Statistical analysis of the results of the carrier states of E. h. in the school children shows no significant correlation with similar states in their corresponding parents, school teachers or school foodhandlers (Tables 9a b and c). This is in apparent contrast to the findings of Nnochiri (1965) where 96 (96%) of 102 parents of children suffering from amoebiasis, were found to be chronic E. h. passers themselves. It is perhaps pertinent

to note that in the series of Nnochiri, under review, only parents of the sick children were examined. The present study, however, included the parents of both groups of children who were carriers, as well as, those of the non-E.h. passers. Furthermore, in the series of Nnochiri, only 21 of the 233 children with positive stools for E.h. were in the age-group (5-10) years. Since the bulk of the primary school children involved in the present survey falls under the latter age group, the chances are that statistical analysis of Nnochiri's (1965) series under this age group may give similar results as those of the present study.

In the light of the results in this part of the studies, one may be tempted to postulate that whilst parents may be the possible sources of infestation by amoebic products under the age group (1-4), as Nnochiri's work tends to suggest, it would appear that other sources, possibly including parents, may account for the similar infestations in older children. The most likely main, or additional sources being playmates, flies and other known agents with which these 'older' children are likely to encounter in their movements from place to place. The chances of food-handlers being a major source of infestation are great since a greater part of the populace depend mainly on these hawkers for their meals.

It should be noted, however, that this suggestion has in no way over-looked the importance of bad sanitation in the epidemiology of amoebiasis. Indeed the finding of only one cyst passer out of 87 children

of the University Staff School highlights the importance of high standard of hygiene in the prevention of the disease.

Finally, the results show that while it is possible to apply different methods concurrently to establish an accurate assessment of the prevalence of amoebiasis in a given community, the parameters may be measuring different but important aspects of the disease. Thus in the present series autopsy study measured level of mortality; coproparasitological methods measures cysts/trophozoites carrier rates while amoebic gel diffusion test probably measured the level of tissue invasion by E. histolytica.

In the light of these results, therefore, it is not an objective in this study to extrapolate findings beyond the material that has been examined but rather to enable the author to examine in greater detail the problems that arise from the results.

TABLE 7

REVIEW OF PUBLISHED "AMOEBIASIS" SURVEY IN NIGERIA

AUTHOR	YEAR of survey	LOCALITY of survey	MATERIAL/ METHOD	TOTAL Analysed	PROVEN CASES	% OCCURRENCE OR REMARKS
JELLIFE	1951	U.C.H., Ibadan	Clinical	1	1	Case Report
FALCONER	1958	G.H., Enugu	Autopsy	1	1	Case Report
MANDAL	1964	Lagos	Coprologic	1816	22	1.2
ADI	1965	U.C.H., Ib.	Clinical review (HEPATIC)	48780	120	0.25
OLATUBOSUN	1965	L.U.T.H., Lagos	Autopsy (children)	250	14	5.6
ESSIEN (a)	1961-64	G.H., Lagos	Autopsy	921	39	4.1
(b)	1961-63	G.H., Lagos	Coprological	19719	1106	4.1
ALELE	1965	M.S., Lagos	Clinical	1400	56	4
MARSDEN	1960	G.H., Lagos	Coprological	500	133	26.6
OKPALA	1961	Lagos	Cop. (workers)	515	56	10.9
NNOCHIRI	1965	Yaba	Clin. & Coprol.	102*	96	96*
COBBAN (a)	1959	U.C.H., Ib.	Clin. & Coprol.	819	75	9.5
(b)	1959	U.C.H., Ib.	(Hosp. staff) Coprol.	105	23	21.80
COWPER & WOOD WOODWARD	1960	Moore Plantation, Ibadan	Coprologic	100	12	12.00
COWPER & WOODWARD	1961	U.C.H., Ib.	Coprologic	21701	898	4.2
ODUNJO	1969	L.U.T.H. Lag.	Autopsy	2743	135	4.9
LEWIS & ANTIA	1969	U.C.H., Ib.	Clin. Review			

* SYMPTOMLESS CYST PASSERS.

TABLE 8a

SCHOOL SURVEY BY STOOL EXAMINATION (AMOEBIASIS IN IBADAN) PUPILS/MOTHERS.

Serial No.	School Pupil	Results	Serial No.	Mother's results	Serial No.	School Pupil	Results	Serial No.	Mother's results
1	F. Obasanya	-Ve	1	-Ve	31	O. Oni	+Ve	31	+Ve
2	R. Iyana	-Ve	2	-Ve	32	A. Idowu	-Ve	32	-Ve
3	J. Kadiri	-Ve	3	+Ve	33	F. Omoniyi	-Ve	33	+Ve
4	I. Bello	+Ve	4	-Ve	34	A. Akintola	+Ve	34	-Ve
5	M. Mowah	-Ve	5	-Ve	35	M. Gbadamosi	-Ve	35	-Ve
6	I. Olubade	+Ve	6	-Ve	36	B. Akintomide	+Ve	36	-Ve
7	A. Aniyo	-Ve	7	-Ve	37	A. Akinsola	-Ve	37	+Ve
8	O. Johnson	+Ve	8	-Ve	38	O. Julius	-Ve	38	+Ve
9	T. Oyelade	+Ve	9	+Ve	39	M. Adeniyi	-Ve	39	+Ve
10	M. Fatoba	+Ve	10	-Ve	40	M. Adeagbo	-Ve	40	-Ve
11	A. Eyo	-Ve	11	-Ve	41	O. Laniyan	-Ve	41	-Ve
12	F. Fatiroti	-Ve	12	-Ve	42	Y. N. Williams	-Ve	42	-Ve
13	A. Nwobi	-Ve	13	-Ve	43	I. Asiru	+Ve	43	-Ve
14	T. Adefarahan	-Ve	14	-Ve	44	R. Alimi	+Ve	44	-Ve
15	M. Dare	-Ve	15	-Ve	45	S. Jimo	+Ve	45	-Ve
16	A. Adewale	-Ve	16	-Ve	46	O. Oladejo	+Ve	46	-Ve
17	S. Salawu	+Ve	17	-Ve					
18	M. Dina	-Ve	18	+Ve		TEST			
19	T. Ayodele	+Ve	19	-Ve					
20	A. Lawal	+Ve	20	-Ve					
21	J. Aruna	-Ve	21	-Ve					
22	R. Dadi	-Ve	22	-Ve					
23	O. Diyaolu	-Ve	23	-Ve					
24	O. Akinpelu	+Ve	24	+Ve					
25	N. Oyedeji	-Ve	25	+Ve					
26	O. Adeyonu	-Ve	26	-Ve					
27	A. Agboluaje	+Ve	27	}					
28	F. Agboluaje	-Ve	28	} +Ve					
29	M. Agboluaje	-Ve	29	}					
30	G. Akintola	-Ve	30	-Ve					

TABLE 8b: SCHOOL SURVEY BY STOOL EXAMINATION TEACHERS/PUPILS

Serial No.	Class Teacher	Results S. E.	PUPILS No. examined	PUPILS No. positive
1	T IIA	+Ve	10	2
2	IIIA	-Ve	10	1
3	IIIB	-Ve	10	3
4	IVA	+Ve	8	0
5	IVB	-Ve	10	3
6	VA	+Ve	8	2
7	VB	-Ve	10	3
8	VIA	-Ve	10	0
9	VIB	-Ve	10	3
10	IA	-Ve	10	1
11	IB	+Ve	10	1
12	IC	+Ve	10	2
13	ID	-Ve	10	5
14	IIA	-Ve	10	3
15	IIB	-Ve	10	1
16	IIC	-Ve	10	5
17	IID	-Ve	9	3
18	IIIA	-Ve	10	3
19	IIIB	-Ve	8	3
20	IVB	-Ve	9	5
21	IVC	-Ve	9	3
22	IVD	-Ve	10	2
23	VA	-Ve	10	4
24	VD	-Ve	10	2
25	VIA	+Ve	8	1
26	VIC	-Ve	10	2
27	VID	-Ve	9	2
28	IA	+Ve	9	1
29	IB	+Ve	9	0
30	IIA	-Ve	10	2

St. Theresa's

St. Stephen's

Elekuro

Serial No.	Class Teacher	Results (S. E.)	PUPILS No. examined	PUPILS No. positive
31	T IIIA	-Ve	10	1
32	IIIB	-Ve	8	0
33	IVB	-Ve	8	2
34	VA	-Ve	10	1
35	VB	-Ve	9	1
36	VIA	-Ve	8	0
37	VIB	+Ve	5	0
38	IA	-Ve	10	5
39	IB	-Ve	9	2
40	IC	+Ve	10	2
41	II	-Ve	10	0
42	III	-Ve	8	2
43	IV	-Ve	8	2
44	V	-Ve	9	1
45	VI	-Ve	9	2
46	IA	-Ve	10	1
47	II	-Ve	10	0
48	IIIA	+Ve	10	2
49	IIIB	-Ve	10	1
50	IB	-Ve	10	1
51	IV	-Ve	10	2
52	V	-Ve	10	0
53	VIA	-Ve	10	3
54	VIB	-Ve	10	1
55				
56				
57				
58				
59				
60				

T Wesley

I. C. C. School, Adeoyo

I. C. C. School, Eleta

TABLE 8c

SCHOOL SURVEY (STOOL EXAMINATION)
(AMOEBIASIS IN IBADAN)
FOODHANDLERS/PUPILS

	SCHOOLS	PUPILS		FOODHANDLERS	
		No. examined	No. positive	No. examined	No. positive
1.	St. Peters (Aremo)	172	33	9	1
2.	St. Brigid's (Mokola)	56	12	3	2
3.	Islamic Sch. (Agugu)	34	12	4	1
4.	Wesley Prac. School (Elekuro)	94	10	8	5
5.	I. C. C. Sch. (Eleta) 1	105	14	3	1
6.	" " " 2	66	9	3	1
7.	St. Theresa's	106	21	3	0
8.	I. C. C. Sch. (Oje)	69	15	3	1
9.	I. C. C. Sch. (Adeoyo)	71	17	5	1
10.	St. Stephen's	226	67	6	0

AMOEBIASIS THROUGH AUTOPSIES

ANNUAL AND SEASONAL DISTRIBUTION OF DEATHS (U. C. H. 1958 - 1967)

YEAR	JANUARY	FEBRUARY	MARCH	APRIL	MAY	JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER	NOVEMBER	DECEMBER	(AMOEBIASIS) TOTAL	ANNUAL DISTRIBUTION OF ALL AUTOPSIES		
														TOTAL	NEONATAL	CORRECTED FIGURES
1958	3	3	-	-	2	3	-	1	2	2	2	-	18	554	159	395
1959	-	-	1	-	1	-	-	2	-	1	-	-	5	732	165	567
1960	1	1	1	2	1	3	1	1	1	2	1	2	17	857	167	690
1961	2	1	-	1	2	1	1	1	2	1	2	1	15	1021	280	741
1962	2	4	1	4	-	1	3	1	2	3	-	1	22	1044	285	759
1963	-	1	1	-	-	1	-	1	1	2	-	1	8	872	270	602
1964	2	2	3	-	1	-	1	1	1	1	1	-	13	843	216	627
1965	3	-	1	2	4	3	-	-	-	2	-	1	16	779	132	647
1966	1	-	1	2	-	3	-	-	-	1	-	1	9	647	23	624
1967	-	-	-	1	2	1	1	-	3	1	1	2	12	573	46	527
(SEASONAL) TOTAL	14	12	9	12	13	16	7	8	12	16	7	9	135			

TABLE 6

TABLE 8d

	TOTAL NO. EXAMINED	TOTAL NO. POSITIVE	%
SCHOOL CHILDREN	1086	209	19.24
TEACHERS	91	20	21.98
FOOD HANDLERS	66	14	21.21
PARENTS	48	10	20.84
	1291	253	19.60

TABLE

COMPARATIVE PARASITE "CARRIER" RATES IN IBADAN (AMOEBIASIS SCHOOL SURVEY 1968/69)

SCHOOLS	PARASITES "LOAD"											Total No. Examined
	E. Histolytica	E. Coli (Cyst)	E. Nana	E. butschii	Ascaris	Trichuris	Hookworm	Strongyloides	Trichomonas	S. haematobium	Girdia-lamblia	
St. Peters (Aremo)	41	34	6	-	169	98	68	12	11	-	3	199
St. Brigids (Mokola)	14	11	3	-	39	13	4	1	-	-	1	59
Islamic School (Agugu)	11	9	1	-	32	17	14	1	1	-	-	38
Wesley (Prac) (Elekuro)	19	27	3	-	68	21	9	2	2	-	2	118
I. C. C. Eleta (i)	18	22	3	1	83	42	42	4	10	-	-	127
" " (ii)	9	7	1	1	54	29	30	3	7	-	-	69
St. Stephens (Inalende)	70	50	7	-	150	56	10	9	5	-	2	254
St. Theresa's	29	12	2	-	73	24	6	2	9	-	1	148
I. C. C. (Oje)	21	15	2	-	78	33	10	5	3	1	-	88
I. C. C. (Adeoyo)	19	17	3	-	68	31	11	5	5	-	1	85
Staff School (UI)	1	2	-	-	8	1	-	-	-	-	-	87
Sacred Heart	-	2	-	-	1	1	2	-	-	-	-	3 F. H only)
Christ Apostolic (Eleta)	-	-	-	-	5	2	-	-	-	-	-	7 "
St. John's (Aremo)	-	1	-	-	3	2	-	-	-	-	-	4 "
Christ The King	-	-	-	-	1	-	1	-	-	-	-	1 "
Methodist (Bodija)	1	1	-	-	1	-	1	-	-	-	-	2 "
St. John's (Eleta)	-	1	-	-	2	1	1	-	-	-	-	2 "
TOTAL	253	211	31	1	832	371	209	44	53	1	10	1291

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TABLE 9: COPROLOGICAL SCHOOL SURVEY CARRIER STATES - TESTS OF SIGNIFICANT ASSOCIATIONS.

1. Between parents and pupils.

		PARENTS		
		+	-	TOTAL
PUPILS	-	9	20	29
	+	4	13	17
	TOTAL	13	33	46

χ^2_c (McNemar's test) = 0.41

$0.75 > P > 0.5$

No Significant association.

2. Between Teachers and Pupils.

	Teachers + ve	Teachers - ve
pupil + ve	9	60
pupil - ve	63	232
Total Examined	72	292
% Positive	12.56	20.60

χ^2_c = 1.94 $0.25 > P > 0.1$

No Significant association

3. Between Foodhandlers and Pupils

School No.	1	2	3	4	5	6	7	8	9	10
% +ve among Foodhandlers	11.11	66.7	25.0	62.50	33.33	33.33	0.0	33.33	20.0	0.0
% +ve among Pupils	19.20	21.4	35.30	10.64	13.33	13.64	19.81	21.74	23.9	29.6

Spearman Co-efficient of rank correlation

$r_s = -0.40$ $P > 0.05$

Thus there is no Significant association.

TABLE 11

SEROLOGICAL (GEL DIFFUSION PRECIPITIN TEST) SURVEY
(AMOEBIASIS IN IBADAN 1968/69)

SOURCE OF MATERIAL	TOTAL NO. EXAMINED	TOTAL NO. POSITIVE	% POSITIVE
I. Primary Schools			
(i) Foodhandlers	66	12	18.17
(ii) Teachers	91	16	13.57
(iii) Parents	60	7	11.67
II. UCH BLOOD BANK			
BLOOD DONORS	89	10	11.24
TOTAL	306	45	14.71

CHAPTER V

ESTABLISHMENT AND PROPAGATION OF LOCAL (IBADAN) STRAINS OF ENTAMOEBA HISTOLYTICA IN IN-VITRO CULTURE

5.1 Introduction

The findings in the preceding chapters suggest that at least in this area the severity of amoebiasis in pregnancy and the puerperium is not fortuitous but that pregnancy predisposes to severe and usually fatal amoebiasis. With this type of preliminary results, the obvious question then would be "By what mechanism(s) are pregnant women and those in the puerperium in this area predisposed to fatal amoebiasis?" It is known that pregnancy modifies the course of various infections (French, 1903; Sprunt, McDearman and Raper, 1933; and Lucas, 1964) but the explanation for this is not known. The most attractive explanation that could be suggested for this selective severity of amoebiasis in pregnancy is the possible lowering of natural resistance to infections which occur in pregnancy (Homer & McNall, 1961).

It was, therefore, considered that an immunological study will probably clarify the nature of the mechanism or factor concerned. However, any immunological concept(s) in a disease entity must involve the question of humoral, as well as, cellular mechanisms. For obvious reasons, the present study is designed so as to include some investigations of the humoral factor in amoebiasis. The study of this aspect undoubtedly demands the use of a reasonably pure antigen. Unfortunately amoebic

antigen is neither easily procured nor available commercially.

The author was, therefore, faced with the problem of obtaining antigen(s). A review of the literature revealed that no established strain of Entamoeba histolytica was available either in Nigeria or anywhere in West Africa up till the time of the present study. Ignoring the obvious flaws in applying "foreign" strain(s) to the study of a disease caused by the local strain(s) of Entamoeba histolytica attempts were made to procure in-vitro cultures from overseas. Unfortunately, these samples of cultures did not survive transportation. It became necessary, therefore, to establish in-vitro cultivation of the local strain(s) of Entamoeba histolytica suitable for the production of antigens. This and related in-vitro experiments on the cultural characteristics of the local strain(s) of Entamoeba histolytica form the basis of this chapter.

5.2 MATERIALS and METHODS

5.2.1 Preliminary Studies

Prior to the establishment of the in-vitro cultivation of the local strain(s) of Entamoeba histolytica some of the available culture media were assessed experimentally for suitability. The suitability of body fluids other than stool for the isolation of Entamoeba histolytica was also explored.

Amongst the culture techniques and body "media" assessed were:

5.2.2 Culture Media

(i) Boeck and Drbohlav's Lock-egg-serum (L. E. S.)

(ii) Dobell and Laidlaw's No. 1 medium

(iii) Dobell and Laidlaw's No. 2 medium

(iv) Cleveland and Collier's medium

(v) Robinson's medium

(vi) Synthetic medium for the associated organism (Esch. coli)

See (Appendix 3) for detailed descriptions.

5.2.3 Body Fluids

(i) Aspirates from cases of liver abscess

(ii) Ascitic fluid sample mainly from cases of amoebiasis complicated by ascites.

(iii) Pericardial and pleural effusion.

From these preliminary studies, were evolved suitable culture media for the isolation of Entamoeba histolytica from stools and occasionally aspirates from liver abscess as described below.

5.3 RESULTS and OBSERVATIONS

5.3.1 Culture Media

There was great difficulty in obtaining sufficient number of chicken eggs for the preparatinn of media in which eggs were the sole constituents.

Furthermore, the chances of contaminating the egg-media during processing and subsequent sterilization were very great. Finally in view of the stickiness of the egg-media within a day of incubation of the in-vitro organisms in egg-media, it was surmized that counting on haemocytometer chambers would almost be impossible.

For the foregoing reasons, attention was diverted to media which were likely to surmount the difficulties imposed by the egg-media.

5.3.2 Body Fluids

Of all the body fluids explored for the possibility of isolation of Entamoeba histolytica only stool and very occasionally contents of amoebic liver abscess yielded growth of the organism. The failure with other body fluids probably reflects the rarity of the physical presence of Entamoeba histolytica in such situations. For instance, only on one occasion during the prospective study was Entamoeba histolytica found in the ascitic fluid obtained from a case of intestinal perforation due mainly to typhoid enterocolitis with superimposed amoebiasis.

5.4. CULTIVATION OF LOCAL (IBADAN) STRAINS OF ENTAMOEBA HISTOLYTICA

5.4.1 Sources of Amoebae

The cysts or trophozoites of Entamoeba histolytica were obtained from stools of patients attending the University College Hospital, Ibadan,

with complaints of various acute diarrhoeal diseases. Specimens of stools were also collected from food-handlers, school children and some teachers, during a brief school survey in Ibadan township. Aspirates of liver abscess obtained from patients admitted to the Department of Medicine, University College Hospital, Ibadan, also form part of the materials available for culture.

Processing of the materials for culture was undertaken immediately after collection. Direct microscopy of faeces was made in saline, followed by concentration according to the technique of Ridley and Hawgood (1956) and other techniques as already described under the section on coprological school survey. Microscopically positive specimens with as many Entamoeba histolytica trophozoites or cysts as possible, but with no cysts or trophozoites of other amoebic species were selected for subsequent cultivation.

5.4.2 Culture Media

The culture media assessed for use in this work are all diphasic ones having agar slope as the solid phase and fluid of different composition forming the overlay. Such media include: Boeck and Drbohlav's (1924-1925) Lock-egg-serum (L.E.S.) medium; Dobell and Laidlaw's (1926); Cleveland and Collier's (1930) and Robinson's (1968) media. The media were prepared in the orthodox manner except for some modifications where

necessary (see appendix 3). The composition of various media were sterilized by autoclaving at 121°C., 15 lb. pressure for 15 minutes. Occasionally, sheep sera were obtained directly from the slaughter houses for incorporation into the culture media. In such cases when sera are not obtained asceptically, heat labile components such as the tissue extracts were seitz-filtered using pressure/vaccuum pump "Speedivac" model RB4 (BTL) (Appendix 4).

5.4.3 Culture and Sub-culturing

About 50mg of fresh untreated faeces were added by wire loop to the fluid over-lay of any of the diphasic media in use. At the same time, the appropriate antibiotics, in some cases as modification of the original medium, rice starch (DIFCO or one prepared by the writer) were added. All cultures were incubated stationary at 37°C. The first few subcultures of Entamoeba histolytica strains were made after 24 hours of inoculation and if a culture was established, thereafter at 48-hour interval. At each transfer a drop of the culture was examined under a "warm environment" microscope (Appendix 5), to identify, confirm the growth, and ensure motility of the organism.

5.4.4 Assessment of Growth

Viable trophozoite counts were carried out at intervals on the primary cultures after each feeding. Viability was based on motility of amoebae

and their ability to exclude 1% eosin dye (Siddiqqui, 1955) in contrast to dead organisms which become intensely stained rose-red. Cultures were regarded as established when the criteria for viability were maintained and when there was progressive increase in viable organisms. Counting was made by simple haemocytometer on a mixture of culture (0.05ml) and equal volume of 1% eosin. Sterility test to ensure the maintenance of "monaxenic" culture medium was performed at intervals by agar plating to confirm that the original concommittant organism (Esch. coli) has not been 'contaminated' by other bacteria. Escherishia coli strain B was used interchangeably with strain K12, as the concomittant organism on different occasions.

5.5 RESULTS and OBSERVATION

Attempts were made to establish local strains of Entamoeba histolytica in in-vitro culture on six occasions using human serum inactivated by heating at 56°C for 30 minutes. On nine occasions, similarly heat-inactivated horse serum (DIFCO and Burroughs Welcome) was used, and on the other five occasions bovine serum was used. In all a total of twenty-five attempts were made to establish continous in-vitro propagations of Entamoeba histolytica (Ibadan) strains from cases of acute amoebic dysentery, amoebic liver abscess, and asymptomatic cysts or trophozoite passers.

A total number of nineteen local (Ibadan) strains of Entamoeba histolytica have been maintained for periods ranging from three months to eighteen months using different culture media to initiate and maintain cultures. Only two - Cleveland and Collier's, and Robinson's media have been found to be most convenient for use in this work. These two media were found easier to prepare and preserve for long-term use than the egg-media of Boeck and Drbohlav's (L. E. S.); and Dobell and Laidlaw's media Nos. 1 & 2. However, for administrative convenience modified Robinson's medium (1968) was subsequently employed throughout this study. This medium was finally modified for use by completely omiting the addition of 10% Lablemco from subsequent subcultures ones a culture is established. Streptomycin was also introduced to replace Erythromycin of the original Robinson's medium.

Failure of culture to establish was in most cases due to the over-growth in some cultures of Blastocystis hominis. This is an organism present in stools of many individuals. It belongs to the Blasto-mycetes and was named by Brumpt, 1912. It was observed that these organisms tend to outgrow Entamoeba histolytica in the same medium employed for the latter. Once profusely growing Blastocystis hominis was very difficult or sometimes impossible to eliminate from cultures. Other sources of failure to establish cultures include inadequate organism inoculum in primary cultures, infrequent or over-enthusiastic feeding, "toxic serum"

and/or other factors yet unknown. In an attempt to eliminate these possible sources of failure, and in order to standardize the culture medium to suit the local need various pilot experiments were set up. The results of some of these in-vitro experiments are included in the following subsection.

Analysis of the success rate, shows that once established, local strains of Entamoeba histolytica grow readily in human, bovine, or horse serum, all inactivated by heating at 56°C for 30 minutes. However, the subsequent work for which the amoebic extracts would be required restricted efforts to the establishment and maintenance of cultures to any other serum but human. Horse serum was, therefore, chosen as the basal medium for both isolation and maintenance of the organism in in-vitro cultures. In instances when any culture is successfully established, positive progressive growth of Entamoeba histolytica was manifested usually by the end of the first or beginning of the second week. Ten per cent lablemco (pH. 7) was found mandatory at this stage, although its addition to culture medium before there was increase in numbers of viable trophozoites appeared to make some difference to the decline in growth population, depending on the culture medium employed.

5.5.1 In-vitro multiplication factor

The most important biological problem concerning Entamoeba histolytica is the identification of the substance or condition furnished

by living cells which is essential to the multiplication of the amoeba. In other words, what makes the nucleus divide? In every environment in which Entamoeba histolytica multiplies there are living cells. Thus in the lumen of the intestine or in the usual laboratory culture there are bacteria. In the deep tissues of the intestinal wall the amoebae advanced beyond the bacteria but are in contact with body cells. In a liver abscess, where there may be no demonstrable bacteria, proliferation of the amoebae takes place in the liver tissue at the periphery of the abscess (Fig. 9).

During the early work on cultivation of the amoeba in test tubes, Cleveland and Sanders (1930) found that continuous propagation was impossible in the absence of living bacteria, and that certain single species of bacteria would support multiplication while others would not. In later years, with the use of micro-isolation of single amoeba (Rees et al., 1941; Chinn et al., 1942) or with the aid of antibiotics to eliminate the original bacterial flora (Jacobs, 1947; Shaffer and Frye, 1948), cultures of Entamoeba histolytica with a single bacteria associate have been widely used in the search for the elusive factor essential to multiplication. Different types of bacteria have been used as concomittant organisms by other investigators for the cultivation of amoebae. Maddison (1965), used Clostridium welchii as the concomittant organism to isolate a strain 0 of Entamoeba histolytica. Shaffer and Frye (1948) established strain F22 in the form of a monaxenic culture of the amoeba and a streptobacillus.

Others have used amongst other bacteria Esch. coli, Bacteriodes symbiosus, and Clostridium perferinges with varying successes and failures. It has also been possible to substitute certain other living cells for bacteria in the amoeba cultures. Philips (1950) found that the amoeba would grow well with Trypanosoma cruzi and Shaffer and co-workers (1953) obtained multiplication with living cells of the chick embryo.

In planning these studies, it was decided that the initial attempts to establish and maintain an in-vitro culture of the local strains of Entamoeba histolytica would be made with Esch. coli as the sole concomittant organism. The most compelling reason for making this choice was that these organisms were easily obtainable, easily handled and unlikely to be harmful, as would others, for example, Clostridium welchii. It was surmized that Esch. coli would be easy to separate from the Entamoeba histolytica cultures when necessary and that its extracts would be easily prepared in exactly the same way as the extracts of Entamoeba histolytica, when required for control studies.

In addition to the fact that other living bacteria may be an essential factor for the multiplication of Entamoeba histolytica, it was also observed that various other environmental elements may also be involved.

Attempts were therefore made to study the existence of such factors in the following sections.

5.6 Growth Characteristics of Ibadan Strains of E. histolytica

In view of the ultimate aim for establishing in-vitro cultivation of Entamoeba histolytica which was to produce the organisms on a large scale for preparation of antigen; it was decided that a thorough investigation be made into the possible factors that may interfere with the growth of the organisms in-vitro.

Simple experiments were then set up to examine the specific effects of serum concentration; the size of inoculum, repeated sampling and other such environmental factors that may be subjected to simple experimental studies.

5.6.1 Growth Experiment I - Serum Concentration and Growth of E. histolytica

Sources of Materials:- Trophozoites of Entamoeba histolytica were obtained from the established strains of the organism in in-vitro cultures. Pooled horse serum was obtained from horses in the local stable (University of Ibadan).

5.6.2 Methods

About 100ml of clotted blood were collected asceptically from each horse. The fibrin was separated from each sample by means of sterilized glass rods and the specimens were kept at room temperature for about one hour and later on transferred to the refrigerator ($4^{\circ}C$) for twenty-four hours. The serum was then separated by centrifugation in the cold using

centrifuge at a speed of 2000 revolutions per minute. The serum from each specimen was then carefully separated using a sterilized Pasteur pipette and pooled in a sterilized flask. The heat-labile components of the pooled serum were seitz-filtered using pressure/vaccuum pump "speedivac" Mode RB4 (BTL). The serum was later on inactivated by heating at 56°C for 30 minutes. Special dilutions of horse serum in fluid overlay of culture medium in a total of 2mls were made as follows.

TABLE 13

TUBE NO.	1	2	3	4	5	6	7	8
SERUM (ml)	0	0.1	0.2	0.6	1	1.4	1.8	2
OTHER FLUID OVERLAY	2	1.9	1.8	1.4	1	0.6	0.2	0
% SERUM	0	5	10	30	50	70	90	100

Twenty-four hour culture of Entamoeba histolytica was harvested and washed in two changes of normal saline by centrifugation at 250g for 3 minutes and resuspended in 1ml of saline.

0.1ml of Entamoeba histolytica suspension was added to each culture bottle and incubated at 37°C.

Viable Trophozoites were counted at 0 hour and again at 48 hours.

The growth Index was chosen from the results of preliminary experiments (Fig15 A) and calculated as follows:

Growth Index = Viable Trophozoites/cu. mm at 48 hours

minus

Viable Trophozoites/cu. mm at 0 hour ie. $\dfrac{V^T_{48} - V^T_0}{V^T_0}$

divided by Viable Trophozoites at 0 hr.

The growth index was obtained separately for each culture and plotted against percentage (%) serum in culture.

5.6.3 Results

The results detailed in table 14 and in graphic form (Figs. 15A&B) show that Entamoeba histolytica (Strain HN:IE5) grows steadily with increasing percentage serum included in the culture medium, attaining the maximum growth in the culture medium containing about 20 - 25% serum. There was a sharp decline in growth in the media containing (30 - 50%) serum with virtually no growth in the media containing higher concentrations of horse serum (Fig. 15B). It was also noticed from the preliminary experiment to the experiment described above that the size of Entamoeba histolytica inoculum and state of serum in the primary culture may have effects on the subsequent yield of the organisms by culture. Thus in (Fig. 15A) where fresh serum and very low number of organisms were employed there was a steady decline and sharp maximum stationary growth phase (no matter the index used), in the culture media containing different

concentrations of serum. The state was however, improved by heathing the serum at 56°C for 30 minutes (inactivation) and by using varying sizes and increased Entamoeba histolytica inoculumi in the culture media.

5.7 Growth Experiment

5.7.1 Size of Inoculum of E. histolytica and Culture Growth

As seen from both the preliminary experiment and experiment (i) above there is an indication that the state of serum included in the culture medium and the size of Entamoeba histolytica inoculum at zero hour (0 hour) may influence the ultimate growth of the organism in-vitro. The experiment described in the following section is therefore, devised to study this problem.

5.7.2 Materials and Method

2 ml aliquots of 25% inactivated human serum as found from previous experiment was measured in a row of culture bottles. Trophozoites of Entamoeba histolytica were harvested, washed twice and resuspended in fluid overlay of the medium.

Two fold serial dilutions of Entamoeba histolytica suspension was made. 0.1ml of each diluted Entamoeba histolytica suspension was added to the culture bottles. The cultures were incubated stationary at 37°C.

Viable trophozoites count was made on each test culture at 0 hour and again at 48 hours.

The growth in each test culture was expressed as a growth index as in experiment (i).

The growth index was then plotted against Viable Trophozoites x 10^5/ml of inoculum at zero hour (0 hr).

Results

Fig. 16 gives an indication that continuous propagation of Entamoeba histolytica in-vitro may not be possible if subcultured organisms are less than 0.5 x 10^5 viable trophozoites per ml of culture. The optimal concentration of Entamoeba histolytica for growth was 3 to 4 x 10^5 viable organisms per ml of fresh culture medium.

5.8 Growth Experiment 3

In order to decide at what stage of the 'culture', future harvesting for preparation of antigen(s) may be contemplated, attempts were made to study the growth cycle of the organisms in-vitro as detailed in the following experiments:

5.8.1 Growth Curves

Pooled horse serum was obtained as described above. Viable trophozoites were obtained from the established strains and processed for culture as detailed in experiment (i) above.

Twenty-four hour old culture of four of the established (local) strains of Entamoeba histolytica was separately transferred into 10ml centrifuge tubes and washed three times in saline by spinning for 3 - 4 minutes at 250g. Viable trophozoites counts were made using simple haemocytometer and the required amount of Entamoeba histolytica was estimated by adjusting the volume of the culture medium initially for each test culture.

Cultures were set up and incubated stationary at 37°C. Viable trophozoite counts using 1% Eosin dye, on each culture were made at roughly 24 hour intervals up to 120 hours (5 days). The growth curves were then obtained by plotting viable trophozoites/ml against time in hours.

5.3.2 Results:

The various growth curves of four of the established strains of E. histolytica in in-vitro cultures are shown in (Fig. 27). The growth curves consist of the various phases shown by any micro-organism in culture. The phases which can be identified consist of the lag phase (A); acceleration (B); exponential (C); retardation (D); maximum stationary and decline phases, are shown by the growth of (strain HN:IB5). It would appear that the distinguishing features of the various strains depend on the rate of growth and to some extent on the growth curves.

5.3.3 Growth 'Exponential' Under Varying Conditions Of Medium:

5.3.3.1 Materials and Methods:

Strain HE:IE5 isolated on the 25th October, 1968, was subcultured for this experiment. A 24-hour culture of E. histolytica (strain HN:IE5) was harvested and subcultured into 'universal bottle' culture medium containing fluid overlay with 25% serum. A total number of six cultures were put up at the same time. After sucking with a pasteur pipette up and down for about three times, viable trophozoite counts were made on the different cultures at 0 hour. Two of the six batches of cultures were repeatedly counted using the simple haemocytometer and supra-vital dye as described above, every 24 hours for 5 days. In these first two cultures, the count was undertaken after both the components of the diphasic medium shall have been replenished. Two other cultures were treated similarly except that the solid portion (agar slope) was kept constant (unchanged) while only the fluid overlay was replenished. The last two sets of cultures were repeatedly counted daily with none of the components; viz. solid or fluid phase of the medium being changed.

The average counts for two cultures were taken and plotted against time in hours.

5.3.3.2 Results

As depicted in (Figs. 18 & 19) results show that E. histolytica has a definite growth cycle in-vitro with maximal population every 3 to 4 days

especially when the culture is replenished every other day. From this it was decided that future harvesting of amoebae for antigen would be made at approximately 48 hours after mass cultivation.

5,9 Repeated Sampling of Culture and Growth Rate:

As part of further search for a means of mass production and good yield of organisms for production of antigen, attempts were made to find what effects, agitation of cultures would have on the growth rate of E. histolytica, and consequently on the population yield of organisms that would be available for use. As a prelude to the introduction of roller culture technique to the mass cultivation of the organisms in-vitro, simple experiment to find the effects of repeated sampling (a form of agitation) was devised as described below.

5.9.1 Growth Experiment 4:

5.9.1.1 Materials and Methods:

Duplicate cultures were set up from two sets of stock cultures on culture medium containing 25% horse serum. Viable trophozoite counts were made on all cultures at zero hour (0-hr.). Serial counts were carried out at 18-hours and 42 hours on one of each pair of test cultures. Again counts of all cultures were made at 75 hours.

5.9.1.2 Results:

The details of viable trophozoite counts are shown in (Table 15). It was observed that in both culture bottles disturbed for serial trophozoites counts one showed slightly more viable trophozoites at 48 hours than its counterpart which was left undisturbed. On the other hand the second disturbed culture gave an exactly the same number of viable trophozoites at 48 hours as the corresponding culture left undisturbed for serial counts.

The repeat experiment gave similar results and it was concluded that either the disturbance employed here was not enough to effect any appreciable change in the growth of the organisms or that agitation of cultures would probably have no growth enhancing effect on E. histolytica. On this basis it was decided that the cultures required for harvesting for the purpose of antigen preparation be incubated stationary at 37°C.

TABLE 15 Effect of Disturbance Due To Sampling On Growth Of E. histolytica (Strain HN:IB 16)

SET OF CULTURES		Viable trophozoite Count x 10^5			
		0 hr	20 hrs.	32 hrs.	43 hrs
CULTURE A	Sub-culture (1)	4	2	4	16
	Sub-culture (2)	4	l.u.	l.u.	12
CULTURE B	Sub-culture (3)	4	2	4	12
	Sub-culture (4)	4	l.u.	l.u.	12

* l.u. = left undisturbed.

5.10. GENERAL WORKING BASIS FOR THE IMMUNOPATHOLOGY OF AMOEBIASIS

5.10.1 Introduction

During the initial studies on the culture and growth characteristics outlined in the preceding section, an incidental finding was that some apparently normal human serum produced immobilization or lysis of the amoebae. The lytic effect sometimes completely disappeared after heat inactivation of the serum at 56°C for 30 minutes. Rabbit anti-serum and serum from amoebiasis patients which had not been inactivated also produced lysis of the amoebae. Such rapid toxic effect of serum on E. histolytica in-vitro had been noted by other workers (Cole and Kent 1953, Zaman, 1960). The lysis of amoebae as a result of treatment with fresh unheated antisera has also been observed, (Brown and Whtby 1955). It was, therefore, necessary to inactivated all the sera before incorporating them into the routine culture medium for E. histolytica.

From the foregoing, it was strongly suspected that apparently normal human serum, as well as, inactivated sera from amoebiasis

patients or anti-amoebic sera raised from rabbits, are injurious to E. histolytica in-vitro. It was, however, observed that sera from patients with the different stages of the disease, have different effect on the in-vitro cultivation of the organism. Thus the presence of serum from known acute amoebic colitis patients was less injurious and in fact, tends to encourage the growth of the organism, when compared with the effect of the sera from patients suffering from amoebic liver abscess.

It would appear, therefore, that the factor responsible for the undue severity of amoebiasis in pregnancy and the allied states, may be inherent in human serum. The search for such factor(s) form the basis of the following aspects of the study. It was, therefore, considered necessary to carry out more careful and systematic studies to determine the effect if any, of human sera on the survival and growth of E. histolytica in-vitro.

5.11 Survival Of E. histolytica In Various Sera:(Preliminary experiment)

5.11.1 Materials:

The serum samples tested were from (i) an adult male Nigerian without symptoms of amoebiasis;

(ii) adult Nigerian patient with amoebic colitis (serum negative on gel diffusion test);

(iii) adult male Nigerian patient with amoebic liver abscess (serum-strongly positive on gel diffusion test);

(iv) 'Normal' pregnant Nigerian woman without symptoms of amoebiasis (gel-diffusion test negative);

(v) Normal human serum + 200 microgram (Ug) of prednisolone (dissolved in water);

(vi) human serum + cyclophosphomide (solution); and

(vii) A pair of 'complete' horse-serum medium as control.

Items (v) and (vi) were included in the experiment as a devise to test the effects of immunosuppresive drugs on the survival of E. h. in-vitro

All the sera had been stored at $4^{o}C$ for periods ranging from three to eight weeks before use.

5.11.2. Method:

Processing of material was as described in the preceding experiments. Viable trophozoite counts were carried out on each test culture at zero hour, and subsequently at 8-hourly for 24 hours. This was then followed by daily counts and pH readings for another three days. The effect of the individual serum samples on the amoebae was estimated as the proportion of trophozoites which survived at 48 hours expressed as a lagarithm of trophozoite numbers present at 0-hour viz:

$$\text{Survival Index} = \log \frac{\text{Viable Trophozoite population at 48 hours } (V^{T}_{48})}{\text{Viable Trophozoite population at 0-hour } (V^{T}_{0}).}$$

5.11.3 Results

The results are shown in Table 16. The histogram (Fig. 20) shows the relative effects of the serum samples tested on E. histolytica. Sera from patients with acute amoebic colitis, as well as, serum from an adult healthy male Nigerian showed the least deleterious effects. Indeed, sera from the former appear to give relative high supportive effects. There was, however, a fairly wide range of effects manifested by sera from patients with amoebic colitis and Normal human serum containing some innunosuppressive drugs, the latter having profound deleterious effects on the amoebae. Serum from amoebic liver abscess patient showed some degree of inhibition of the growth of E. histolytica.

5.11.4 COMMENTS

The influence of the storage on the effect of serum samples is not known and may well be responsible for the differences between the effects of the various sera used in the experiment. Furthermore, the correct interpretation of the drug effects in-vitro is complicated by the fact that one of the drugs tested (prednisolone) is only partially soluble in water and the "dose effects" of both drugs are not known. Thus the apparent deleterious effect may be due to the insoluble drug particles in the case of prednisolone, while the dosage used in cyclophosphamaide may be higher than normal.

However, corticosteroids are known to enhance the susceptibility of experimental animals to infection by E. histolytica (Teodrovic, Ingall and Greenbergh, 1963); and it is possible that similar effect will operate in human cases. In view of this in-vivo behaviour of corticosteroid, despite the obvious flaw in the present in-vitro experiment, it is still quite possible that the "in-vivo" enhancing effects of steroid may be different from the direct effects of the drug on the amoebae in-vitro.

Before drawing an inference that the effects of pregnant serum on amoebae in-vitro is comparable with that of steroids in-vivo, it was decided to devise some working hypothetical basis for the immunopathology of human amoebiasis. A culture system to investigate the effect of sera from patients suffering from wide range of condition on the growth of the organisms in-vitro forms part of such basis attempted in the next chapter.

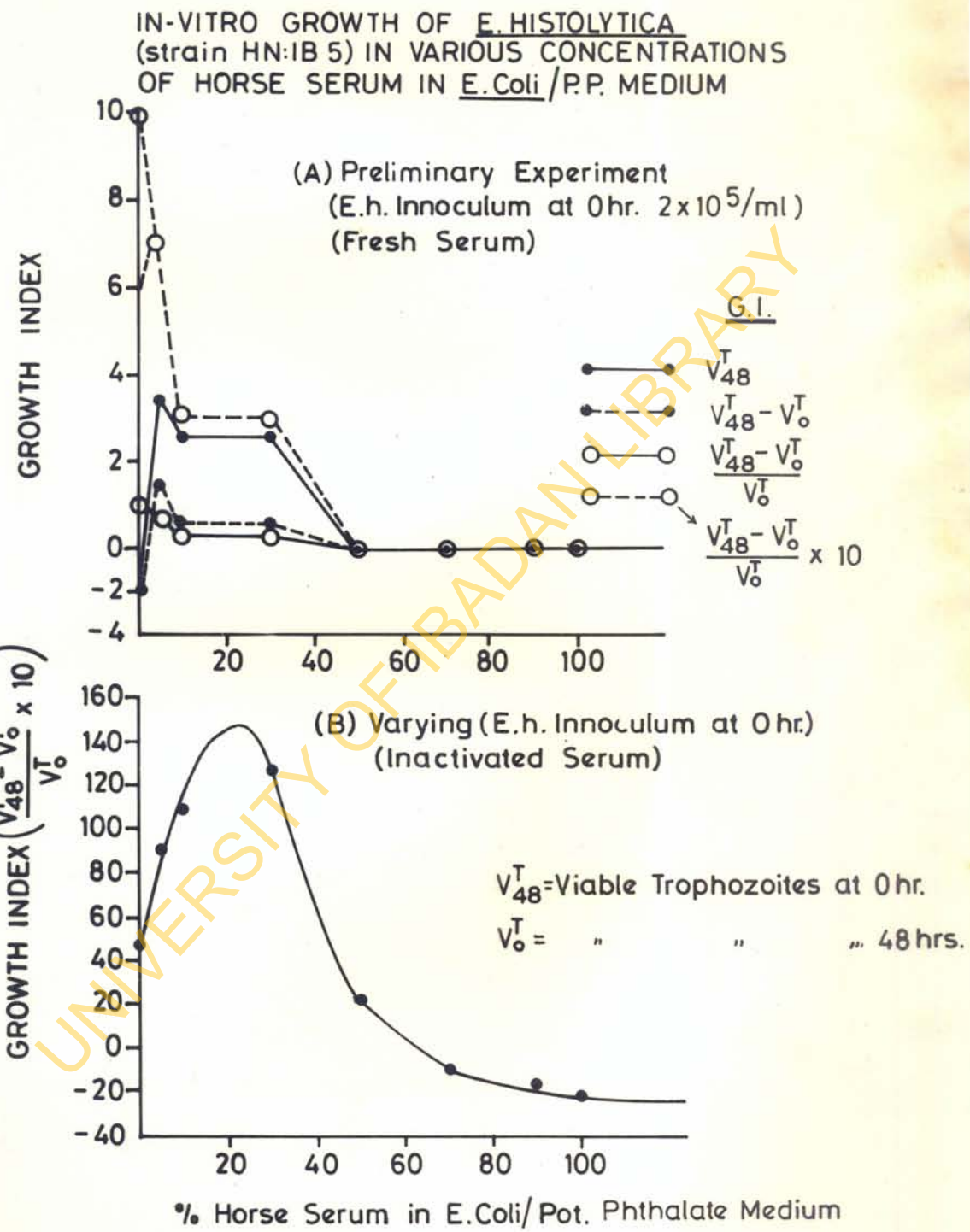


Fig. 15A.

IN-VITRO GROWTH OF E. HISTOLYTICA (strain HN:IB 5)
IN VARIOUS CONCENTRATIONS OF HUMAN SERUM
IN E. Coli/P.P. MEDIUM.

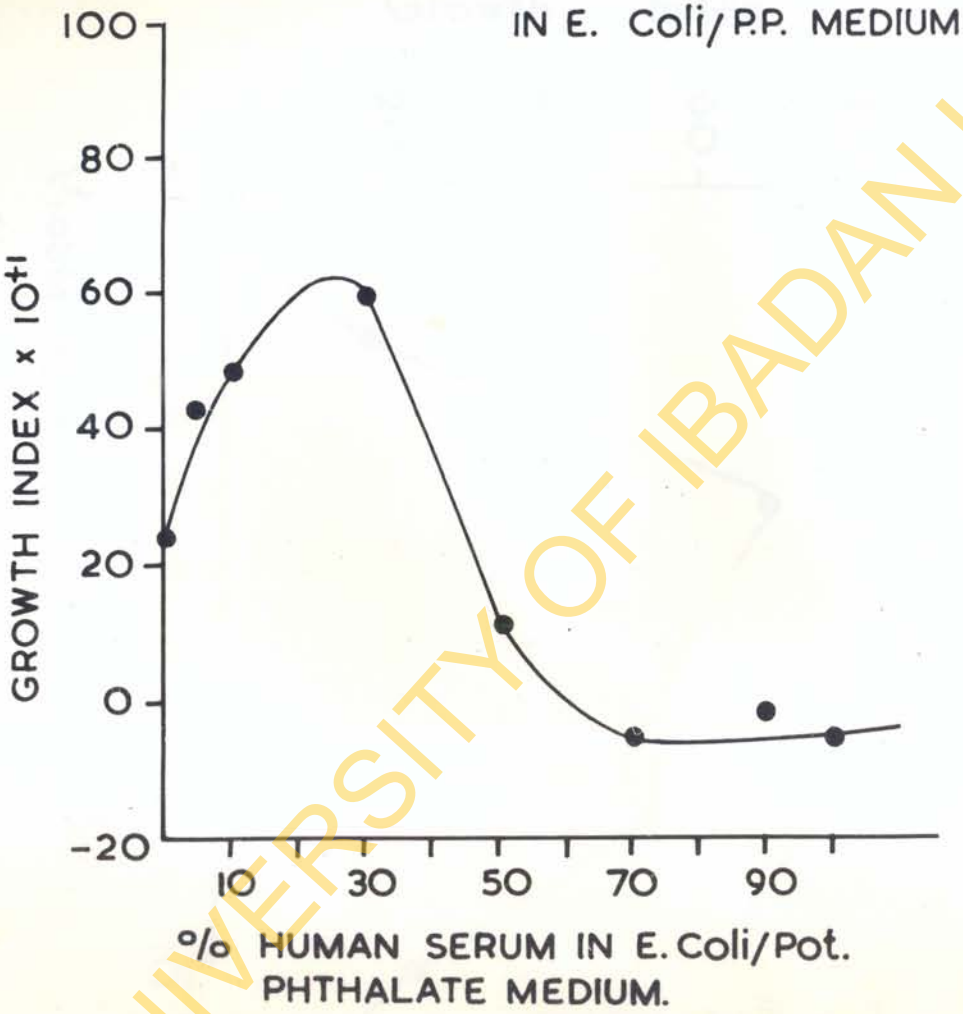


Fig. 15B

Effect of size of innoculum on rate of growth of Entamoeba histolytica strain HN: IB 9 in In-vitro culture

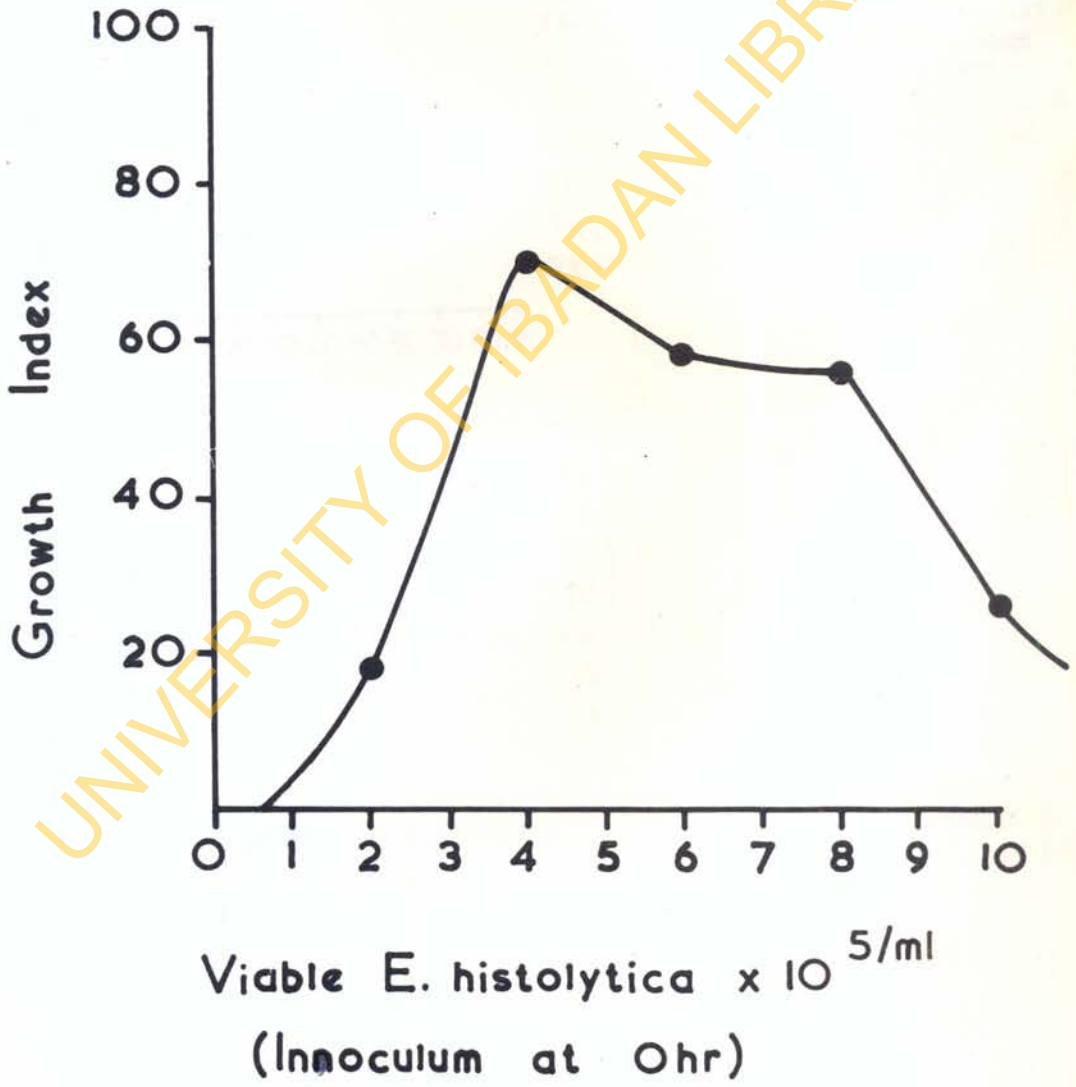
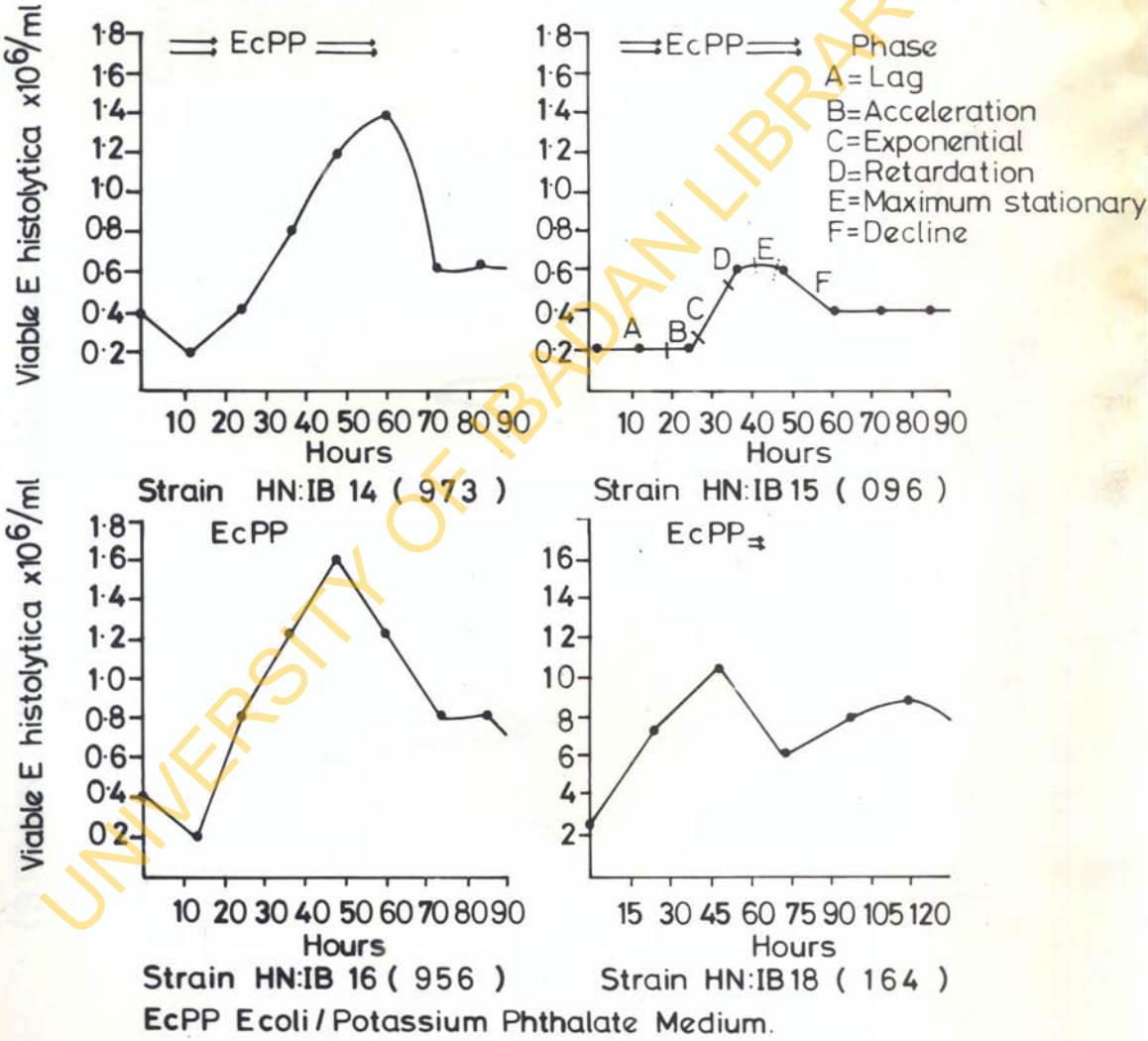


Fig. 16

GROWTH CURVES OF PRIMARY CULTURE
OF ESTABLISHED E.h. IN IN-VITRO CULTURE



EcPP Ecoli / Potassium Phthalate Medium.

Fig. 17

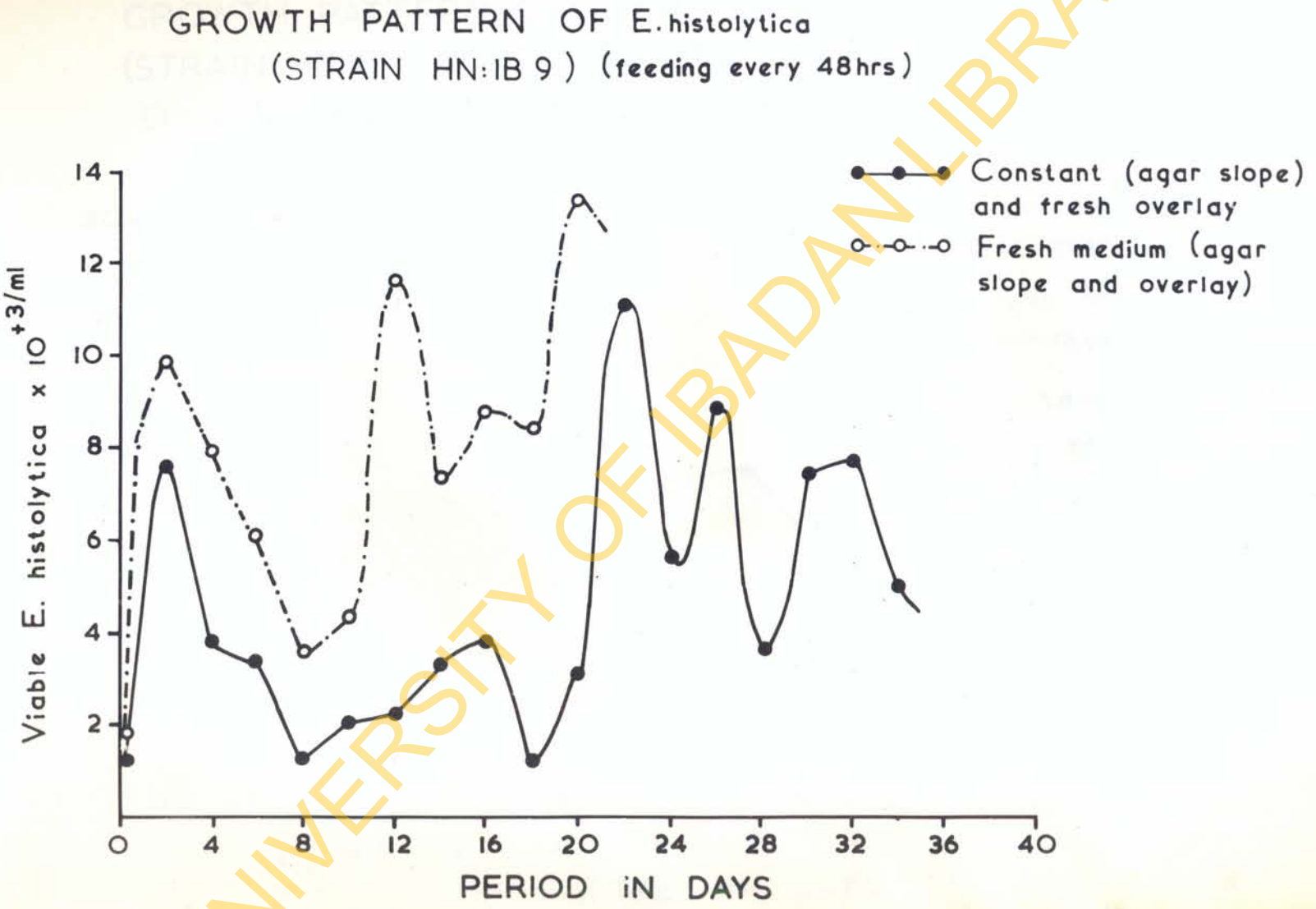


Fig. 18.

GROWTH PATTERN OF E. histolytica.
(STRAIN HN:IB 9) UNDER 'VARYING' CONDITIONS
OF CULTURE MEDIUM (Daily feeding)

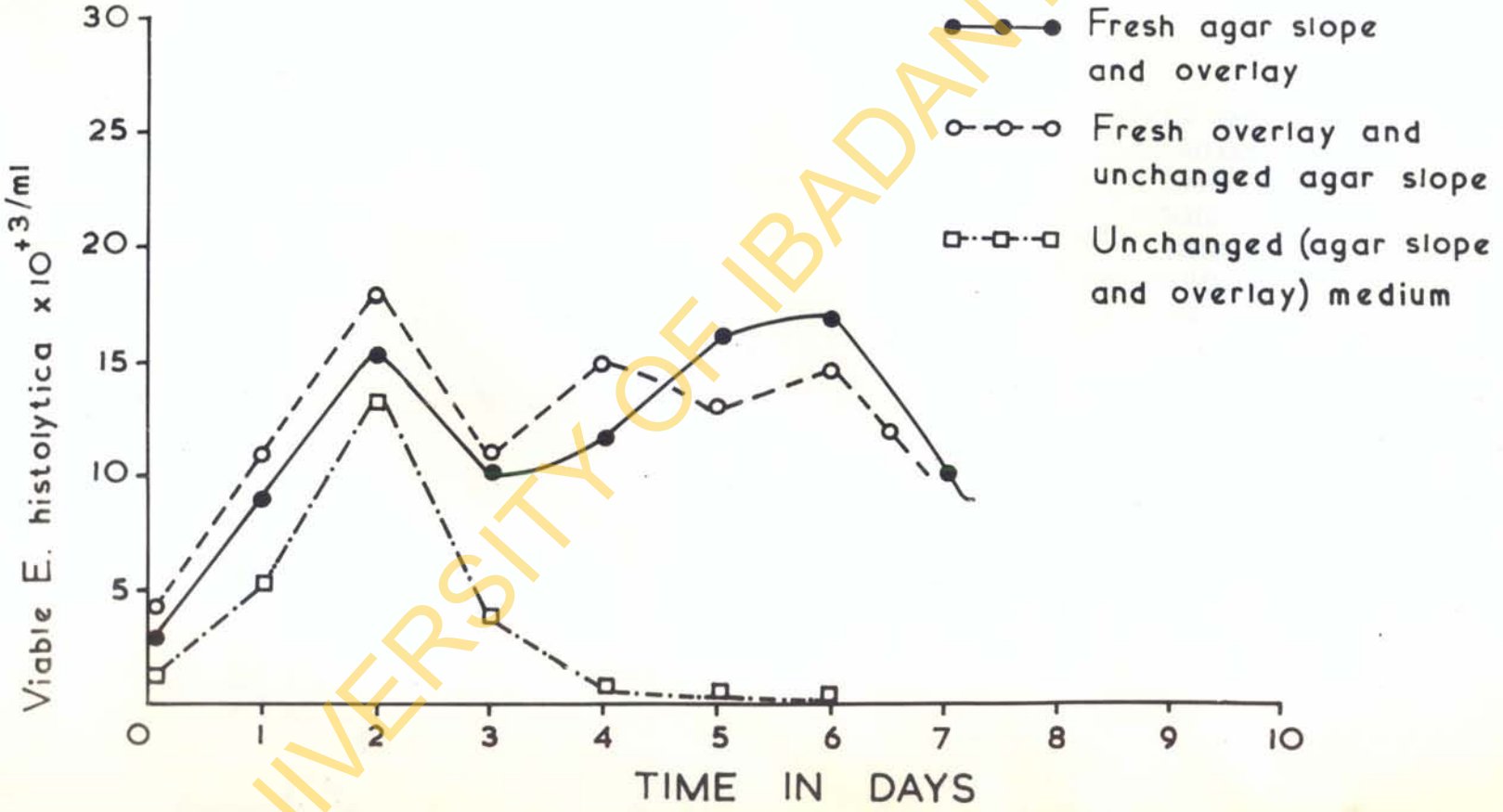
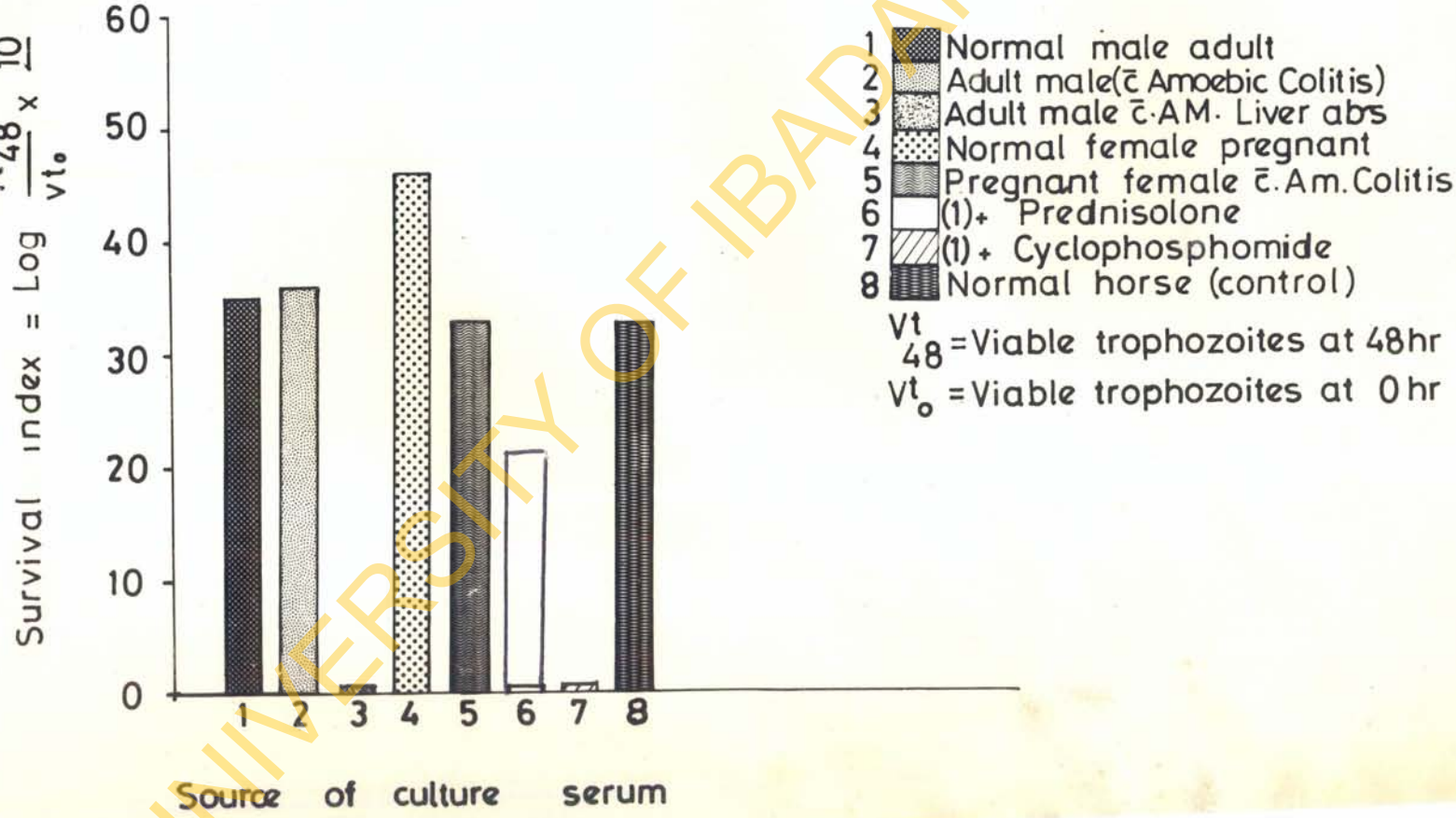


Fig. 19

SURVIVAL OF ENTAMOEBA HISTOLYTICA (HN:IB 5)
IN VARIOUS SERA (MEDIUM E.coli/P.P)



(Fig. 20)

TABLE 14 GROWTH OF ENTAMOEBA HISTOLYTICA (Strain HN: IB5) IN VARIOUS CONCENTRATIONS OF HUMAN SERUM IN E. COLI/P. P. MEDIUM

Tube No.	Per cent Serum in Culture Medium	Viable Trophozoite at 0 hr $V^{T}0$ x 10^5		Viable Troph. at 48hr ($V^{T}48$) x 10^5		GROWTH INDICIES (G. I.) ($V^{T}48 - V^{T}0$ $V^{T}48 - V^{T}0$ VOT				G. I. x 10	
		Prelim	Expt. 1	Prelim	Expt. 1	Prel.	Exp. 1	Prel.	Exp.	Prel.	Expt.
1	0	2	12	0	68	- 2	56	-1	4.66	-10	46.6
2	5	2	16	3.4	122	1.4	106	0.7	6.66	7	66.6
3	10	2	8	2.6	124	0.6	116	0.3	14.5	3	145
4	30	2	14	2.6	164	0.6	150	0.3	10.71	3	107.1
5	50	2	10	0	36	-2	26	-1	2.6	-10	26
6	70	2	20	0	8	-2	-12	-1	-0.6	-10	-6
7	90	2	8	0	10	-2	2	-1	0.25	-10	2.5
8	100	2	18	0	2	-2	-16	-1	-0.38	-10	-3.8

TABLE 16: SURVIVAL OF ENTAMOEBA HISTOLYTICA (Strain HN:IB5) IN VARIOUS SERA (MEDIUM E.COLI/P.P.) (Preliminary Expt.)

No	Source of Serum	Viable Trophozoite V^T (hr) x 10^6					log V^T_0	log V^T_{48}	Survical Index log V^T_{48}/V^T_0 x 10
		0	24	48	72	96			
I	Normal adult (male)	2	2.8	11.4	9.4	4.4	0.30	1.04	35
II	Adult male with acute Amoebic Colitis	2	4	12.0	11.4	10.6	0.60	1.08	36
III	Adult male with Amoebic Liver Abscess	2.2	0	0	0	0	0.34	0	0
IV	'Normal' pregnant (without Am. colitis)	1.5	4.0	6.6	4	1.4	0.18	0.82	46
V	Pregnant (W) with Amoebic Colitis	2	4	9.6	8.3	6.6	0.30	0.98	33
VI	N.H.S.(1)+ Prednisolone	2	0.2	4.6	0.3	0.1	0.30	0.66	22
VII	N.H.S. (No. 1)+ Cyclophosphamide	0.2	0	0	0	0	0.30	0	0
VIII	Control horse Serum	1.8	6.2	8.0	1.8	0.8	0.25	0.90	35

CHAPTER VI

EFFECTS OF HUMAN SERA ON THE GROWTH OF E. HISTOLYTICA IN-VITRO

6.1 Introduction

Nutritional requirements of organisms can be considered as of prime importance in the consideration of factors responsible for the deleterious effects of some serum samples on *E. histolytica* in-vitro. It is however, possible that other known biological effects of sera on organisms survival generally, can be postulated for the deleterious effects. It is very likely that *E. histolytica* requires one or several essential nutrients for survival, the concentration of which vary widely in serum of different individuals.

It is also possible that the effects manifested by the serum samples tested are those usually attributed to "toxic" sera in culture work. However, apart from thermolability, and perhaps effects due to nutritional deficiency, little is known about the properties and the true nature of the serum factor(s) responsible for non-specific toxicity of sera to *E. histolytica* in-vitro.

It is well established that complement plays an important part in the lytic activity of antisera against various bacteria (Boyd, 1956), and this is also probably so in the case of *E. histolytica*, because the lytic activity disappeared on inactivation of the serum. Its different concentration in the serum of different individuals may be partly responsible for the

varying degrees of survival of susceptible E. histolytica in sera from various individuals. The absence or presence of specific antibodies to antigen(s) present in E. histolytica may be playing an important part in the outcome of the observed growth promotion or growth suppression of E. histolytica in various serum samples.

Of relevance to this last possibility is the peculiar severity of amoebiasis in debilitated children and in patients previously treated with immunosuppressive drugs such as corticosteroids as already noted above. The apparent severity of amoebiasis in pregnant women and in women in the early puerperium (Lewis and Antia, 1969; and Abioye, 1970) offers the attractive concept of "high susceptibility to infection in the non-immune" or in patients with some derangement in their immune mechanism.

The foregoing observations, and speculations led the author to search for experimental evidence in support of the premisis that pregnant women and women who have recently delivered of babies may have some growth promoting factor(s) or lack growth inhibiting factor(s) for E. histolytica in their respective sera.

The rationale, methods, and results of the experimental studies are presented in this chapter. Two systems were proposed for the confirmatory detection of humoral anti-E.h. factors, one of which is an experimental model for in-vitro detection of growth promoting and/or culture inhibiting factor(s) in sera from various human sources. The other is the

applicability of various immunological techniques to the detection of any humoral factors using antigen(s) produced from the local strains of E. histolytica.

6.2. Preliminary Studies:

6.2.1 Growth Effects of Sera from Various Individuals on E. h.

The studies on survival of E. histolytica in human sera included in chapter 5 precluded generalisations and as such attempts were made to study the definitive effects of any of the sera on the growth of the organism in-vitro as opposed to its mere survival.

6.2.2 Materials:

E. histolytica (Strain HN:IB9) employed in this study was obtained originally from fresh specimen of stool containing large number of active trophozoites, from an adult male Nigerian suffering from amoebic colitis. The original specimen of stool contained predominantly active trophozoites although a good number of cysts of E. histolytica were also present.

Human Sera: Serum samples were obtained from the following sources:-

(1) 5 male adult indigenous blood donors;

(2) 6 cord blood samples at the labour ward UCH, Ibadan;

(3) 8 male patients with amoebic colitis;

(4) 6 blood samples from normal deliveries at the labour ward (UCH, Ibadan);

(5) 2 adult male patients with malignant hepatoma;

(6) 2 female patients with amoebic colitis and staphylococcal septicaemia.

(7) 6 male patients with amoebic liver abscess.

6.2.3 Methods:

Processing of material and the culture techniques are similar to those employed in the definitive experiments described below. The only difference was in the counting of viable trophozoites every 24 hrs for 72 hours and then taking the average of culture counts per ml for cultures from individuals with common ailments. A histogram of such averages was then constructed as shown in Fig. 21.

6.2.4 Results:

It was observed that human sera from different groups of individuals may have wide variation in their effects on the growth of established strains of Entamoeba histolytica in-vitro.

The strain of amoebae used in this preliminary experiment also appeared to grow relatively poorly in serum from patients with amoebic liver abscess. On the other hand, the amoebae tend to grow comparatively better in sera from patients suffering from acute amoebic colitis than in the sera from patients with amoebic liver abscess. The most obvious and striking growth effects noticeable from the results of this preliminary

experiment was the apparent supportive growth effect of sera from women who have recently delivered of babies (Fig. 21).

6.2.5 Comments:

The results of the preliminary investigation give an indication that probably, there is a growth promoting factor or less growth inhibiting factor(s) in the sera of women soon after delivery of babies. However, since it was considered that firm conclusions would be difficult to make from these preliminary findings, it was decided to repeat the experiments with increased number of human sera and arrange the results in a manner subjectable to statistical analysis. Such attempts were made in the following definitive experiments below:

6.3. Definitive Experiment:

Effect of Sera from Various Groups of Individuals on E.h. In-Vitro.

Having established, at least from the results of the preliminary studies, that inactivated human sera support the growth of E.histolytica to variable extents, it was considered that complement alone cannot be responsible for the suppression of growth by some of the sera used above. The significance of any other factors that may be responsible for this differential growth rate of the organism in various human sera, posted a question to be answered.

EFFECTS OF HUMAN SERA FROM VARIOUS INDIVIDUALS ON THE GROWTH OF E. histolytica (Strain HN : IB.9)

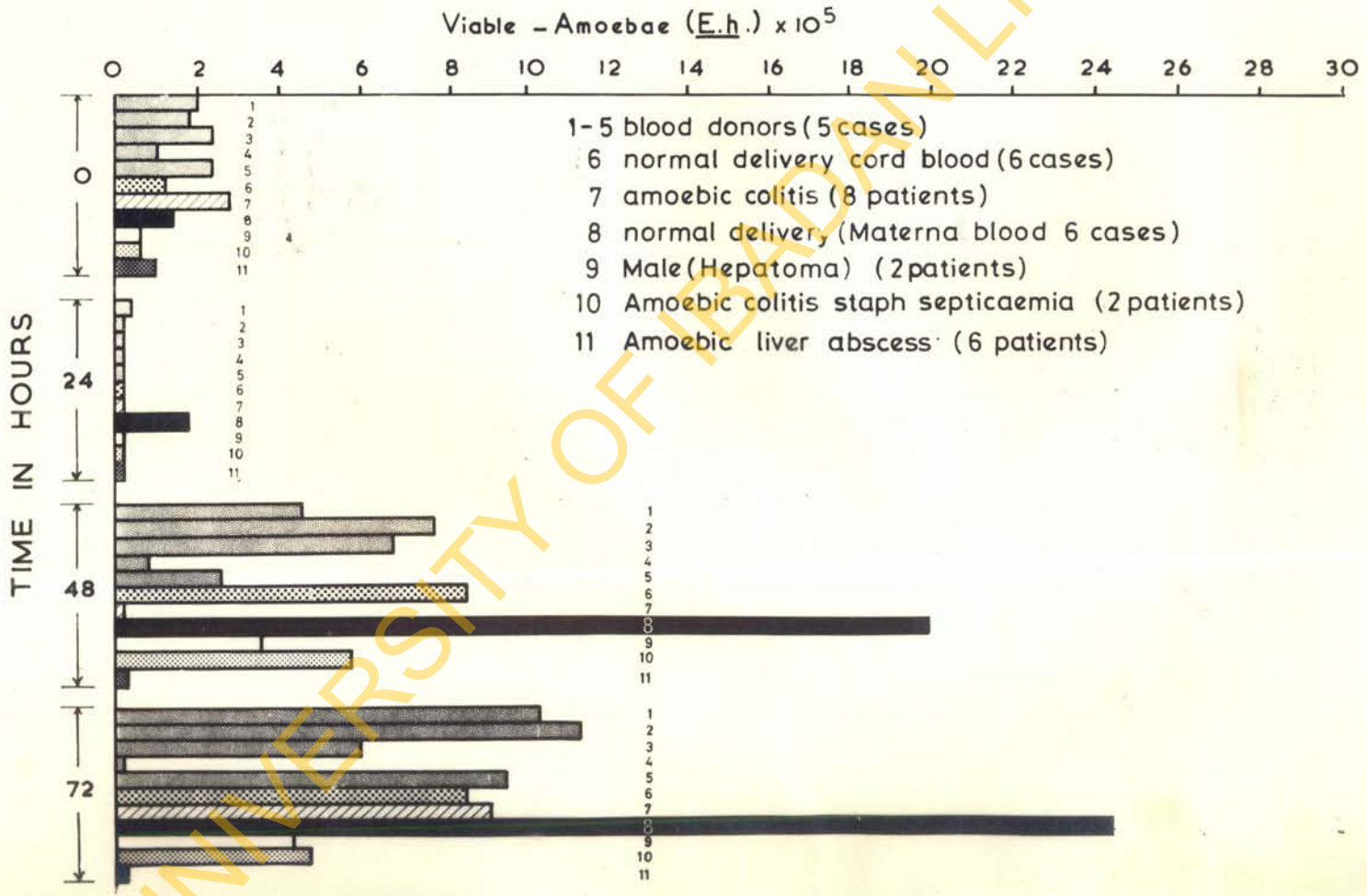


Fig. 21.

6.3.1 Materials and Methods:

E. histolytica (Strain HN:IB16) was used in this experiment.

Test Sera: Five to twenty mls of blood was collected from each of:-

(a) 10 adult Nigerian blood donors at the Blood Bank (UCH);

(b) 2 untreated male amoebiasis patients;

(c) 8 untreated female non-pregnant (of child-bearing age) amoebiasis patients -

(d) 5 untreated pregnant women with amoebiasis;

(e) 7 pregnant women without symptoms of amoebiasis;

(f) 36 blood samples from normal deliveries at the labour wards (UCH and Adeoyo Specialist Hospitals, Ibadan);

(g) 22 cord blood samples from normal deliveries at the same sources as (f).

(h) 7 untreated male patients with amoebic liver abscess;

(i) 3 amoebic liver abscess patients in remission.

Serum was separated from all samples within 36 hours of blood collection, preserved with erythromycin (0.12 ml of 0.05% solution) and stored at - 20°C until used.

Standard Serum: This was 150ml serum separated from 400ml clotted blood collected from a healthy adult Nigerian at a single bleeding. The serum was preserved as above, and stored at -20°C in 2 ml aliquots.

6.3.2 Cultures:

The system used was that developed in the preliminary (Standardisation) growth experiments. Serum samples were tested in batches of 10 to 20. Duplicate cultures of the standard serum was included in the batch of test cultures. Two millilitres of each serum was measured into culture bottles and 6ml (solution) of the other constituents of the fluid overlay of the culture medium added. An aliquot of the appropriate strain of E. histolytica culture calculated to contain enough viable amoebae to give an initial 3-4 x 10^5 amoebae/ml in each bottle, was removed. The contained amoebae was spun down at 250g for 3 minutes.

The organisms were washed by centrifugation in two changes of normal saline and resuspended in appropriate volume of other contents of the fluid overlay (less the serum). 0.5ml of the trophozoite suspension was then added to each culture bottle. The bottles were tightly stoppered and incubated stationary at 37°C. Viable trophozoite counts were carried out on each bottle at 0-2 hours and again at 48 - 50 hours.

6.3.3 Assessment Of Culture Growth:

Culture growth in each bottle was measured by the difference in viable trophozoite counts at 48 hours (V^t_{48}) and at zero hour (V^t_0). The growth effect of each test serum was expressed as a growth index, which

is the ratio of growth in test culture to growth in the standard serum culture; the latter having a growth index of 1.0. The growth effect of each test serum on strain HN:IB 16 of E. histolytica was therefore calculated as follows:

$$\text{Growth Index} = \frac{\text{Culture Growth In Test Serum } (V^t_{48} - V^t_0)\ TC^*}{\text{Culture Growth In Standard Serum } (V^t_{48} - V^t_0)\ SC^*}$$

$$= \frac{\text{Increase In Viable E.h. population in test culture } (V^t)TC}{\text{Increase In Viable E.h. population in standard culture } (V^t)}$$

i.e. V^t_{TC}/V^t_{SC}. (N.B. TC = TEST CULTURE* and SC = STANDARD CULTURE*)

6.3.4 Results:

From both the scattergram (Fig. 22) and Table 17 showing the relative growth effects of Strain HN:IB 16) emerged the following results:

(i) That sera from cases of acute amoebic colitis tend to support the growth in-vitro of the strain of amoeba used in this experiment more than any other form of amoebiasis - notably amoebic liver abscess.

(ii) That the amoebic liver abscess group showed the lowest growth promoting effect or greatest growth inhibiting effect on strain HN:IB 16 trophozoites, with a growth index range of 0.25 to 0.75 and a median of 0.33. Sera from adult (indigenous) blood donors, males,

and non-pregnant females (of child-bearing age) with or without symptoms of amoebic colitis, had a growth index range of 0.25 to 1.5 with a median of 0.75. The range of growth indices for cord serum samples was 0.20 to 1.00 with a median of 0.50.

(iii) That the widest range of growth effect was encountered in the sera of pregnant women and women who were in the immediate puerperium, the latter showing the greatest growth promoting effect than any other group. Indeed the observed difference between the means of the growth indices of sera from pregnant/ women in the puerperium and those of adult indigenous sera (0.26) is 13 times greater than the standard error of difference between the means (0.02). Thus showing that E. histolytica (Strain HN:IB 16) growth is significantly higher in the sera from pregnant/ puerperal women than in the sera from any other groups with a level of significance $p < .001$.

6.3.5 COMMENTS:

The results of both the preliminary and definitive experiments posed important questions as to the nature of the factor(s) enhancing the growth of strain of amoebae employed in this study, in the sera of pregnant women and those in the early puerperium. The other question is on the nature of the factor which may be responsible for the marked deleterious effect of the sera from the patients with amoebic liver abscess.

Considering the early speculations regarding the possible factors, the fact that the sera were heated to destroy the complement, immediately removed the possibility that the effects are those due to complement. The possible presence of antibody to amoebae may account in part for the deleterious effects of sera from amoebic liver abscess patients; on the growth of the organism in-vitro. The most attractive explanation for the growth promoting effects will be nutritional factors. Concerning the role of nutritional factors, one cannot but agree wholly with Edington and Gilles (1969) that despite all the experimental and clinical observations, the role of dietetic factors in amoebiasis is not at all clear and that the conclusions offered on it are conflicting (Alexander and Meleney, 1935; Hegner, 1924; Griffin and McCarter, 1943). In the present study, therefore, attempts were made to answer the two questions posed by examining both the role of humoral antibodies in the growth effects of sera and also of nutritional factors.

Since one cannot estimate humoral antibody to an antigen unless the antigen is available, further discussion on the role of antibodies is left to the next chapter where the immunological aspects are studied in detail.

As regards the possible role of any nutritional factor(s), a brief mention was made while discussing the possible role of serum cholesterol as being responsible for the rare association of amoebic liver abscess and hepatic cirrhosis. At that stage, it was suggested that the rare association was probably due to the fact that in hepatic cirrhosis, the liver

contains only a small quantity of cholesterol (Ralli et. al., 1941). In the light of the latter observation, it was decided that serum cholesterol be taken as both experimental model and also as an indirect clinical evidence in the investigation of the role of the nutritional factor responsible for the growth promoting effect of some of the human sera employed in the above experiments.

The role of serum cholesterol as a possible growth promoting factor for Entamoeba histolytica form the basis of the investigation in the next definitive experiment below:

6.4 Cholesterol and Growth of E. histolytica In-Vitro:

Several workers (Snyder and Meleney, 1942 & 1943; Rees et. al, 1944; and Anderson, 1948) have suggested that cholesterol acts as a growth factor for E. histolytica. It is however, not known whether there is a definite quantitative correlation between the growth index of the organism in-vitro and the level of cholesterol in human sera from various sources. Hence it was of interest to investigate this aspect of the problem.

6.4.1 Materials and Methods:

Study Population: Human sera for this experiment was obtained from the following sources:

(i) 3 Male Nigerians (2 adults & a 3-year old boy);

(ii) 23 Maternal blood samples from normal deliveries at the labour wards (UCH, & Adeoyo State Hospitals, Ibadan);

(iii) 16 cord blood samples from normal deliveries at the same sources as (ii).

(iv) 21 adult male blood donors from the (UCH, Ibadan) B. Bank

(v) 12 pregnant/puerperal women with amoebic colitis (3-2nd; 3-3rd; 4-last trimester; and 2-in the puerp.)

(vi) 12 symptomless pregnant women (2-2nd; 2-3rd; 7-last trimester; & one E.h.-cyst passer).

(vii) 5 Non-pregnant women (of child-bearing age) with amoebic colitis;

(viii) 4 Adult males with amoebic colitis;

(ix) 2 Adult male Nigerians with conditions other than amoebiasis (one with hepatoma and the other with Hodgkin's lymphoma).

6.4.2 Cultures And Assessment Of Growth:

The system used was that devised in the preliminary (standardisation) growth experiment. The assessment of culture growth was the same as that chosen for the definitive growth experiment above.

6.4.3 Estimation Of Serum Cholesterol:

Total serum cholesterol was estimated by the Cephalin Cholesterol Flocculation technique. The method was that of Hanger (1939). Commercially obtained cephalin - cholesterol antigen (Difco Laboratories, Detroit, Michigan) was used. Flocculation was read after 24 hours in the dark.

The growth indices of the organism in the various human sera included in this study together with the corresponding serum cholesterol levels were subjected to statistical correlational analysis.

6.5 Results:

Table 18 lists the mean values of the total serum cholesterol levels and the corresponding growth indices for E. histolytica in sera from the various groups of individuals examined. Figures (23a & 23b) give the scattergram of the indices and serum cholesterol levels together with the linear regression equations. The results of this experiment show that the sera from women in the early puerperium and pregnant women, especially those in the last trimester have the highest serum cholesterol levels. It was also observed that the same sets of sera have greater growth indices with mean values of 1.97 and 0.97 respectively.

A statistical correlational analysis of the data gives a linear regression between the serum cholesterol levels and the growth indices of the organisms in human sera .

The regression equations being:- *(X = .0063y - .1683)

$$*b_{y.x} = 0.0063y - 0.1683 \text{ (Regression of } x \text{ on } y)$$

$$b_{x.y} = 96.4x + 76.6 \text{ (Regression of } y \text{ on } x)$$

where y represents the serum cholesterol level, and x the growth index. The coefficient of correlation r = 0.8, shows that the serum cholesterol levels and the growth indices are highly positively correlated.

The correlation coefficient is very significantly different from zero ($t = 12.34$; $p < .001$) on 93 degrees of freedom.

The results thus show that high levels of serum cholesterol enhance the growth of E. histolytica (Strain HN:IE 16) in-vitro employing sera from various sources.

6.6. Comments:

For some time it has been known that cholesterol is a nutritional factor for E. histolytica (Griffin and McCarten, 1943; Rees, Bozicevich, Reardon, and Daft, 1944; Snyder and Meleney, 1943). It has been found that it also increase virulence (Sharma, 1959). Singh (1959) was able to induce a non-pathogenic strain to become invasive in the intestine by adding cholesterol to the culture. This he did by washing and incubating the cultures for 24 hours in the presence of cholesterol and also by inoculating the washed parasites into rats which had previously been fed with cholesterol.

The data outlined in the introductory paragraph to this chapter provide the basis for this aspect of the study, but there are also other related observations. First, there is clinical impression of some positive correlation between cholesterol and haematogenous extra-intestinal amoebiasis (Biagi et. al., 1941). The results of this experiment confirm and substantiate this clinical impression as shown in the accompanying scattergram and the equations of linear regression (Figs 23a and 23b).

Secondly, the organs in which extra-intestinal amoebiasis is frequent are the liver and brain, both of which contain high concentration of cholesterol in the tissues (Rathmann, 1958). Also the greater part of the synthesis of cholesterol takes place in the liver (Could and Taylor, 1950). Thirdly, during the period of life in which hepatic amoebiasis occurs with greatest frequency, the blood level of cholesterol is higher in men than it is in women (Flores-Barroeta, Nunez, and Biagi, 1959; Lewis, Olmsted, Page, Lawry, Mann, Stare, Hanning, Lauffler, Gorden, and Moore, 1957). Lastly, ever since the report by Jimenez (1874) it has been observed that the beginning of hepatic abscess occurs most often after a "disorderly" meal with large alcoholic intake. Dock (1957), and Kinsell (1958), state that the intake of large quantities of animal fat raises the level of cholesterol in the blood. In alcoholics without hepatic cirrhosis, the concentration of hepatic cholesterol is elevated (Ralli et. al., 1941). Possibly this also occurs in the undernourished, since their liver has a greater quantity of fat than does the liver of normal persons (Sepulveda, Hernandez, and Rojas, 1957).

All the evidences adduced in the foregoing paragraph point to the possible role of cholesterol in the pathogenesis and perhaps severity of amoebiasis in general. It would, however, be more interesting to know whether cholesterol levels can in any way explain the undue severity of amoebiasis in the puerperium and pregnant states. An indirect evidence

for this possibility lies in the finding of high cholesterol levels in pregnant and women in the early puerperium. This finding agrees with that of Watson (1957) and Tyler and Underhill (1925). These workers found total serum cholesterol to increase progressively throughout pregnancy and remained elevated through parturition and the early puerperium.

At this stage, it is tempting to equate the severity of amoebiasis during pregnancy to the elevated serum cholesterol especially in the light of findings in this chapter. On the other hand, it is equally tempting to suggest that the severity is due to the generally increased concentration of various steroids known to occur during pregnancy.

The latter observation is prompted by the findings that steroids in general tend to aggravate or increase the severity of amoebiasis (McAllister, 1962; Mody, 1959; Eisert et. al., 1959; Kanani and Knight, 1969). It is most likely that both cholesterol and steroids have the same or similar influence on amoebic lesions. This is not surprising, since cholesterol is the source of the steroids found in increased amounts in the circulation of normal pregnant patients (DeAlvarez, Gaiser, Simkins, Smith, and Bratvold 1959). Furthermore, Adlersberg and co-workers (1950 and 1955), showed that the administration of cortisone produces an increase in total serum cholesterol.

From the foregoing results it would appear that serum cholesterol level is one of the factors responsible for the severity of amoebiasis during pregnancy and the puerperium. However, it is well accepted that pregnancy produces a state of nutritional, hormonal, circulatory and emotional stress, the quantitative importance of any or all of which is yet to be determined. It is, therefore, possible that other serum factors may still be operating in the severity of amoebiasis during pregnancy. For this reason, a study of the effect of sterols other than cholesterol will also be of interest to determine whether they also possess the property of altering the virulence or whether it is solely confined to cholesterol. For instance, sitosterol which has a chemical constitution like that of cholesterol but possesses the property of binding the latter in the intestine, should also be investigated.

Meanwhile, the finding of the possible role of cholesterol in the severity of amoebiasis can only be regarded as tentative. A more direct possible factor is therefore, being sought in the immunological studies in the following chapter.

TABLE 17: RELATIVE GROWTH EFFECTS OF SERUM SAMPLES FROM VARIOUS HUMAN SOURCES ON ENTAMOEBA HISTOLYTICA (STRAIN HN:IB 16) IN-VITRO

GROUPS OF INDIVIDUAL	No. Tested	GROWTH INDEX ***			
		RANGE	MEDIAN	MEAN ± S.D	% with G.I. Less than 0.9
Adult female Preg. / Puerperium	48	0.25-2.66	1	0.99 ± 0.07	40
Adults (Indigenous Male/ FNP)*	20	0.25-1.50	0.75	0.73 ± 0.06	60
CORD Blood	22	0.20-1.00	0.50	0.44 ± 0.44	80
AMOEBIC Liver Abscess	10	0.25-0.75	0.33	0.32 ± 0.22	100

* FNP = Female Non-pregnant
** S.D. = Standard Deviation
*** Growth Index (GI) Culture growth in test serum $(V^t_{48} - V^t_0)$ TC

Culture growth in standard serum $(V^t_{48} - V^t_0)$ SC

TABLE 18: SERUM CHOLESTEROL AND GROWTH OF ENTAMOEBA HISTOLYTICA IN HUMAN SERA

GROUP OF INDIVIDUALS	No. of Samples	SERUM CHOLESTEROL LEVEL		GROWTH INDEX	
		Mean Value	Standard Deviation	Mean	Standard Deviation
Maternal Blood (normal deliveries)	25	198	47	1.97	0.94
Pregnant Women	19	179	44	0.97	0.50
Miscellaneous (Sick Males and 5 non-preg) normal females	15	166	50	0.72	0.36
Adult Male Blood Donors	21	117	40	0.53	0.24
CORD BLOOD (Normal deliveries)	15	80	50	0.51	0.35

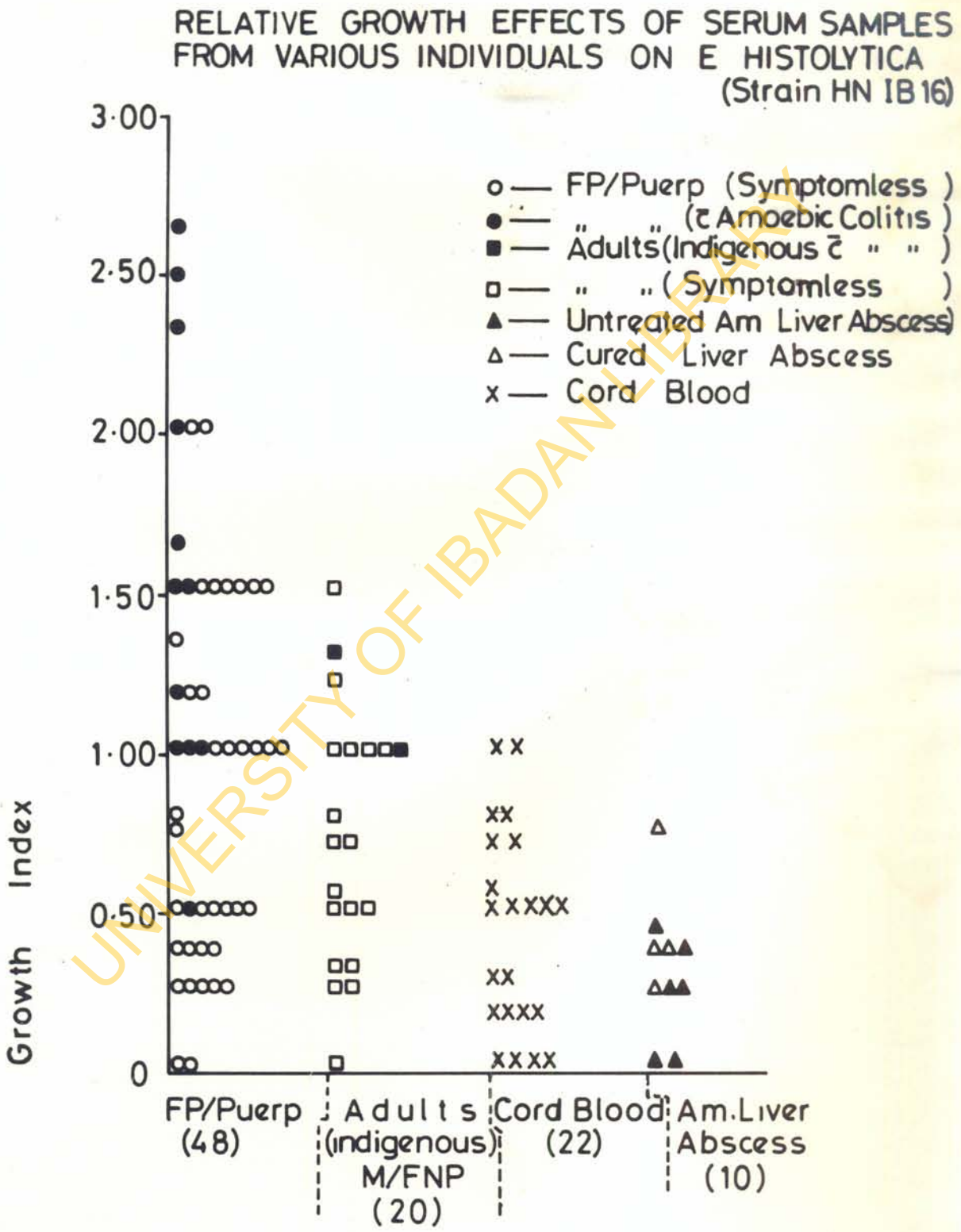


Fig. 22

SERUM CHOLESTEROL LEVEL AND GROWTH EFFECTS OF SERUM-SAMPLES ON E · HISTOLYTICA (STRAIN HN : IB 16)

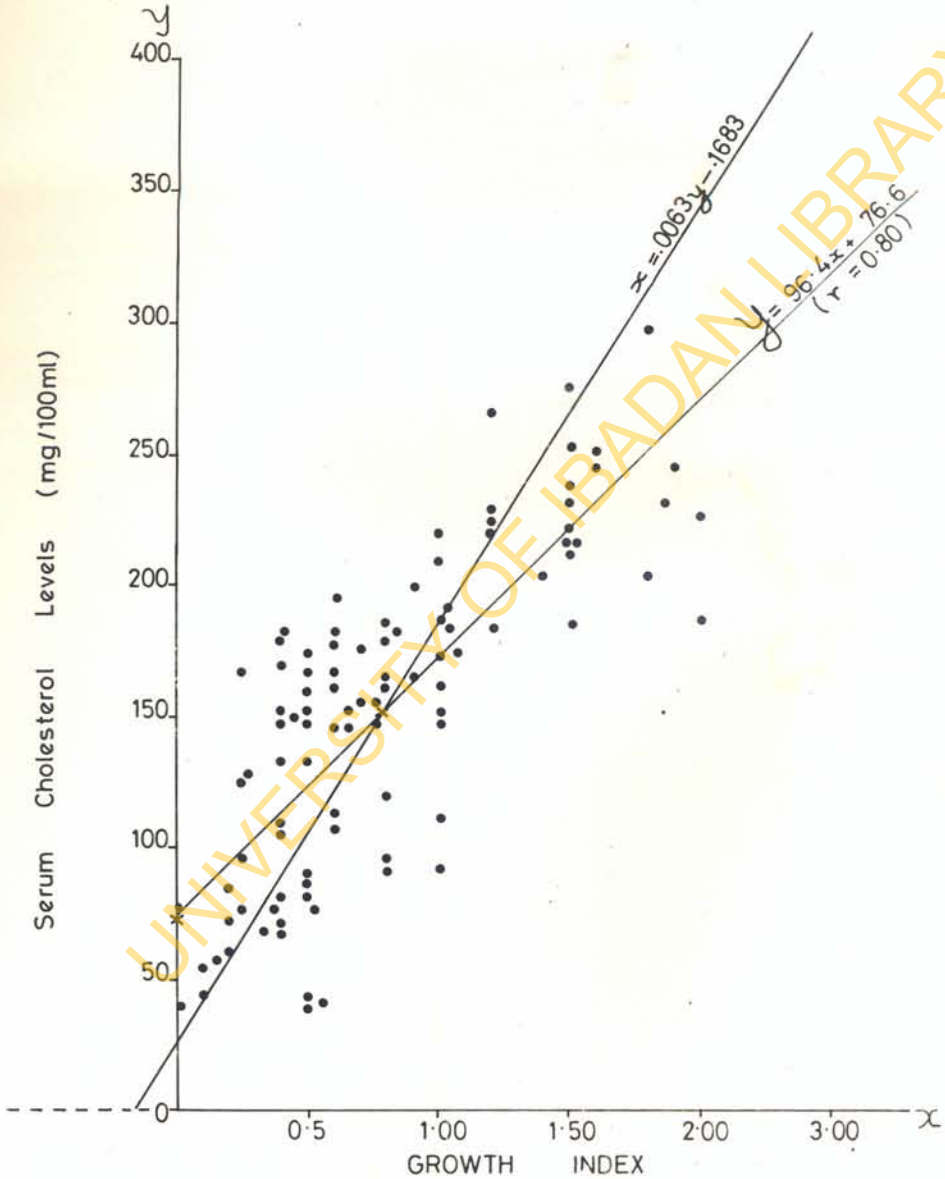


Fig. 23a.

SERUM CHOLESTEROL LEVELS AND GROWTH EFFECTS OF SERUM SAMPLES ON E · HISTOLYTICA (STRAIN HN : IB 16)

Regression Equation $y = 96{\cdot}4x + 76{\cdot}6$

y = Serum cholesterol level

x = Growth Index

Coefficient of Correlation $r = 0{\cdot}80$

(Significance of r) $t = 12{\cdot}34$ (98 degrees of freedom)

$P < .001$

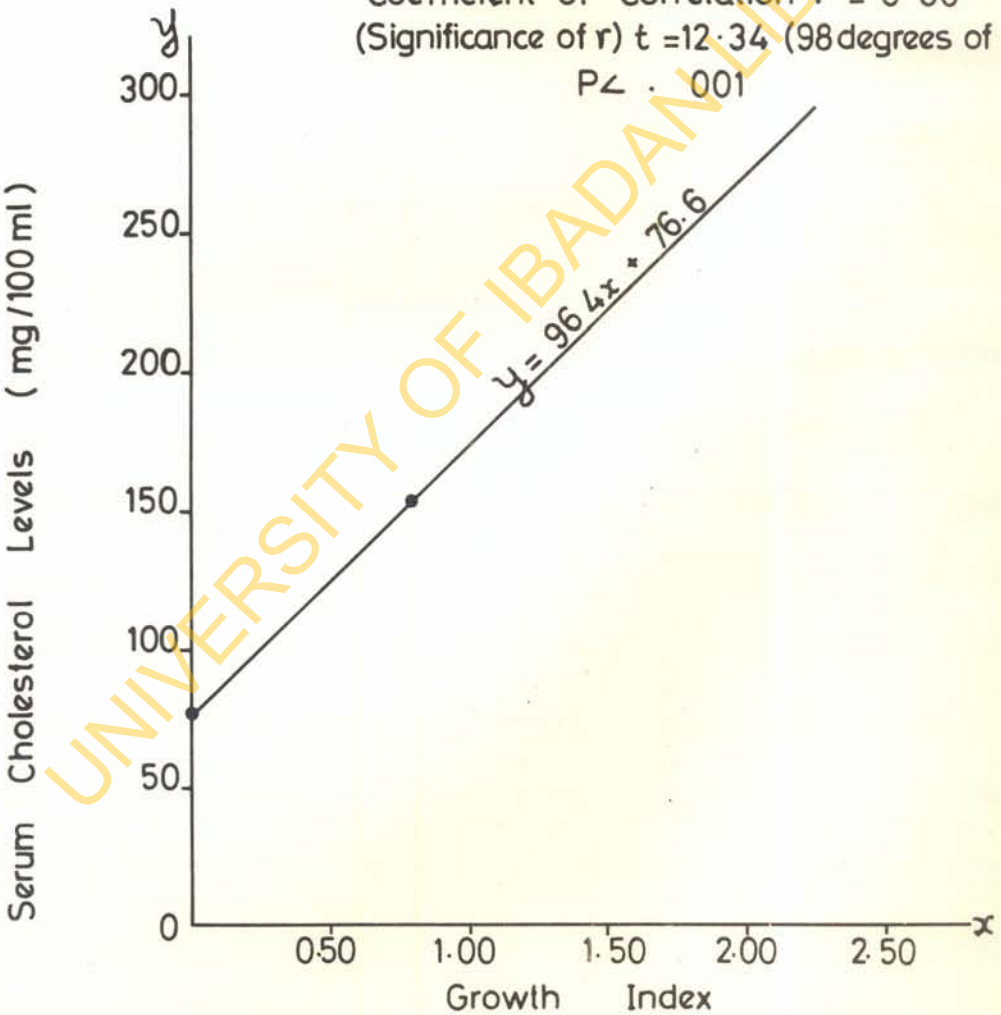


Fig. 23b

CHAPTER VII

IMMUNOLOGICAL ASPECTS OF AMOEBIASIS

SECTION I: Characterization And Antigenic Reactivity Of E.h. Extracts:

7.1 Introduction:

The findings in chapter six give an indirect indication that serum factors may be of importance in the consideration of the cause of the severity of amoebiasis during pregnancy and in the puerperium. The suggestion made in the chapter concerning steroids which are known to aggravate the severity of the disease is also worthy of note. This is so because, steroids belong to a class of agents labelled as "immuno-suppressive agents". The fact that cholesterol which was found to be the indirect agent, mentioned in the chapter under reference, is related to the so-called immuno-suppressive agents (steroids), encourage a further search for a more direct serum factor in immunological studies.

Attempts, are therefore, made in the present chapter to evaluate the role of humoral immunity in the severity of amoebiasis in general and during pregnancy and the puerperium in particular. It has been shown that a wide spectrum of antigenic substances must be released into circulation during the course of amoebic infection (Sen, Mukerjee, and Ray, 1961; Kessel et. al., 1965; Auernheimer, et. al., 1962; Meerovitch et. al., 1967; and Goldman et. al., 1967). It is also clear that many of these substances are capable of promoting immune response, but the nature and potency of these antigenic substances are still controversial.

Various speculations and suggestions have been made regarding these controversial aspects of amoebic antigens. Thus Lewis and Kessel (1967), suggested the use of high molecular weight antigens for equally high antigenic activity; Atchley and his co-workers (1962), advocated for the strong standardisation of antigens before utilizing serological tests for the diagnosis of amoebiasis. Ali Khan (1968) indicated the need for fractionation of amoebic antigens for more precise results of similar tests in amoebiasis.

Considering all the foregoing points together with the fact that amoebic antigens are not available on the market at the time of this work, it was decided that amoebic antigens be prepared and characterized as much as practicable before use. This is in accordance with the suggestion and the general belief that it is important to characterize the antigenic complexity of any diagnostic and immunogenic materials.

7.2. Preparation Of Amoebic Antigen(s)

7.2.1. Materials And Methods:

Amoebae Strains, Culture Media And Cultivation:-

Four strains of *E. histolytica* isolated from human faeces as described in chapter five, were used for the preparation of antigen(s). These local strains are (i) HN:IE 5, isolated from the stool of an eleven year old girl with acute amoebic colitis. (ii) Strain HN:IE 8 (Hospital No. 10736) - an adult male patient with amoebic colitis; (iii) Strain

HN:IB 10 (AO 109359)-isolated from the stool of a five-month pregnant woman suffering from acute amoebic dysentery and (iv) strain HN:IB 4 cultured from the contents of an amoebic liver abscess from an adult female patient admitted to the Medical Unit of University College Hospital, Ibadan.

The method and medium for cultivation were those of Robinson's with some modifications as already noted in chapter 5. Horse serum obtained from Burroughs Wellcome (Wellcome Reagents Limited), was used in the mass cultivation of the organism. The cultures were monitored periodically for the presence of contaminants. Examination for Esch. coli was included as an integral part of the preparation of the antigen(s). Agar-plating on McConkey medium was used to confirm Esch. coli as the sole concomittant organism in the culture medium.

The strains were maintained in screw-capped bottles (16 mm x 65 mm) containing 10 ml. of medium incubated upright at 37°C. Transfers and doubling subcultures were made on alternate days until sufficient numbers of cultures were available for subculturing into larger bottles ready for mass cultivation. Growth was visible macroscopically as greyish white deposit.

Large scale cultivation was carried out similarly in 200 ml screw-capped Erlenmeyer flasks containing 50 mls of medium; and/or 250 ml screw-capped bottles containing similar amount of medium. The cultures were incubated at 37°C. The flasks/bottles were inoculated with the content of two smaller bottles with approximately 1×10^6 to 2×10^6

amoebae from 48 hour old cultures and cultures 'harvested' 48 hours later (Fig. 24)

Extraction Of Antigens:

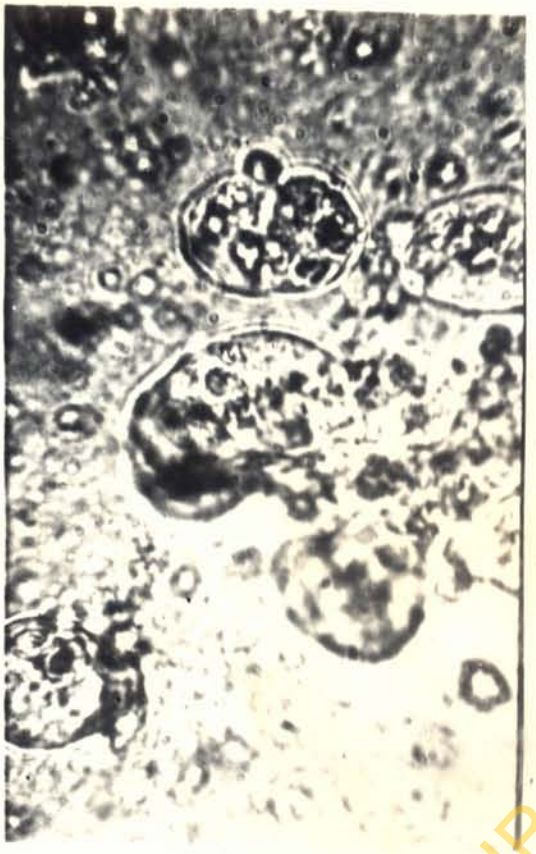
The culture bottles were chilled quickly by immersion for 10 mins in iced ethyl-alcohol to dislodge amoebae adhering to the glass. Trophozoites from 48 hour-cultures were pooled and washed four to six times by mixing with 0.25M sucrose. The amoebae were concentrated by centrifugation (at 250g for 10 mins.) and resuspended in pH 7.2 phosphate buffered saline. Duplicate counts of amoebae were made by means of haemocytometer. The suspension was adjusted to concentration of 8×10^6 amoebae/ml. on the basis of the haemocytometer counts of viable organisms.

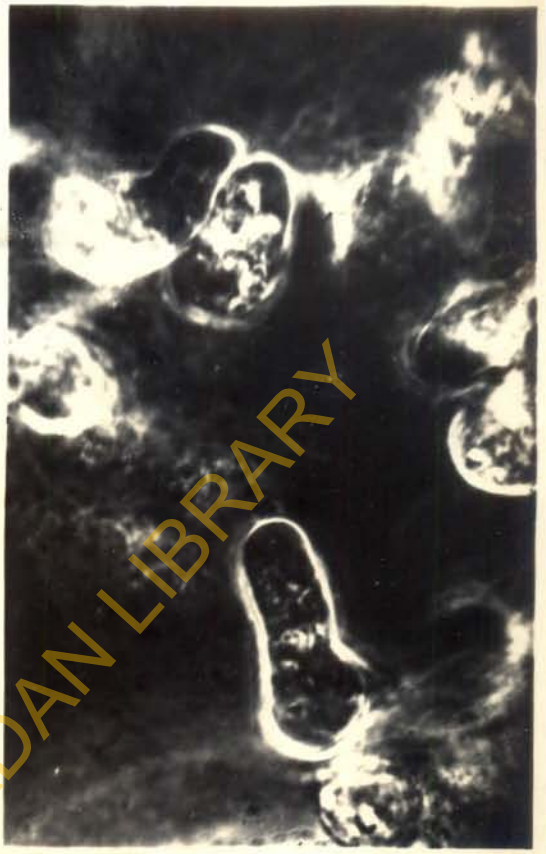
Harvesting during the period of exponential growth as determined during the establishment and propagation of E. h. in-vitro, was among the precautions taken to ensure that the greater number of the amoebae included in the antigen preparation were viable. Others include examination for motility, normal cytology and a reaction to a supravital stain as detailed under the culture methods in chapter 5.

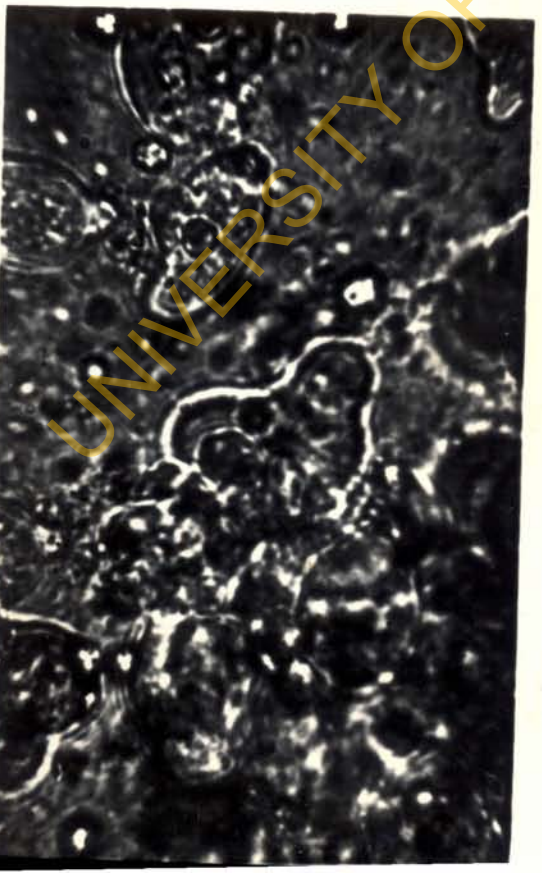
Initially, the amoebae suspension were ultrasonicated for 1-2 minutes in an iced-water bath by the use of an MSE ultrasonic disintegrator (20 K Hz, 100-W; Measuring and Scientific Equipment Ltd., London, England). Preparations were centrifuged at 4500 revolutions per min. for 30 minutes in the cold, to remove the remaining particles which were

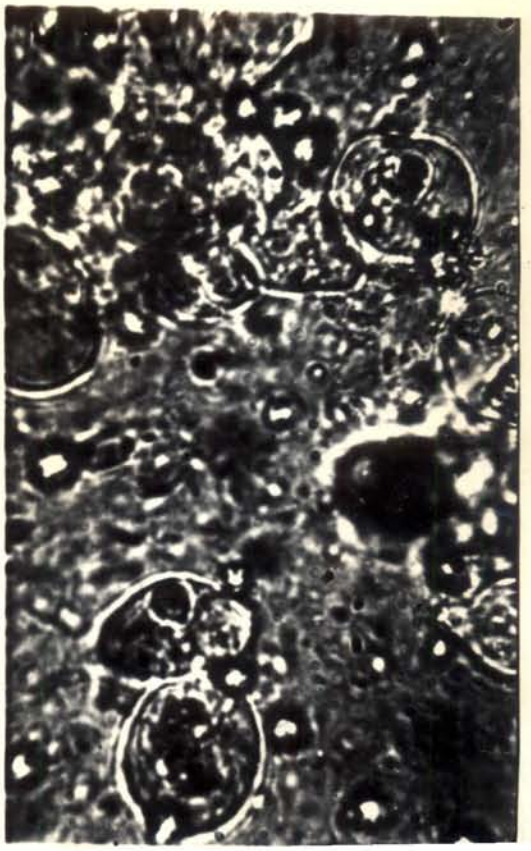
(To Face Page 168).

Fig. 24. Different (Local) Strains of E. histolytica In In-Vitro Cultures









discarded. The opalescent supernatants were quickly frozen and stored at -20°C to -70°C.

Alternatively, the washed sediment was transferred to small rubber capped bottles and stored temporarily at -70°C. Further preparation of antigen was thereafter carried out by thawing the material in iced alcohol bath so as to disintegrate the amoebae. The material was then centrifuged at 4500g revolutions per minute for 45 minutes in the cold. The supernatant was ultrafiltered through cellulose dialysis tubing of 3/32-inch diameter under vaccuum of 5 to 10 cm Hg at 4°C (Fig. 25). The extract was centrifuged once more for clarification and stored in a vial at -70°C.

The extract is dispensed in 2.5 ml ampoules, for long-term preservation, and the contents frozen in a freezer at -70°C. Freeze-drying was done in Edwards Centrifugal Freeze Drying Apparatus (Speedivac Model 5PS Gallenkamp), the shelf temperature was maintained at 0°C for eight hours, after which the ampoules were sealed under vaccuum.

The total peotein contents of the antigens were determined by the Biuret methods (Kabat, 1964).

The extracts of Esch. coli - the sole concomittant organism in the E.h. culture medium; was prepared and disintegrated by some oscillation 20 Kc/8cc for 30 minutes. After centrifugation at 4500 revolutions per minute, the supernatant was concentrated by ultrafiltration as described above for amoebic antigen(s).

Fig. 25

Vaccuum Pump showing ultra-filtration of E.h.antigen using cellulose dialysis tubing 8/32-inch-diameter. (Appendix 4 refers).

7.3 Antigenic Specificity And Characterization Of E.h. Extracts:

7.3.1 Rabbit-Anti-Amoebic Sera: Immunization of rabbits with E.h. antigens was carried out by the use of E.h. antigens prepared from the local strains established and described in the course of this study. The amoebae strains grown in in-vitro culture for 36 to 48 hours consisted of trophozoites and occasional cysts.

Both disintergrated amoebae (of known protein contents) and 'intact' organisms obtained from in-vitro cultures (a total number of 3 to 4 millions amoebae) were used as antigens for the purpose of raising anti-amoebic serum from rabbits. The antigens were separately injected into paired adult rabbits (R_1 - R_8) according to the following programme:

In each case, after harvesting the amoebae from culture, they were washed in normal saline and concentrated by gentle centrifugation 500 to 800 rpm for 10 minutes. This process was repeated three times.

(a) On day 1, after withdrawing 1 cc of blood intra-cardially, a rabbit from each pair received 1 ml. of 2.5×10^6 amoebae, given in both footpads and subcutaneously in the thigh. In the 4th week the same rabbit was given a second injection of 0.5 ml. of packed amoebae containing 2×10^6 amoebae/ml, subcutaneously and three days later given 0.5 ml of packed amoebae containing 1×10^6 amoebae ml. intramuscularly.

(b) The second rabbit from each pair was given a total of 3-4 million amoebae given as five intravenous injections spaced at 4-day intervals. On the 7th day after the last injection a test bleeding of 3-4ml of blood was taken from the marginal vein of ear from each rabbit to obtain antiserum.

Four weeks after the last injection each rabbit was given a booster dose 1 ml of 2 mg/ml antigen thoroughly emulsified in an equal volume of complete Freund's adjuvant (Appendix 7); and administered in multiple sites. Blood taken on each occasion, was allowed to clot and sera were separated in the cold by centrifugation. The potency of the antisera was tested by agar-gel diffusion against the locally prepared E.h. antigen(s) of different protein concentrations (Fig.26). Satisfactory blood sera were preserved by adding few drops of 0.1 merthiolate and then stored at 4°C until used.

Specificity of each batch of antigen and anti-amoebic rabbit sera were tested against reference antigens obtained from abroad. The latter included few ampoules from Dr. Powell and about a total of 1 ml of antigen from Park Davis (prepared respectively from DKB; and 200:NIH/HK 9 strains of E. histolytica. Further studies on specificity were carried out by absorption methods as would be described later on. All these studies were made before the antigens were used for immunodiagnosis of human amoebiasis.

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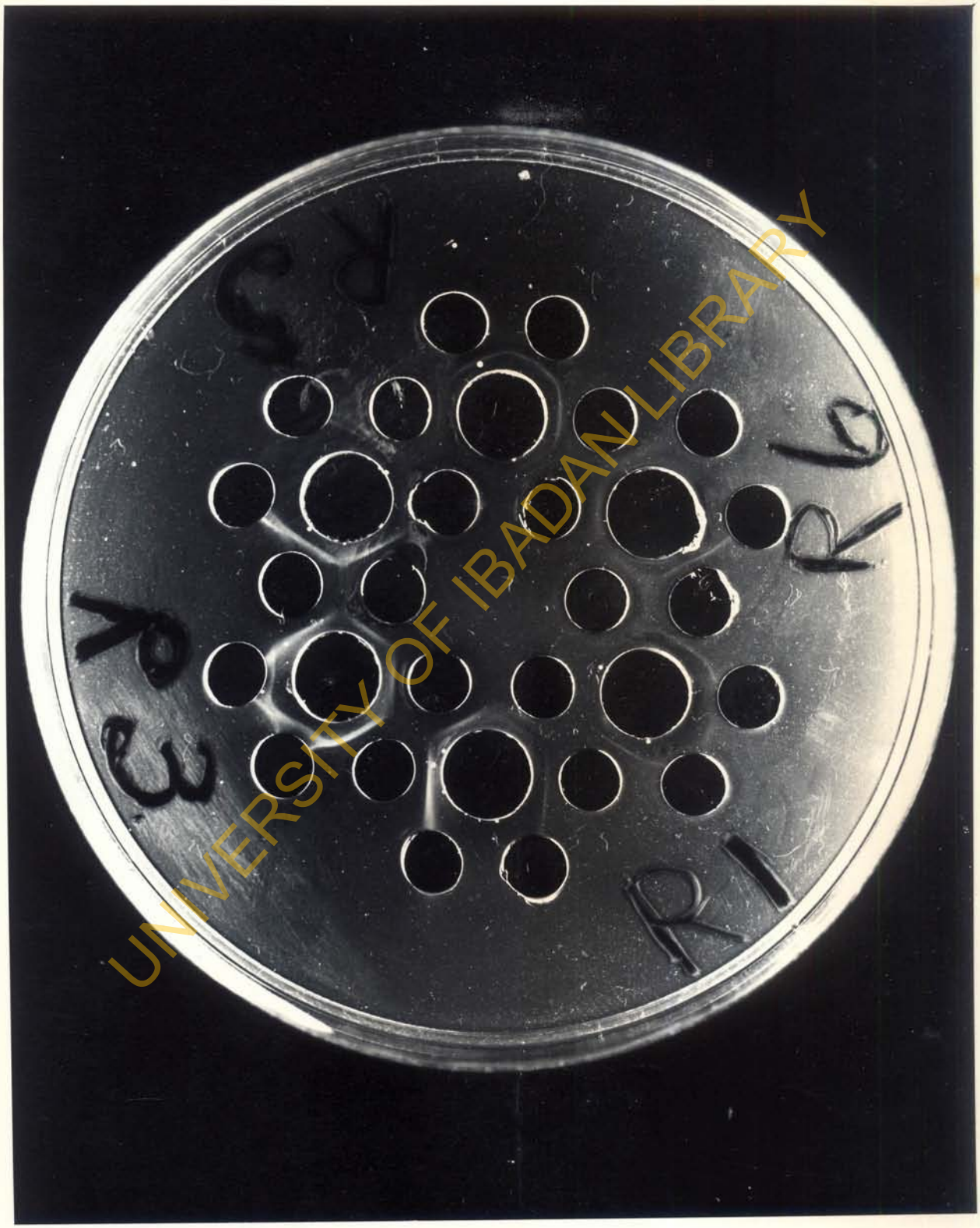
Fig. 26a.

Agar-gel showing reaction of Rabbit - anti-E. h. sera with E. h. antigen in central wells.

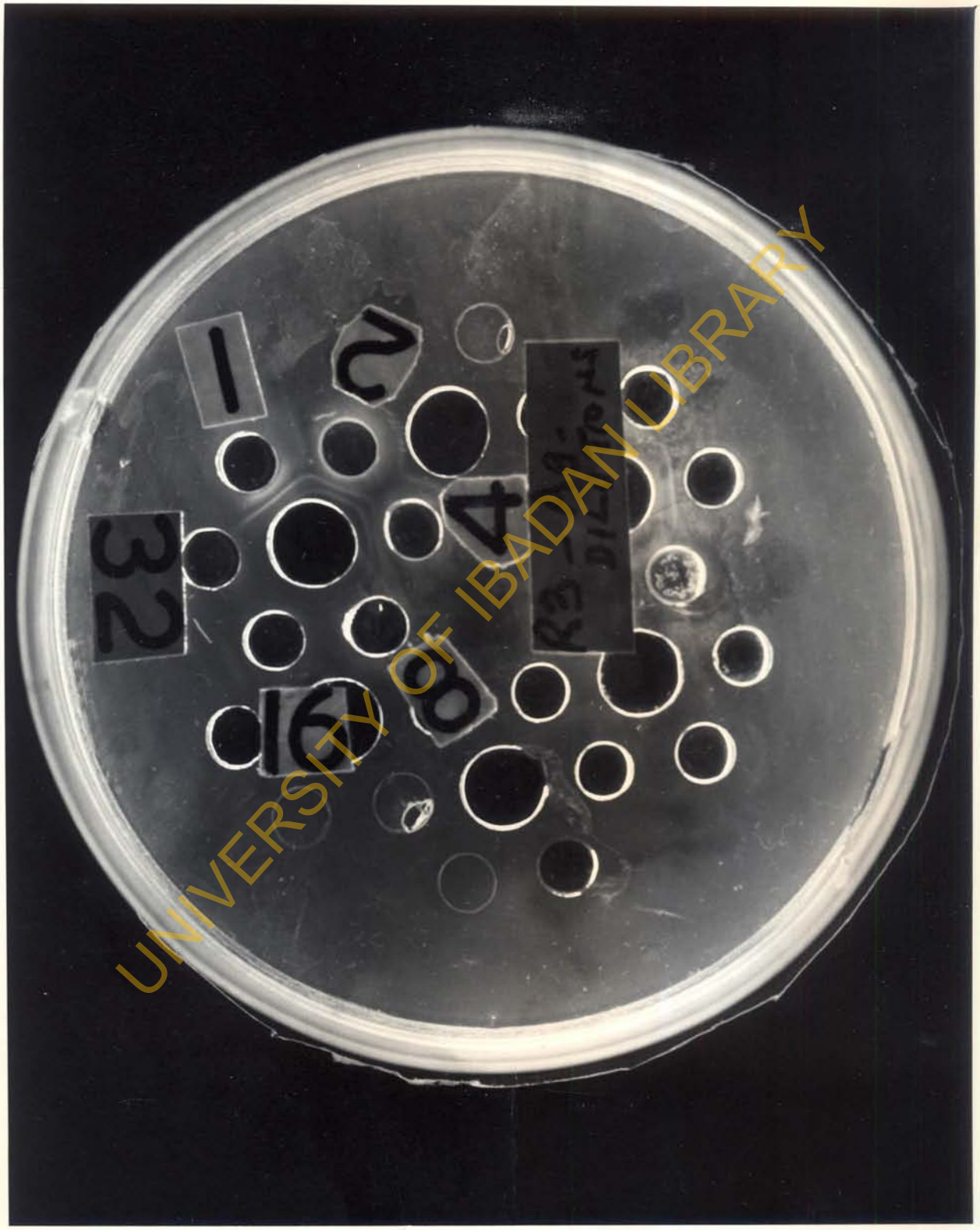


(Fig. 26a)

(To Face Page 174).

Fig. 26b

Agar-gel plate showing reaction of Rabbit - anti-Eh Sera at different dilutions.



(Fig. 26 b)

7.3.2 Absorption Studies:

These were carried out in four different stages as follows:

(a) Absorption of rabbit antiserum and sera from cases of amoebic infection with Esch. coli antigen. Human and rabbit anti-sera were absorbed with each antigen for one hour at 37°C and overnight at 8°C. About 1 ml of serum was added to a thoroughly washed Esch. coli culture, mixed thoroughly, and centrifuged after having been kept at the above mentioned temperatures. The absorbed sera were later tested with E. histolytica antigen(s) using gel diffusion technique. An unabsorbed serum specimen was included as a control. Normal human serum was also used as another control. The antigen was replaced by normal saline as control in some of the tests.

(b) Absorption Of Antisera With Amoebic Antigen(s):

Amoebic antigens were added to both sera from known cases of amoebiasis and also to rabbit anti-E.h. sera, kept at the above mentioned temperatures, and centrifuged. The supernatant in each case was then tested against amoebic antigens side by side with unabsorbed sera and normal human sera and unimmunized rabbit sera as control. Other experiments were performed using anti-Esch. coli rabbit sera against locally prepared amoebic antigens to exclude possible cross-reactivity and thereby ascertain the specificity of E.h. antigens.

7.3.3 Physico-Chemical Analysis Of Amoebic Extracts:

7.3.3.1 Sephadex G- 200 Fractionation Of Antigen(s):

Antigen of strain HN:IB 5 (total protein 10 mg) equivalent to 200 x 10^6 amoebae/ml was fractionated by gel filtration on sephadex G - 200 (Pharmacia, Uppsala, Sweden). The fractionation procedure was performed thrice using 2 ml of antigen each time. This volume of antigen was passed through a column (1.5 x 55cm) of sephadex G-200. The column was equilibrated and eluted with 0.01M phosphate buffer pH 7.4 containing 0.1% merthiolate. The flow rate was adjusted to 10ml/hour and 5ml fractions were collected by means of the LKB fraction collector equipped with a Uvicord assembly, set at 259mμ and a recorder. The eluted fractions were read at 260 and 280 mμ in a Beckman Sp 500 Spectrophotometer. The readings were plotted against tube numbers/volume in mls.

Using the graph, the contents of the tubes were pooled to form the fractions. After pooling, the fractions were concentrated to the original whole antigen volume by ultrafiltration using 8/32-inch visking tubing. The various fractions were compared with each other and with the whole antigen by means of agar-gel diffusion test. The effects of total protein concentrations on the elution pattern was examined by repeating the fractionation with different batches of antigen(s) prepared from the same strain of E. histolytica, but containing 5 mg and 12mg/ml protein instead of 10mg/ml. The last batches were kept longer with the resultant collection of greater numbers of tubes.

7.3.3.2 Estimation Of Diffusion Coefficient Of E.h. Antigens:

(Using Double Diffusion From Troughs At Right Angles)

An agar layer 4mm deep on a flat glass plate 10 x 10cm was prepared with two thin rectangular troughs at right angles to one another, but separated by 1mm. Stainless steel template bars 1.25cm in width and 10cm in length were placed at 90° face downwards on the glass. Difco agar 1.5% w/v prepared in M/15 phosphate buffered saline pH7.2 containing 0.1% merthiolate was poured onto the glass with the templates in position. The templates were then carefully removed without disturbing the agar after it had set. The arrangement resembled that described by Elek (1949).

Equal volumes of amoebic antigens and rabbit anti-amoebic serum were then placed in the trough. These were allowed to diffuse in moist chamber for 48 hours and photographs taken. The angle between the precipitin line and the antigen trough was measured with a protractor to within $\frac{1}{2}^{\circ}$ on an immage enlarged several times, projected on to a walled cardboard screen (Fig. 28). With a known value of diffusion, coefficient of antibody (D_{ab}) rabbit globulin (Kabat, 1964), the coefficient of diffusion (D_{ag}) of amoebic antigen was calculated.

7.3.3.3 Diffusion Method For Estimating Size Of E.h. Antigen:

Flat sheets of 1.5% agar gel were prepared as in experiment (...2) above. A row of holes 7-8mm diameter, and about 5mm apart were cut with a cork borer. These holes were filled with solutions of molten agar

at 100°C made up at various concentrations (ranging from 2 - 7%) in the same buffer as the agar sheets. After setting at room temperature, cores 3-4mm diameter were cut from the centre of each agar plug, leaving cylinders with walls approximately 2mm thick. A narrow trough for antiserum was next cut in the agar sheet about 7.5mm from the row of cylinders and the plate set aside for 24 hours in moist chamber in the cold for the gels to 'mature' and attain stable and reproducible properties.

Antigen solution was then placed in the cylinders and antiserum in the trough. Reaction was allowed to take place in most chamber for ' 2-3 days.

7.3.3.4 Ultra-Violet Absorption Spectra Of E. histolytica Antigens:

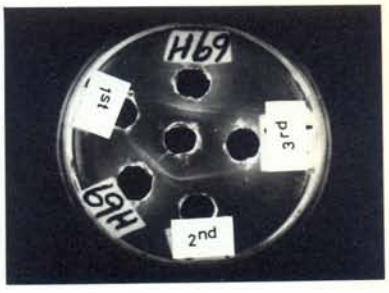
The ultra-violet absorption spectra of the whole antigen(s) and fractions were examined between 200 and 340mμ using the same Spectrophotometer Sp 500 mentioned above. This was done by taking readings at various wavelengths starting from 200mμ running through the stated range and then plotting the results as the 'Absorbance' against wavelengths.

7.3.3.5 Immunoelectrophoretic Separation Of E. histolytica Antigen:

The locally prepared E. h. antigens were subjected to immunoelectrophoretic analysis using 1.2% Oxoid Noble agar and Veronal buffer pH 8.6. The technique of Williams and Grabar (1955) and Grabar (1959) was used with only slight modification. Agar was prepared at a concentration of 1.2% in Veronal buffer pH 8.6 and ionic strength 0.5. A potential

29b. SUMMARY OF IMMUNODIFFUSION TESTS FOR AMOEBIASIS.

AGAR – GELL REACTION IN AMOEBIASIS



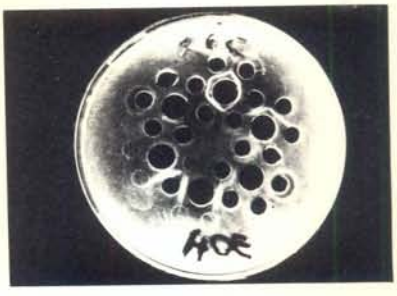
1. Immunodiffusion Analysis of Chromatographic Fractions/whole Amoebiasis Serum.
1st = 1st Fraction
2nd = 2nd Fraction
3 = 3rd Fraction
69H = Whole Serum

3 REACTION OF IDENTITY
Local Ag/Hk 9

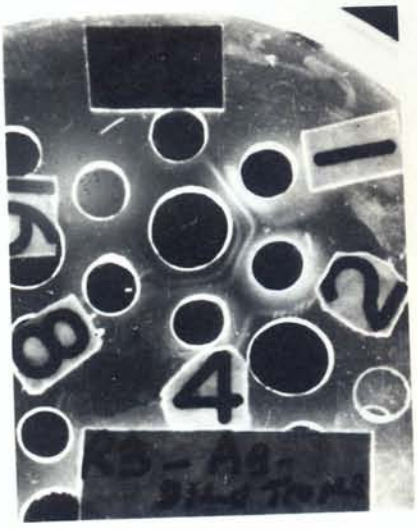
Vs 67H - Amoebic Liver Abscess Serum
29H - Amoebic Liver Abscess Serum
R3 - Rabbit - anti-E.h. Serum

2. DIFFERENTIATION Of Amoebic Liver Abscess from Pyogenic Abscess by gel diffusion test.
PM42 = Pyogenic Abscess (PM)
PM51 = Amoebic Liver Abscess (PM)
67H = Clinical Amoebic Liver Abscess
R23 = Rabbit anti-E.h. Serum
RAEC = Control E.coli Ag.

Vs E.h. antigen



4. STANDARDISATION OF LOCAL E.h. Antigen REACTION OF IDENTITY.
Antigens Vs Amoebic Liver Abscess Serum.

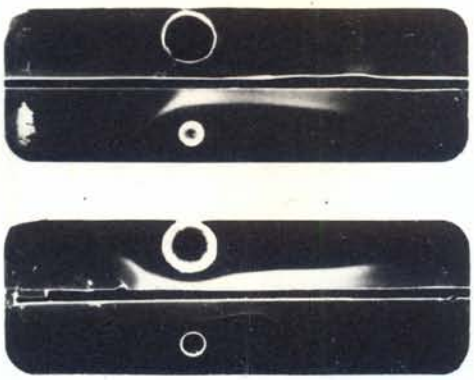


9

STANDARDISATION OF E. h. ANTIGENS
BY IMMUNODIFFUSION
Ag Dilutions Vs Rabbit Anti-E. h. Serum

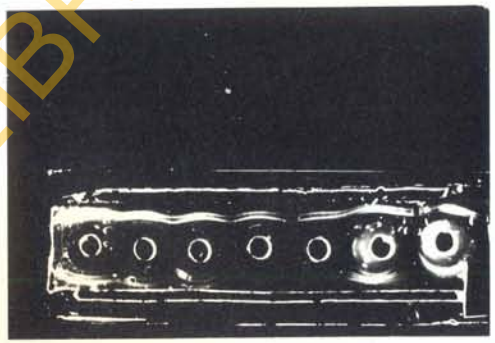
10. REACTION OF IDENTITY
WHOLE E. h. ANTIGEN
AND CHROMATOGRAPHY
FRACTIONS 1 and 2

1 = 1st Fraction
2 = 2nd Fraction
5 = Whole Antigen



11. Immunoelectrophoretic analysis .
of E. h. antigen Vs Amoebiasis Serum.

5. E. H. Antigen Control Studies
1. DKB (E. h.)
2. E. coli Antigen
3. Strain HN:IB 5
4. HK 9
5. Horse Serum
6. Strain HN:IB 16

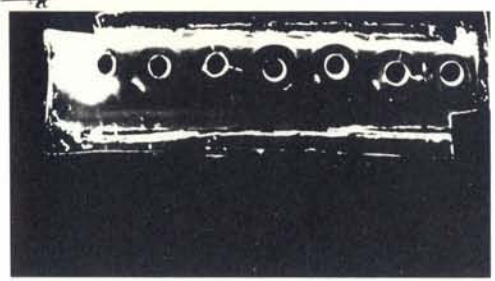


↑ Human Amoebiasis Serum = Upper Trough

↓ Rabbit-anti-E. h. Serum = Lower Trough

% Agar ring

1 2 3 4 5 6 7



6.

SIZE OF ANTIGEN
COMPARISON OF DIFFUSIONS
Ag/Ab. Trough. Agar gel

DETERMINATION OF CO-EFFICIENT OF DIFFUSION OF E.HISTOLYTICA ANTIGEN BY TWO DIMENSIONAL DOUBLE DIFFUSION PLATE TECHNIQUE WITH RECTANGULAR BASINS AT RIGHT ANGLES

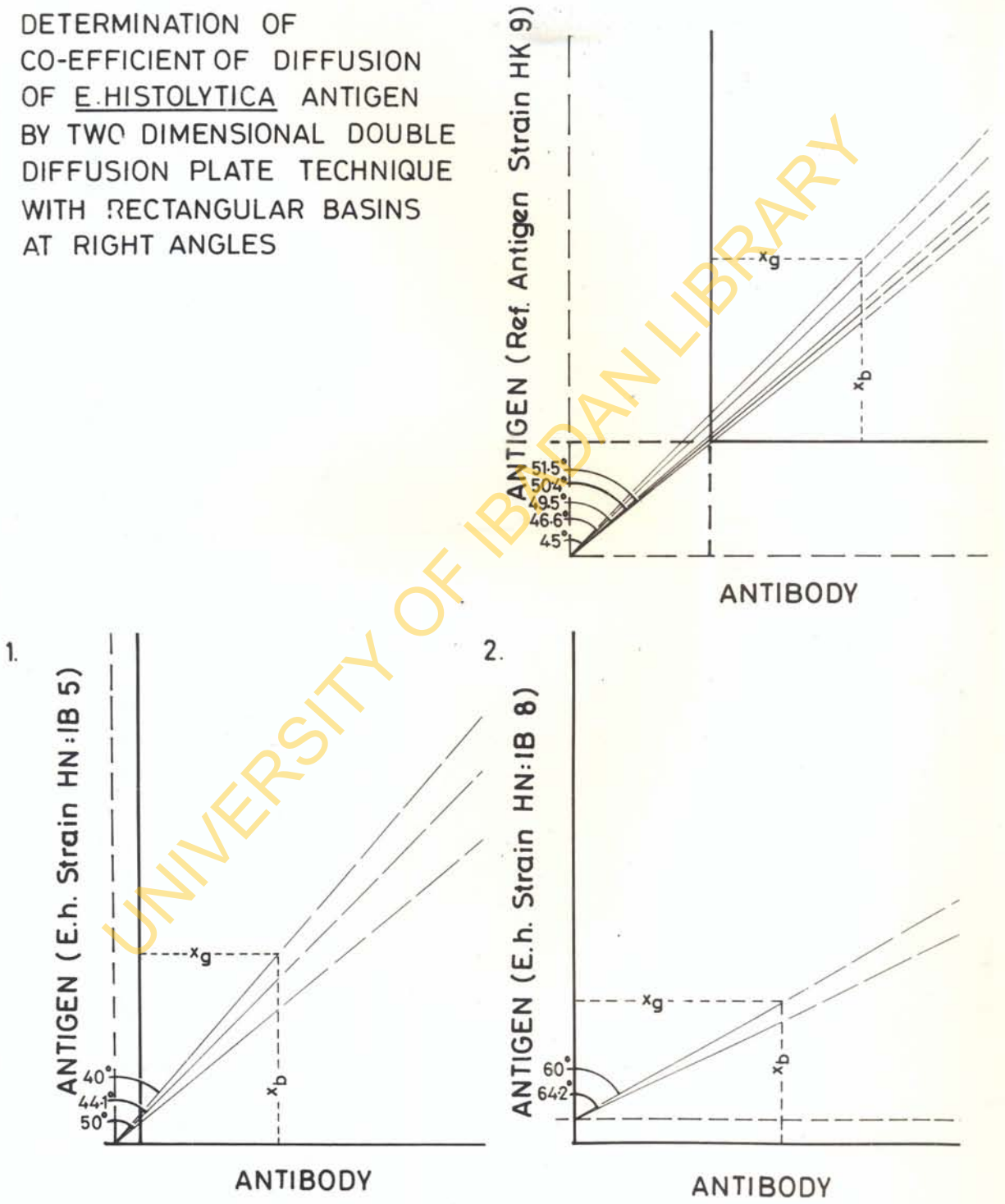
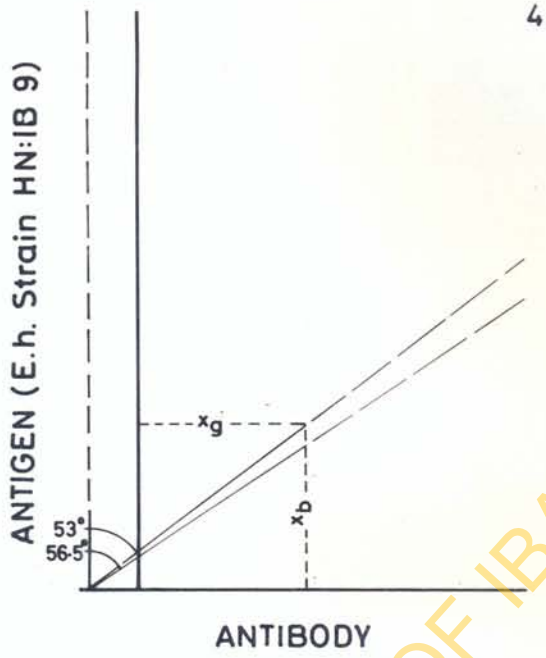
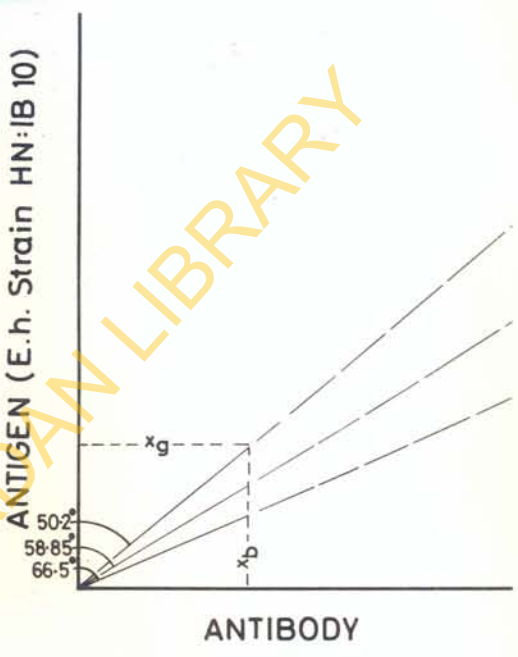


Fig. 28.

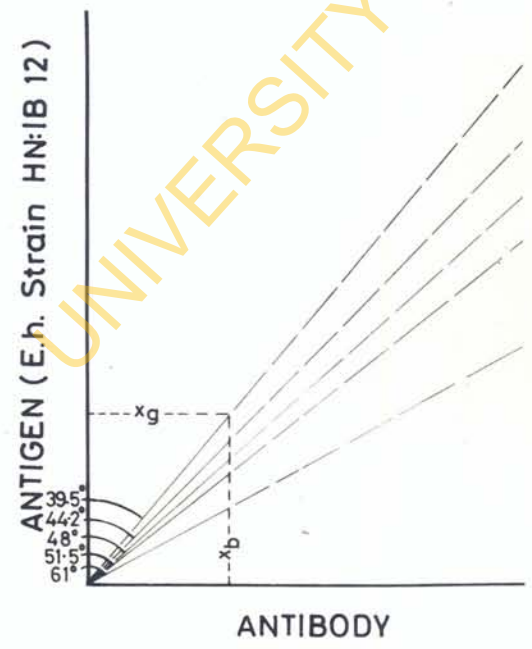
3.



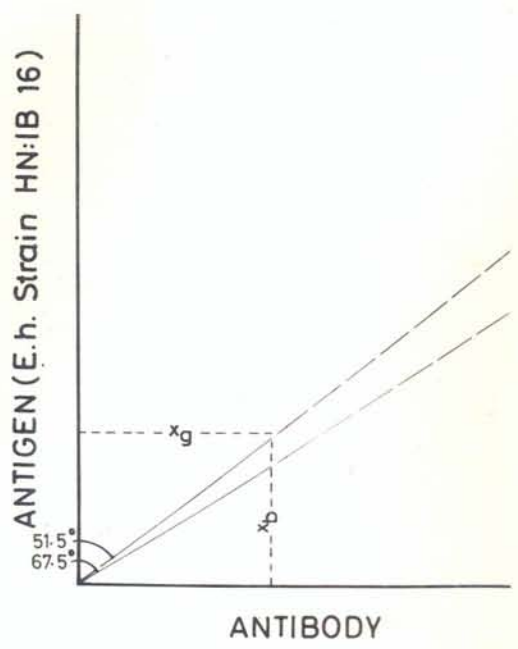
4.



5.



6.



7.

RT. Angled Troughs = (Gel diffusion) Method
For the determination of coefficient of diffusion of E. h. antigen

Vertical trough - E. h. Antigen
Horizontal Trough - Rabbit-anti-E. h. Serum.

8. Characterization of E. h. Antigen
Absorption (Heat Test)

Rt = Ag. at room temp.,
56° = 100°
E1 = E. coli absorbed antigen at room temp.
E2 = E. coli absorbed E. h. antigen at 56°
E3 = E. coli absorbed E. h. antigen at 100°
69H = Amoebiasis Serum.

difference of 6 volts/cm of agar over a period of 2 hours at room temperature allowed adequate differentiation of the α_1 and α_2, and B-globulins of human serum (included as a marker).

Lines were developed with the anti-amoebic sera raised in rabbits. The agar slides were kept in moist chambers at room temperature ($19^{\circ}C \pm 2^{\circ}C$) for 3-4 days. Most of the precipitation arcs developed by this time but a few required up to 5 days. Slides were washed in saline for 5 days and photographs taken afterwards.

7.3.3.6 Ouchterlony Immunodiffusion Test (Agar:gel:)

Locally prepared E. histolytica antigens were diffused in agar gel against various dilutions of sera from different sources:

(i) human sera from patients with liver abscess/colitis;

(ii) human sera from symptomless cyst carriers;

(iii) human sera from blood donors (UCH, Ibadan, Blood Bank);

(iv) Antisera raised in rabbits against E.H. in cultures;

The double diffusion in agar was performed as described by Ouchterlony (1953) using a solution of 1.2% purified agar (Diffco Laboratories, Detroit, Michigan) with 0.1% Merthiolate in PBS pH 7.2. Patterns were cut out in the agar gel with a template similar to that described by Gell (1957) and Southill (1962). Approximately 0.5 µml of appropriate antisera were placed in the large central wells and about 0.25 µml of test sera or amoebic antigens were placed in the peripheral wells. The precipitin

lines were allowed to develop in a moist chamber at 18-20°C. They were read on a viewing box at 24, 48, and up to 72 hours.

7.4 Results:

7.4.1 Serological Activity Of Antigens:

Adequate precaution was taken in choosing each step in the preparation of the antigens to ensure the retention of maximum antigenic activity in the extracts. Ultrasonication, freezing and thawing are among the different methods of disintegration of amoebic trophozoite or cysts which lend themselves to easy comparisons. For the purpose of such comparisons, agar gel diffusion test was selected.

Although this method is said to be insensitive, it is specific and gives information about different antigen-antibody systems. Using this serological technique it was shown that the extracts from the local strains of *E. histolytica* are immunogenic and capable of exciting antibody formation in rabbits. Comparison with antigens obtained from other strains from abroad notably strains HK 9 and DKB give reactions of identify on agar gel with the local extracts (Fig. 39). There was no appreciable difference in this reaction as regards the method of isolation of the antigens.

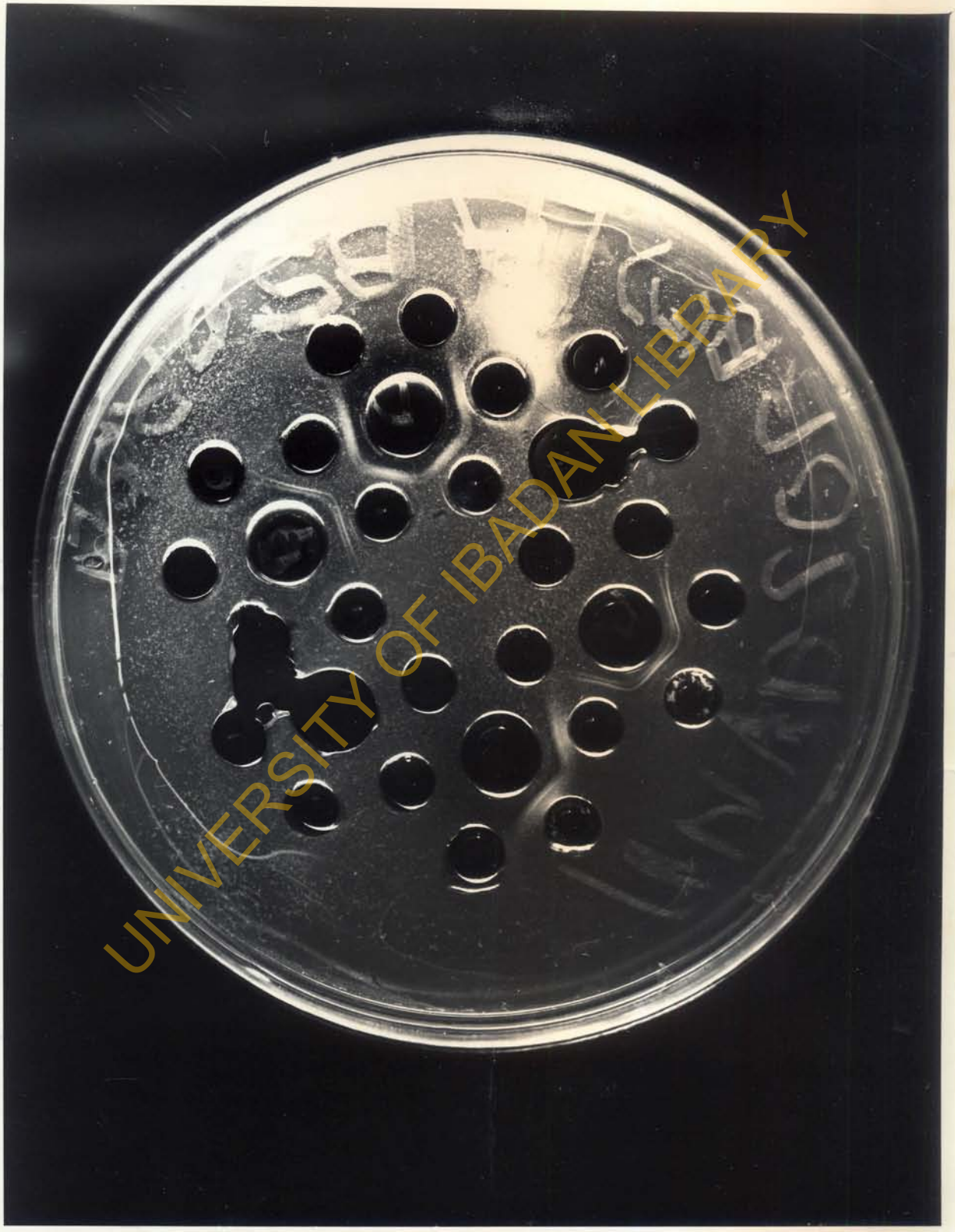
Extracts obtained using both ultrasonication and the method of freezing and thawing, give precipitin patterns consisting of lines ranging from two to five in number depending on the batch of antigens or sera employed in the tests.

(To Face Page 186).

Fig. 29 a

Agar-gel plate showing the immuno diffusion reaction of E. h. antigens absorbed with rabbit anti-E-coli serum

Fig. 29b - Summary of Immunodiffusion test for Amoebiasis.



7.4.2 Absorption Studies:

Absorption of both rabbit antisera and sera from patients suffering from amoebiasis with antigens, removed the precipitating antibody in agar gel diffusion test. On the specificity of the E.h. antigens, both the patients sera and anti-sera raised from rabbits gave no precipitin reaction to bacterial associates in the culture medium (Fig. 29a).

7.4.3 Physico-Chemical Analyses Of Amoebic Extracts:

7.4.3.1 Column Chromatography:

Sephadex G-200 separation of E.h. antigens gave five peaks of proteins (Figs 30 a&b). Only two of these have major 280mu absorbing peaks; a relatively sharp peak fraction I and a broader peak fraction V. Agar gel diffusion tests show that fractions IV and V are devoid of any precipitin activity (Fig. 30b). Fractions I, II, and III have one common precipitin line ( ).

7.4.3.2 Ultra-Violet Absorption:

In addition to the distinct physico-chemical properties which were utilized in separating the various components, E.h. antigens also exhibit marked differences in their absorption of ultra-violet light. The ultra-violet absorption spectra of the antigens and their fractions examined between 200mμ and 400mμ using Beckman's Spectrophotometer are shown in Table 19 and (Figs. 32). The absorption spectra differed both

qualitatively and quantitavely. The two fractions that are reactive in gel diffusion test, as well as, the whole antigens have optimum absorption between 260mμ and 230mμ. The curves of these reactive elements in the antigens reveal reasonably well-defined minima at about 258 mμ and maxima at about 278mμ. On the other hand, the curves of the least or non-reactive fractions (in agar gel) revealed only broad and gradual changes in slope similar to that exhibited by conjugated proteins. In general the curves of the absorption spectra of the intact E. histolytica antigens and the reactive fractions are similar (separately) to the absorption spectrum of Deoxy-5-methylcytidylic acid at pH 6 - 12 (Beaven, Holiday, and Johnson, 1955).

7.4.3.3. Coefficient Of Diffusion:

In general, it appears that the number of precipitin lines is dependent on the protein contents of the antigens and also on the antibody concentrations of the sera tested. The coefficient of diffusion of the different components of amoebic antigens range between

3.1×10^{7} and 28×10^{7} cm^{2} sec^{-1} (Appendix 8 refers).

ELUTION OF E. HISTOLYTICA (STRAIN HN:IB 5) ANTIGEN FROM SAPHADEX G 200

Sample - 2mls E. histolytica antigen
(Strain HN:IB 5) - 6mg/ml
TOTAL PROTEIN 12mg (BIURET)
Resin Saphadex G 200 (140 x 400 μ mesh)
Column dimension : 1·5 x 56 cm
Eluant: PBS PH·7·4
Temperature : 19°c ± 2°c
Flow rate : 10ml/hr
Fraction collected : 10 ml / tube

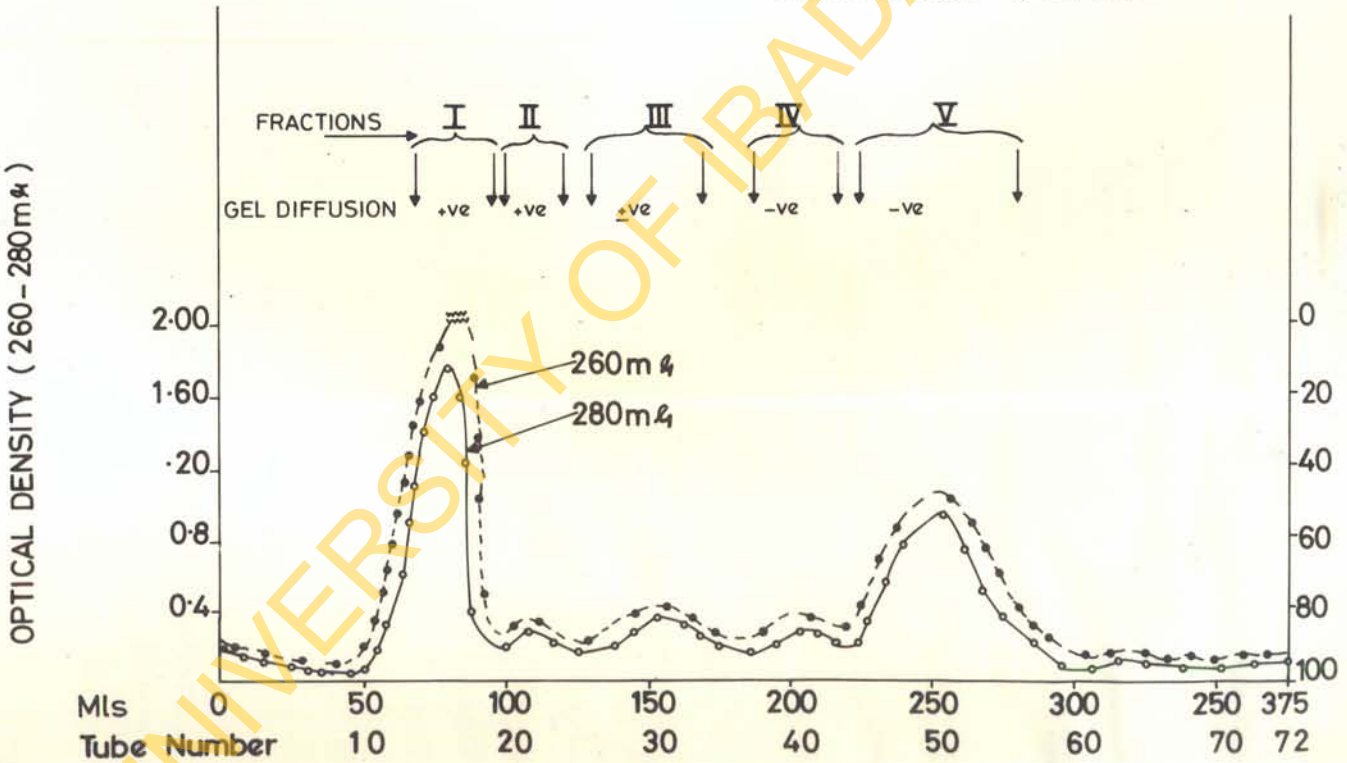


Fig. 30a.

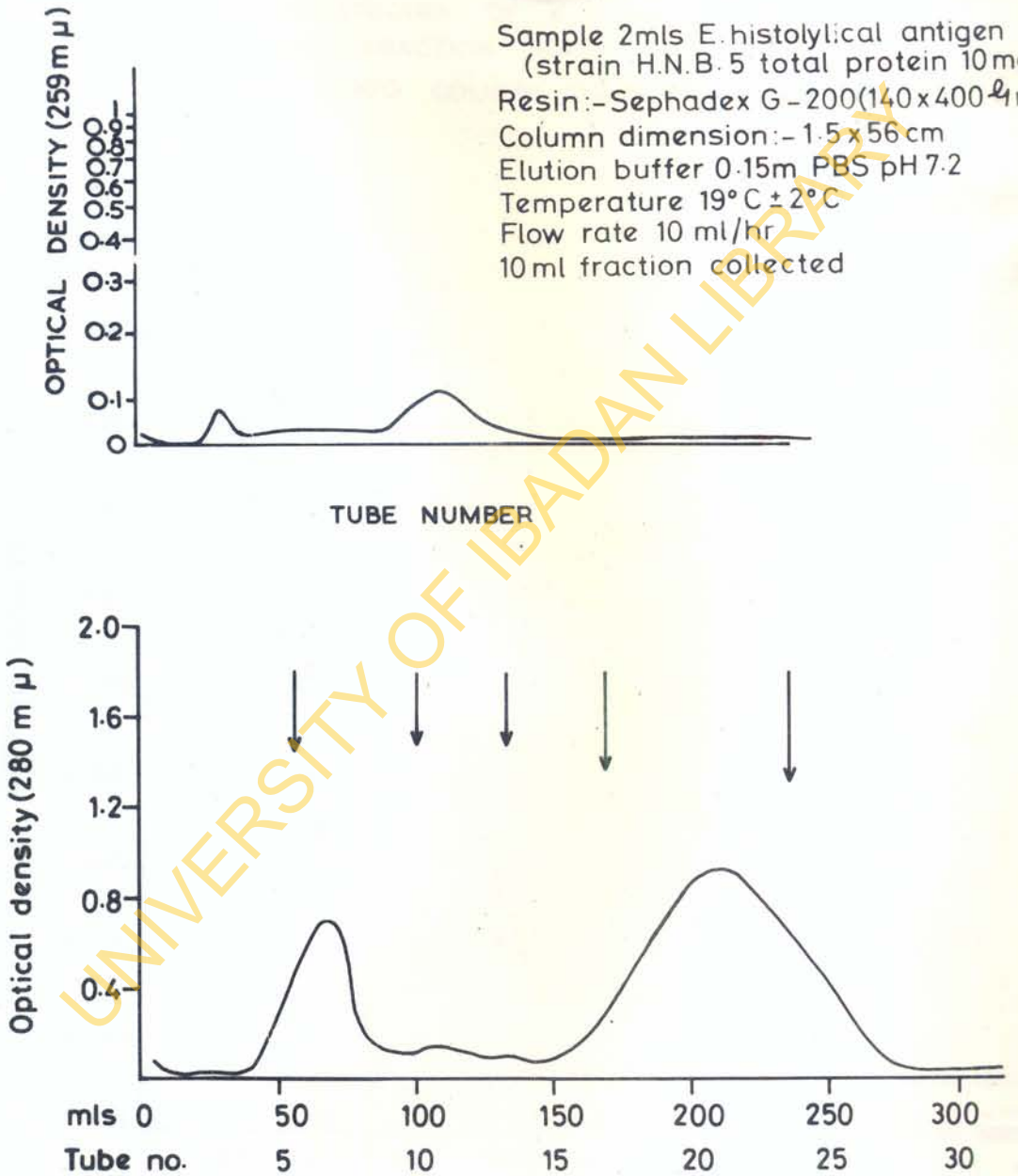


Fig. 30b.

ABSORPTION SPECTRA OF E.histolytica (WHOLE) ANTIGEN AND FRACTION ——
(SEPHADEX G 200 COLUMN CHROMATOGRAPHY HN-IB5

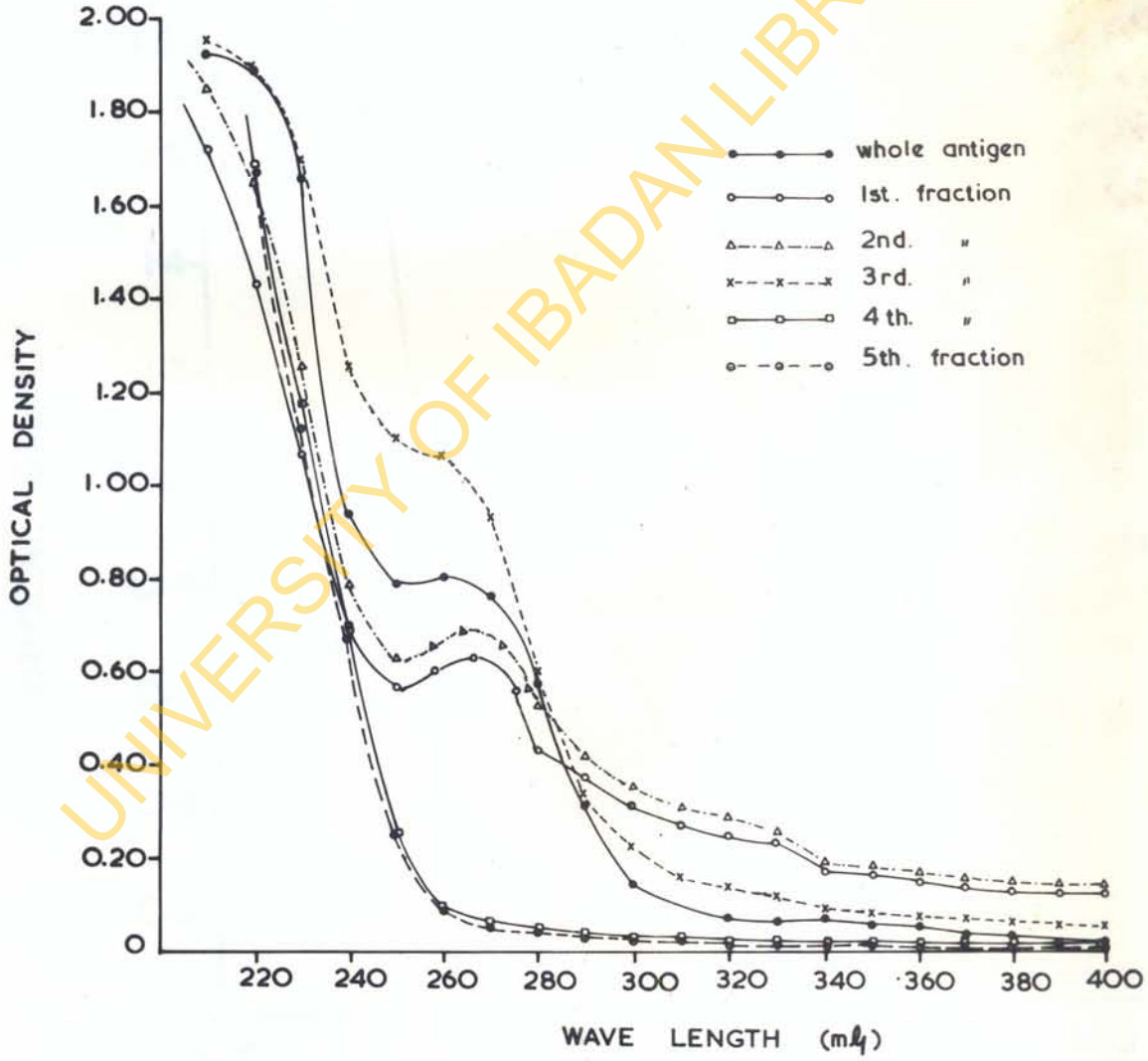


Fig. 32a.

ULTRAVIOLET ABSORPTION SPECTRUM OF ANTIGEN

TR 25 (STRAIN) PREPARATION 1mg/ml

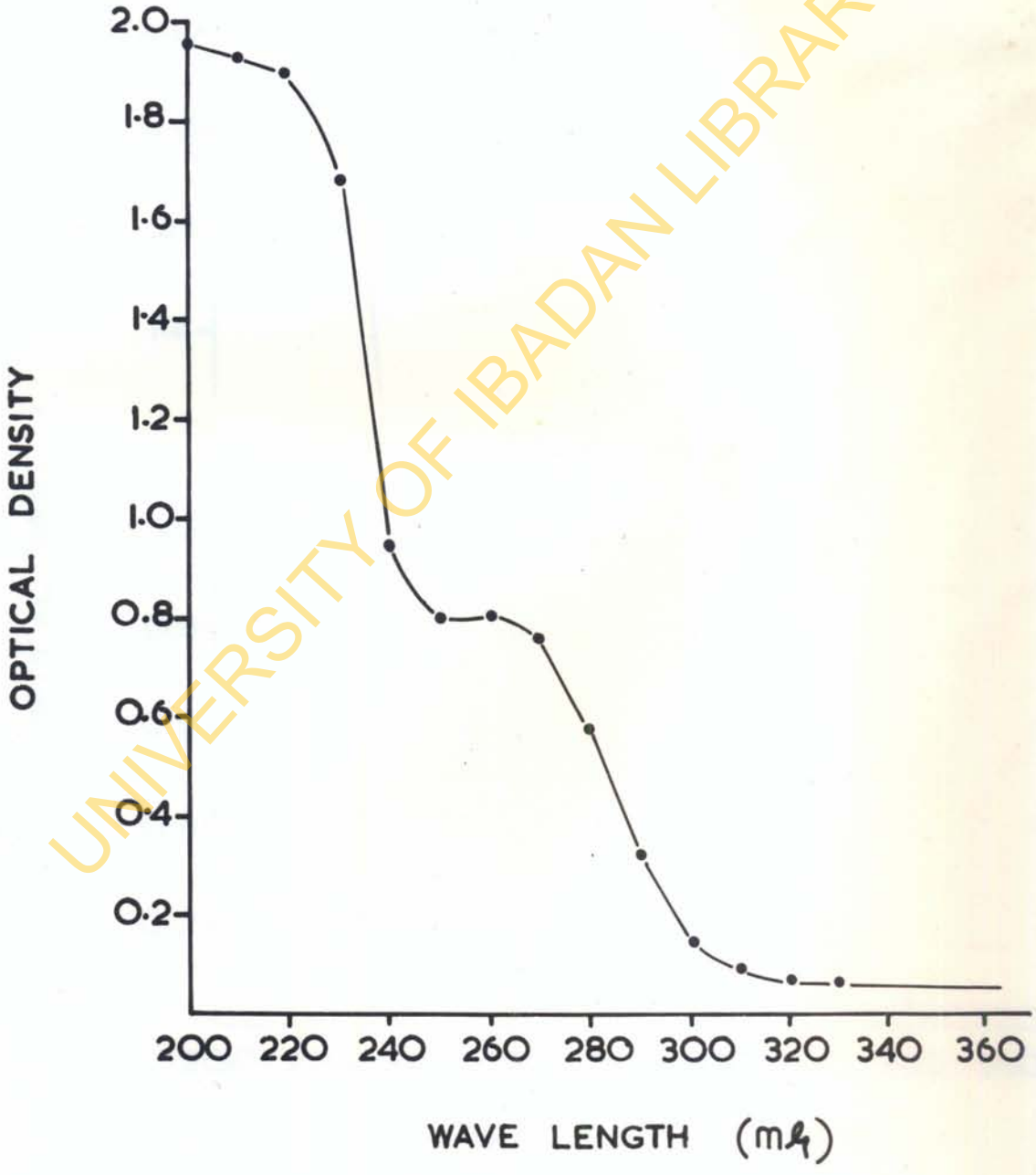


Fig. 32b

ULTRAVIOLET ABSORPTION SPECTRUM OF ANTIGEN

STRAIN AM 736 PREPARATION 1 mg/ml

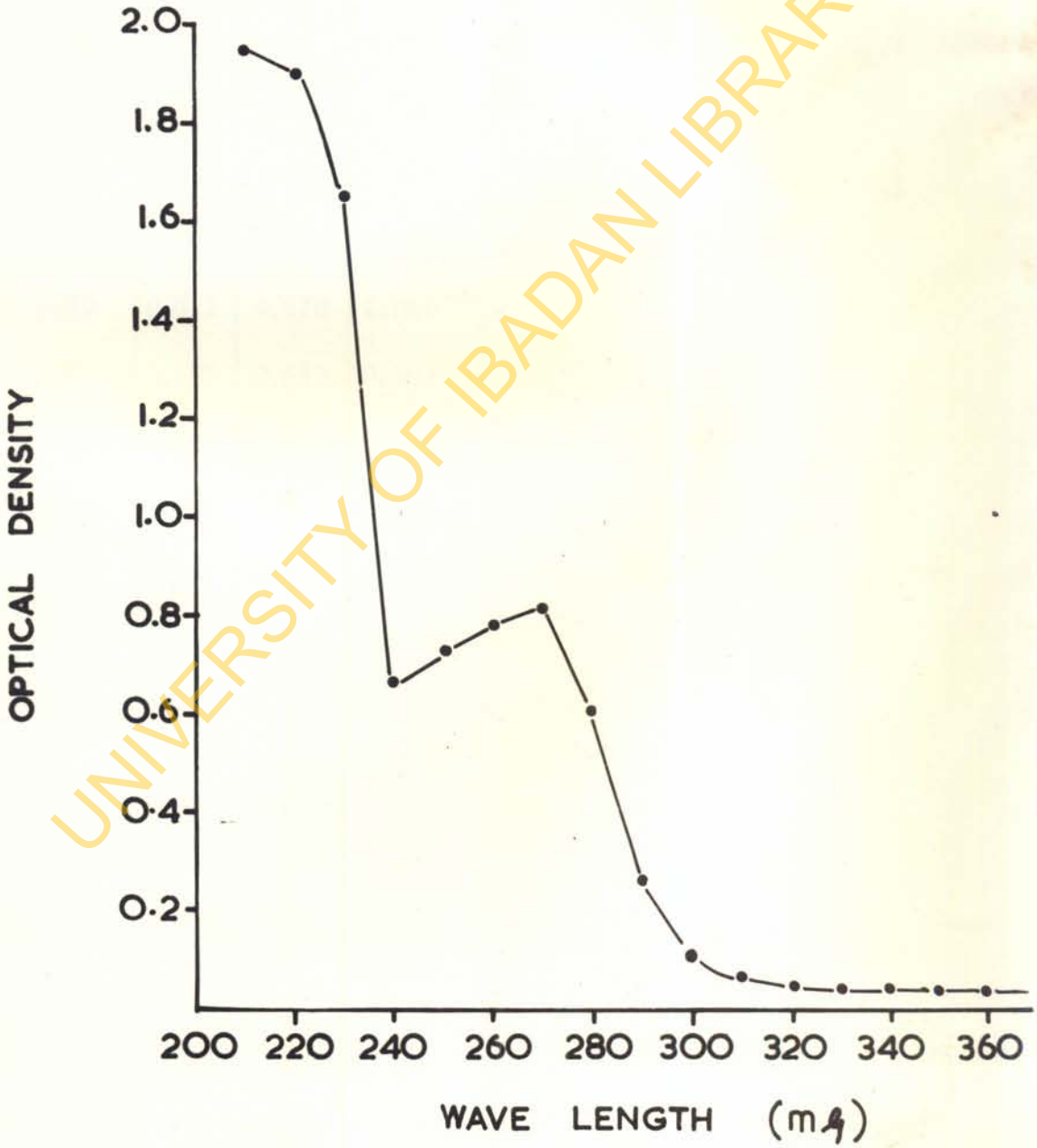


Fig. 32c.

TABLE 19

ABSORPTION SPECTRA OF ENTAMOEBA HISTOLYTICA (STRAIN HN:IB 5) WHOLE ANTIGEN AND FRACTIONS.

Wave Length	Whole	1st	2nd	3rd	4th	5th	6th
210	1.93	1.72	1.85	1.950	2.00	2.00	2.00
220	1.90	1.43	1.65	2.00	1.70	1.68	1.90
230	1.68	1.07	1.25	1.70	1.17	1.12	1.38
240	0.94	0.695	0.785	1.25	0.695	0.675	0.824
250	0.79	0.565	0.630	1.090	0.252	0.248	0.298
260	0.80	0.511	0.570	1.060	0.097	0.095	0.097
270	0.76	0.477	0.540	0.903	0.062	0.057	0.049
280	0.57	0.438	0.525	0.598	0.055	0.046	0.035
290	0.31	0.378	0.418	0.323	0.040	0.036	0.025
300	0.14	0.317	0.347	0.202	0.033	0.030	0.022
310	0.09	0.277	0.307	0.157	0.028	0.025	0.019
320	0.07	0.250	0.282	0.135	0.025	0.023	0.018
330	0.06	0.227	0.258	0.117	0.022	0.020	0.016
340	0.062	0.177	0.187	0.087	0.021	0.020	0.016
350	0.058	0.163	0.173	0.075	0.018	0.008	0.002
360	0.053	0.152	0.163	0.067	0.017	0.007	0.001
370	0.033	0.140	0.152	0.065	0.014	0.007	0.001
380	0.032	0.135	0.145	0.059	0.013	0.005	0.001
390		0.125	0.137	0.052	0.012	0.005	0.001
400		0.120	0.132	0.049	0.012	0.005	0.001
410		0.113	0.125	0.032	0.010	0.004	0.001
420		0.092	0.120	0.028	0.010	0.003	0.001

7.4.3.4 Size of Antigens:

The molecular sieve method for determining the size of antigen show that some components of amoebic extracts still pass through the pores of 7% agar. This suggests that the extracts have components with molecular weights far below one million (Polson, 1956). Since the size of an antigen consists of the number of antigenic determinants (Mates and Schulman, 1967) it is not known whether the passage of more precipitin lines through high percentage of agar when rabbit-anti-amoebic serum as opposed to serum from amoebiasis patient (Fig. 43) indicates that E.h. is a stronger immunogen and therefore, of stronger reactivity in rabbits than in the human.

7.4.3.5 Immunodiffusion Technique:

This test shows that:

(i) The extracts from the local strains of E.h. are antigenic. They are capable of producing or stimulating antibody formation in rabbits and of reacting with the homologous antibody in-vitro in agar gel.

(ii) The antigens, irrespective of the strains of E.h. used in the preparation, tend to produce more precipitin lines in agar gel when reacting with sera of patients with amoebic liver abscess.

TABLE 20: COEFFICIENTS OF DIFFUSION OF ENTAMOEBA HISTOLYTICA ANTIGEN(S) USING THE TECHNIQUE OF RIGHT ANGLE AGAR GEL ANALYSIS

	STRAIN	PRECIPITIN LINES										
			ANGLE 0^{o}					DISTANCE X_b				
		X_g	1	2	3	4	5	1	2	3	4	5
	Reference Antigen HK9	4.4	51.5	50.4	49.5	46.6	45	3.55	3.8	4.1	4.75	5.4
1	HN:IB 5	4	50	44.1	40	-	-	3.9	4.9	5.6	-	-
2	HN:IB 8	6	64.2	60	-	-	-	3.6	4.2	-	-	-
3	HN:IB 9	4.4	56.5	53	-	-	-	3.3	4.4	-	-	-
4	HN:IB 10	4.4	66.5	58.85	50.2	-	-	1.9	2.7	3.8	-	-
5	HN:IB 12	3.8	61	51.5	48	44.2	39.5	2	2.9	3.3	3.3	4.5
6	HN:IB 16	5	67.5	51.5	-	-	-	3.2	4.0	-	-	-
	MEAN (1-6)	4.9	69.95	53.16	46.10	-	-	3.1	3.85	4.23	-	-
	S.D. (1-6)	0.69	9.55	5.27	4.38	-	-	0.82	0.79	0.99	-	-

0^{o} = Angle between Antigen trough and Precipitin lines.

Xg = Distance between the farthest point on ppt line and Ag trough

Xb = Distance of ppt. line from Antibody trough.

The reaction in cases of acute amoebic dysentery and in occasional cyst passers, is confined mainly to the production of one or two precipitin lines which may be quite faint in most cases.

(iii) There is no quantitative or qualitative difference in the reactivity of amoebic antigens in the sera of various groups of amoebiasis patients. In other words, the sera of male patients, non-pregnant female (of child-bearing age) patients; as well as, those of pregnant amoebiasis patients, show the same reactivity in terms of the number of precipitin lines in gel diffusion test. This is so in as much as, these various group of patients suffer from the same 'form' of the disease. It would thus appear that the number of precipitin lines obtained in the gel diffusion test would depend on the concentrations of the amoebic material in the test antigens, and also on the concentrations of corresponding antibody present in the test sera. The immunogenicity of amoebic antigens in various animals may be another factor to be considered in the interpretation of these results.

7.4.3.6 Immunoelectrophoresis:

This test shows that amoebic antigens contain some proteins of α_2 electrophoretic mobility (Fig. 34).

7.4.4 Clinical Application:

An analysis of the results of the clinical assessment of the locally isolated E. histolytica antigens shows that about 90% of proven cases of amoebic liver abscess; 48% of patients with symptomatic amoebic colitis; and about 8% of symptomless cyst passers, have detectable serum antibody in agar gel diffusion tests (Table 30).

7.5 Comments:

The findings in this section suggest that the extracts prepared from the local strains of E. histolytica are antigenic. They also give an indication that these extracts are weak antigens in detecting antibodies to human amoebiasis, particularly in amoebic dysentery. However, in terms of potency, it can be stated that the extracts are reasonably potent immunogens in rabbits.

The difference in the number of precipitin lines produced in agar gel diffusion tests, using E. histolytica antigens to detect homologous antibodies in the sera of patients with amoebic liver abscess on the one hand, and in those with amoebic dysentery on the other hand, can be explained on several hypothetical basis. The strong antigenicity of the extracts in the rabbits, as shown by the greater number of precipitin lines passing through higher concentrations of agar when rabbit-anti-E.h. sera, as opposed to sera from patients with clinical amoebiasis, are used, can also be considered on similar basis.

First, the interaction of the infecting organisms with the intestinal mucosa in the case of amoebic dysentery, may have been so transient, possibly as a result of the rapid development of the acute diarrhoeal disease, so much so, that there may have been little or no time for host immunological response. This mechanism may account for the detection of greater number of precipitin lines, and therefore, detectable antibodies to E. h. in greater number of patients with amoebic liver abscess than in those with acute amoebic dysentery.

Secondly, the concept of antigenic disparity postulated for the metazoan endoparasite, may similarly be employed to explain the phenomenon of this differential antigenic reactivity. In this situation, it has been postulated (Dineen, 1963), that immune response is likely to favour the survival of only those variants of the metazoan parasite which display a sufficiently reduced antigenic disparity with the host.

The carrier state in amoebic infestation can be considered as an "intimate association" between the parasite or its products, with the host, thereby rendering the former less antigenically foreign to its host. However, despite the fact that E. h. is extracorporeal and intraluminal in its host, if the foregoing analogy with the metazoan endoparasites can be accepted then the process of immunological selection postulated by Dineen (1963) can be used as an explanation for the occurrence of carrier states in amoebiasis. It has also been suggested (Dineen, 1963), that the

foregoing process be based on either simple loss of an appropriate antigenic character by the organism or an acquisition of the antigenic character of the host or both. In such a circumstance, the host fails to recognize the parasite as "foreign" following possibly, the long and perhaps 'intimate association' and, therefore, fails to produce antibody, to an agent (namely : E.h. or its products) which are no longer "foreign" to it.

Lastly, the strong antigenicity of E. histolytica extracts in rabbits deserves some comments. It is deduced as a priori consequence of the hypothesis in the preceding paragraphs that the natural host will show relatively little immunological response to antigens showing less antigenic disparity with the host. For the sake of argument, the latter antigens may be regarded as "homologous" to the host antigens. Conversely, it is expected that with the rabbit, which is not a natural host for E. histolytica, there will be serological responses to a greater extent. This probably, may be due to immunological selection postulated for human amoebiasis above. This view is in accordance with the general concept that the more distant is, the species relationship between antigen and animal to be immunized, the greater will be the immune response.

In the light of the foregoing, therefore, amoebic extracts may be considered as homologous antigens in human amoebiasis and as such very likely to be weak immunogens.

Ultra-Violet Absorption Spectroscopy:

In the present study, absorption spectroscopy has been employed as an analytical tool and as a basis for deductions concerning structure. The curves of the absorption spectra of the intact E. histolytica antigens and the reactive fractions are similar (separately) to the absorption spectrum of Deoxy-5-methylcytidylic acid at pH6-12 (Beaven, Holiday, and Johnson, 1955). In addition the intact extracts of E.h. show selective absorption in the wavelength 260mμ region at pH 7.2. These properties suggest that E.h. antigens may have amongst others some physico-chemical properties of the class of polynucleotides. It is well known that this class of compounds are very prone to rapid enzymic degradation in the body (Humphrey and White, 1970). It has been suggested by Plescia (1969), that it is unlikely that the immunogenicity of a protein is either dependent on or is adversely affected by enzymes such as proteases. In contrast, however, as noted by these workers, the immunogenicity of polynucleotides might well be limited as a result of their degradation by host nucleases.

On this basis, the spectrophotometric study which suggests a polynucleotide structure for amoebic extracts, may be taken as a possible additional premises which can be used to explain the weakness of E.h. antigens.

Size Of Antigen And Co-efficient Of Diffusion:

Size of antigen is the number of determinants it contains. An antigenic determinant is defined as any chemical group or constellation of chemical groups to which an antibody can be made. There is evidence that the greater the number and diversity of the determinants on an immunogenic molecule, the greater is its immunogenicity (Simonson, 1970). Accepting that immunogenicity is a function of the number of determinants carried by an antigen as suggested by the work of Mates and his co-workers, (Plescia, 1969), then there must be an explanation for the difference in the gel diffusion reactivity of E. h. antigens when interacting with the rabbit antiserum and human patient's serum. If it can be shown that every precipitin line on agar gel represents the reaction due to separate antigenic determinant, then one can suggest that E. h. antigens producing a greater number of lines in the rabbit antiserum than in the amoebiasis patient's serum, is a more potent antigen in the former situation than the latter. This is in accordance with Plescia's (1969) view that the greater the number of determinants an antigenic molecule has, the greater is the response likely to be.

It appears at this stage, that the immunological system employed in this section does not give any conclusive proof as to the low immunogenic or antigenic activity of E. h. antigens. It has also been impossible to

account for the severity of amoebiasis in the puerperium and the allied states, by the use of the same system. This is so, even after the characterization of the antigens. It is, however, known that in intestinal amoebiasis the trophozoites will be exposed to naturally accurring fluid media such as mucous and faeces. It would be interesting to speculate and perhaps show by future studies, that host resistance against the disease might be a function of the coefficient of diffusion of the amoebic "secretion" or "excretion" products in the intestinal contents.

It should, however, be emphasized that all the foregoing speculations are not in any way, meant to solve or explain the problem of the severity of amoebiasis in certain clinical states. It would appear therefore, that the characterization of antigenic components of amoebic extracts embodied in this section is more or less an end in itself. Consequently, it has not given any direct evidence for the role of immunity in the severity of amoebiasis in the puerperium and allied states. Attempts are therefore, made in the following chapter to search for a possible direct immunological evidence which can be used to explain the unusual severity of amoebiasis in pregnant women and women in the puerperium.

Table 20 Gel Diffusion Reaction In Human Amoebiasis
(i) Symptomatic Amoebiasis (Acute Colitis)

Serial No.	Name	Sex	Hosp. No.	Age	Date	AGD (No. of L)	Remarks
1	Amusa Rafatu	F	171168	A	6.2.68	0	28/52 Preg.
2	Human Adeyemi	F	1357273	A	18.7.68	2	Ca. rectum
3	Adelabu Ishola	M	204231	A	-	2	-
4	Adisa Sikiratu	F	198441	A	13.7.68	1	Nephrotic Syn.
5	Murano Olaoye	M	208338	A	31.12.68	0	-
6	Salami Kudiratu	FP	202874	A	11.9.68	(1)*	Abortion 5/12P
7	Ogungbemi Moses	M	198090	53	6.7.68	(2)*	Hyp. H. Disease
8	Alabi James	M	197987	65	22.7.68	(3)*	Ca. Ethmoid
9	Ige Olusola	M	-	A	-	2	-
10	Ogunrotime Richards	M	-	A	4.7.68	1	Chronic diarrhoea
11	Umuagba Matthew	M	-	21	28.8.68	0	Acute colitis
12	Oseni Risikatu	FP	110866	A	8.8.68	(1)*	6/12 Pregn.
13	Akinola Bisi	FP	105696	A	7.8.68	1	5/12 Pregn.
14	Otobhill John	M	185975	A	12.8.68	2	Am. Pos. Dys. Col.
15	Ogunsina Agbeke	F	-	A	-	(1)*	-
16	Bello Adisa	M	-	A		2	-
17	Akande Lawani	M	-	A		0	-
18	Victoria Kazeem	F	-	A	5.7.68	2	-
19	Adeosun Lamidi	M	189608	24	12.12.68	0	-
20	Adeyemi Layi	M	-	A	-	0	-
21	Adeoye Ramonu	M	213802	A	20.3.69	0	E.h. troph. ++
22	Amole Busari	M	LN7633	A	30.4.69	2	
23	Akanni Gbadamosi	M	203902	A	30.4.69	0	
24	Davies Bamiaku	M	216129	35	-	3	Amoeboma & Cut.
25	Oduntan Elijah	F	220008	A	17.6.69	0	
26	Fawole Abigail	F	115767	A	17.6.69	(2)*	

Serial No.	Name	Sex	Hosp. No.	Age	Date	AGD (No. of L)	Ramarks
27	Akanno Tijani	M	219193	A	24.6.69	0	Ac. Am. + Hodgkins
28	Owolabi Kehinde	F	194523	A	25.6.69	(1)*	6/12 P.
29	Ladepo Victoria	F	154998	A	26.6.69	0	
30	Ayeni Oluremi	FP	109359	A	26.6.69	0	
31	Obisesan Salawu		180163	A	1.7.69	0	
32	Yashe Gaya	M	219647	A	4.7.69	2	C.M.E.
33	Fajuyigbe Victoria	F	218732	A	9.7.69	(2)*	
34	Yaro Dogo	M	221400	A	11.7.69	0	
35	Ogunsanwo Ayodele	M	149167	A	18.7.69	1	
36	O. Olalekan	M	222062	A	20.7.69	0	
37	Esan Salami	M	221833	A	20.7.69	0	
38	A. Dorcas	F	206890	A	22.7.69	0	
39	Fayisitan Samuel	M	-	50	20.10.69	0	
40	Adesina Olufunlayo	F	107893	A	23.3.70	0	
41	Adaramodu Florence	FP	200634	21	17.7.69	(1)*	27/12 Preg.
42	Fashina Bola	F	231588	A	27.1.70	(2)*	
43	Soyode Elizabeth	FP	213685	A	29.1.70	0	6/12 Preg.
44	Adeniyi Emily	F	-	A	2.2.70	0	
45	Odukale Adenike	FP	199139	A	-	0	28/52 Preg.
46	C. O. Adesina	F	-	A	20.2.70	2	
47	Adeniyi Grace	F	236028	A	5.3.70	1	
48	Raji Sunmonu	M	205315	34	11.3.70	0	
49	Layiwole Sule	M	25136	A	1.4.70	0	
50	Ogundipe Braimoh	M	162016	51	2.4.70	0	
51	Basha Yakubu	M	237949	A	2.4.70	0	
52	Famose Rufus	M	218744	A	7.5.70	0	
53							
54							
54							

* Parenthesis Indicate Very Weak Reaction.

(ii) AMOEBIC LIVER ABSCESS

Serial No.	Name	Sex	Hosp. No.	Age	Date	AGD (No. of L)	Remarks
1	Olalere Adeyemi	F	215442	A	-	2	-
2	Ojo Paxzon	M	314634	A	20.5.69	3	
3	Ojo Adeniyi	M	219192	A	6.6.69	2	
4	Eriyo Abiodun	M	220308	3	27.6.69	3	
5	Afolabi Falilatu	F	192747	33	4.8.69	3	Aspirate E.h. ++
6	Alimi Yesufu	M	224774	A	22.9.69	5	
7	Oyebanji Kazeem	M	198210	$3\frac{1}{2}$	20.10.69	4	
8	Shishi Dennis	M	221647	A	15.7.69	5	
9	Dansuki Ibrahim	M	220630	A	28.6.69	0	Aspirate yielded E.h. ++
10	Omoreghe Samuel	M	236006	A	13.3.70	(2)	Extensive Fibr. wall.

(iii) SYMPTOMLESS CYSTS/TROPHOZOITE PASSERS

1	Oloshunde Salawu	F	191509	A	5.3.68	0	
2	Salawu Muniratu	F	19961	A	14.8.68	0	Chronic Cholecyst
3	Fawomisan Olusola	F	189676	19	26.2.68	0	
4	Amuwe Victoria	F	219372	A	10.6.69	0	
5	Okuyemi Victor	M	216338	23	10.6.69	0	
6	Izegache Anthony	M	153757	29	10.6.69	0	
7	Akinbola Jacob	M	170643	A	17.6.69	0	
8	Adebayo Mary	F	168224	A	20.6.69	0	
9	Salami Fatimo	F	164752	A	20.6.69	1	
10	Salami Sabitiyu	F	206859	A	1.7.69	0	
11	Adeboya Joseph	M	218105	A	29.7.69	0	
12	Adeyemo Bimpe	F	219891	A	17.6.69	0	

Parenthesis indicate very faint reaction.

SECTION II

REACTIVITY OF HUMAN IMMUNOGLOBULINS IN AMOEBIASIS

7.6.1 Introduction:

As shown in the preceding section it was not possible to use the gel diffusion test as an index of investigation for the differential severity of amoebiasis in different states particularly pregnancy and the puerperium. Further possible evidence was therefore sought in the antibody stimulation to *E. histolytica* infections.

In connection with this search, it is interesting to note that in recent years, multiple immunological tests have become available for the laboratory diagnosis of human amoebiasis. Among these tests are complement fixation (Fulton, Joyne and Price, 1951); amoebic gel diffusion test (Siddiqui, 1961; Sen, Ghosh, Mukerjee and Ray, 1961; Atchley, Auernheimer, and Wasley, 1963; Maddison, 1965; Maddison, Powell and Elsdon-Dew, 1965); haemagglutination test (Kessel, Lewis, Pasquel and Turner, 1965; Maddison, Powell and Elsdon-Dew, 1965; Milgram, Healy, and Kagan, 1966; Krupp, 1970); Flourescent antibody test (Goldman, 1966; Jeanes, 1966; and Boonpucknavig and Nairn, 1967); and immunoelectrophoresis (Savanat and Chaicumpa, 1969); and bentonite fluocculation test (Tupasi and Healy, 1970). Although these various serological tests are designed to detect specific antibodies in amoebiasis, relatively little is known about the nature and significance of such antibodies.

There is a growing impression based mainly on animal experiments (Ali Khan and Meerovitch, 1968), that certain immunoglobulin classes may be involved in the host response to E. histolytica infections. The report by Maddison and her co-workers (1968) on the study of the reactivity of human immunoglobulins in the sera of two patients with amoebic colitis provided some evidence for the possible role of humoral immunological factor(s) in human amoebiasis. It is known that about ninety per cent of the immunoglobulin producing cells of the lamina propria of the duodenum and jujunum produce IgA (Grabbe and Heremans, 1966), but the nature and types of immunoglobulins produced during response to intestinal parasites are virtually unknown. Furthermore, in-vitro culture studies by Nakamura (1959), and Abioye (1970), suggested the presence of growth inhibiting factor(s) for E. histolytica in the sera of human subjects with current or past histories of amoebiasis.

Various workers indicated that amoebiasis may be unduly severe when it occurs during pregnancy and the puerperium (Homer and McNall, 1961; Edington and Gilles, 1969; Odunjo, 1969; Lewis and Antia, 1969; Duque, 1969 and Abioye, 1970); there are no data indicating whether or not antibody plays any role in such clinical manifestations.

The study in this section is designed to evaluate the role of humoral antibody responses in different individuals with special reference to pregnant/women in the puerperium, suffering from amoebiasis.

7.6.2 Materials And Methods:

7.6.2.1 Study Population:

One hundred and nineteen specimens of human sera, obtained from eighty-nine indigenous male and female adult Nigerians as detailed below were studied:

(1) Forty-three (43) patients with amoebic colitis consisting of:

(a) Fifteen (15) adult males

(b) Thirteen (13) non-pregnant females of child-bearing age;

(c) Fifteen (15) pregnant women at various stages of pregnancy

(2) Eleven (11) adults suffering from amoebic liver abscess. Two of these were females.

(3) Seventeen (17) adult symptomless amoebic E. h. passers and

(4) Eighteen (18) adult patients suffering from conditions other than amoebiasis (sick controls).

The remaining set of specimens used in absorption and follow-up studies were obtained from the individuals in groups (1), (2), and (3) above.

7.6.2.2 Characterization Of Antibody In Amoebiasis:

(a) Chromatography

Column effluent were passed through ultra-violet absorptiometer, and the absorption measured at a wavelength of 259mU (Uvicord LKB produkter Ab-Stockholm, Brommel, Sweden).

5ml of each of the three sera from amoebiasis patients used in this study was fractionated separately by passage through Sephadex G-200 according to the method of Flodin (1962).

Sephadex G-200 Column: 30 mg of dry sephadex G-200 (Pharmacia Uppsalla, Sweden) was weighed into about 2½ litres of ion-free distilled water in 3-litre beaker. The sephadex mixture was stirred continously for 15 minutes and allowed to settle; the fines were strained off by carefully decanting the supernatant. More distilled water was added to the stirring and the straining were repeated until no more visible fines on the top of the water. The beaker containing the sephadex was then filled with ion-free water and placed at 4°C for three days to allow maximum swelling of the gel.

Columns (2.7cm. x .150cm) were used to chromatograph 4 to 5ml of serum. For use the water was completely decanted and the sephadex equilibrated with 0.1M Tris-HCl in 0.5M Nacl with 0.1% merthiolate added at pH 8.0. The sephadex was then carefully loaded into the column and allowed to settle. A serum sample (5ml) with 750mg sucrose added, was gently layered on the upper surface of the sephadex in the column. The serum was allowed to absorb completely into the sephadex. About 5ml of the buffer was then carefully layered on the sephadex to a height of about 2 inches. The column was then connected to a buffer reservoir which contained the same buffer as was used for equilibration.

The flow rate was adjusted to 15 - 20 ml/hour and 5ml fractions were collected by means of an LKB fraction collector equipped with a uvicord attachment set at 259mU. The chromatographic separation was carried out at room temperature($19^{\circ} \pm 2^{\circ}C$).

Each collected fraction was read at 280mU to determine the protein content. The readings were plotted against tube numbers (Fig. 43). Three distinct peaks were regularly obtained from each sample. The appropriately numbered tubes were selected to cover the whole range of the three peaks. Fractions were pooled, dialysed against distilled water at $4^{\circ}C$ and then concentrated by negative pressure ultra-filtration using visking tubing 8/32-inch. These fractions were subjected separately to immunodiffusion test and immunoelectrophoresis, developing lines with anti-whole human serum and anti-specific human immunoglobulins (Hyland Labs).

(b) Gel-diffusion Test:

Sera and fractions were tested for anti-amoebic activity using the locally-prepared antigens, in micro-Ouchterlony plates similar to those, already described above. Attempts to identify the precipitinogens of reactive fractions were made by immunoelectrophoresis when the precipitation patterns formed by whole serum and fractions with electrophoresed E. histolytica antigens were compared.

(c) Flourescent Antibody Test:

Cultured amoebae similar to those used as a source of antigenic extract, was washed three times in phosphate-buffer saline (PBS) pH 7.2, resuspended in sufficient buffer to contain 30-50 amoebae per c.mm Drops of this suspension were placed on alcohol-cleaned slides to cover an area of approximately 5mm diameter. The slides were then dried rapidly on a hot plate at 56°C and stored at -20°C until required for use.

Anti-human globulin conjugated with flourescein isothiocyanate and flourescein-labelled anti-human γ^M and anti-γ^A were obtained from the institute of Sera and Vaccines Praha, Czechoslovakia, and Burrough Welcome.

The optical system used was a Reichert Zetopan flourescence microscope fitted with a dark-ground condenser, a UV-passing primary filter (Schott UG1/1.5mm), and a colourless UV-absorbing secondary filter (C. Reichert, Optsche Werke, AG Wien XVII, Hernalser Hauptstrasse 219, Austria).

Titration of the patients' sera was performed by the following method, based on the techniques developed by Coons et al. (1942) and Weller and Coons (1952). The sera were diluted in PBS (Chadwick and Fothergil, 1962) to give a range of two fold dilution from 1:8. A single drop of each dilution was applied to a separate smear of E. histolytica

and the slides incubated in a moist chamber at 37°C. After 30 minutes, the slides were washed in buffered saline for 30 minutes in 3 x changes of PBS, Conjugated anti-human globulin was next applied to each smear and allowed to react for 30 minutes. This was followed by washing in PBS for one hour in 3 x changes of buffer. Finally, each smear was counterstained with 0,. 05 per cent (1:200) solution of Evan's blue (Nichols and McComb, 1962) for 10 minutes to block non-specific flourescence. The preparation was washed again and mounted under cover slips in a mixture of nine parts glycerol and one part of PBS, prepared about two hours before the test.

The smears were examined under the flourescent microscope and the intensity was recorded according to the scale adopted by Wilkinson (1961). The highest dilution of serum was taken as the end point of the titration. Positive control sera from known cases of amoebiasis and negative control sera (obtained from healthy babies under two years of age) were included in every series of test.

7.6.2.4 Immunoglobulin Measurements:

The serum immunoglobulin concentrations were determined by a modification (Fahey and McKelvey, 1965), of the single radial diffusion method of Mancini, Carbonara, and Heremans (1965). The plates were obtained from Hyland Laboratories, Carlifornia, U.S.A. About six standards obtained from Behringwerks and Hyland Laboratories, were

included in each set of tests and used for the calibration curve for each immunoglobulin class. The diameter of the precipitation ring was plotted on semi-logarithm paper against known antigen concentrations.

When any immunoglobulin level of the test serum approached or exceeded that of the highest value of the reference serum standard, the test serum sample was diluted with normal saline to reduce the level of the protein fraction to within the range of the particular reference serum. The test was then repeated and the result multiplied by the dilution factor. For the IgG estimation, all sera were initially diluted 1:10 and the results were multiplied by 10.

The IgM and IgA plates were read after 16-hour incubation at room temperature (19°C ± 2°C). The IgG plates were read after incubation for four hours at 37°C.

7.6.3 Absorption Studies:

In the initial absence of information regarding the specificity of the immunoglobulin involved in antibody response in human amoebiasis, attempts were made to remove specific antibodies for E. histolytica by absorption of the immune sera with different strains of the organism. For this purpose, E. histolytica culture were washed three times and used as the absorbent antigens. A packed volume of washed E. h. culture estimated to contain 2 x 10^6/ml E. histolytica (trophozoites) was mixed with 0.5ml of serum (V/V). The mixture was then incubated for 1 hour at

37°C and at 4°C for 16 to 24 hours. After incubation, the mixture was centrifuged at 2000G for two hours and the sedimented absorbent antigens discarded. The sera, prior to absorption, as well as, the absorbed supernatant were tested on agar gel diffusion using locally prepared amoebic antigens. The serum immunoglobulins were estimated both prior to and following absorption, and the concentrations as well as, the immunoelectrophoretic patterns compared (Fig 44 and Table 20).

7.6.4 Clinical Follow-Up:

Fifteen of the amoebiasis patients listed above and ten symptomless controls were followed up for a period ranging from 5 to 10 weeks starting from the first day on which the diagnosis and confirmation of amoebic colitis or amoebic liver abscess was made. Sera for the base-line immunoglobulin estimations were usually collected before the patients were started on specific amoebicide (metronidazole). Subsequent blood specimens were obtained within two weeks after the first collections and within the 5th and 6th week from the first day of the diagnosis being made. Immunoglobulin estimations were carried out within 48 hours of the collection of blood.

7.6.5 RESULTS:

7.6.5.1 Column Chromatography and Immunoelectrophoresis:

The immune sera mainly from amoebic liver abscess patients showed on column chromatography and gel diffusion test, that the antibodies to E.h. reside mainly in the second (IgG-rich) fractions of the separated serum proteins. Diethylaminoethyl (DEAE)- cellulose column was not used to elute IgG. The tests here, only showed that the bulk of the antibody resides mainly in the IgG-rich fraction 2(35).

7.6.5.2 Flourescent Antibody Test:

Table 21 shows the serum distribution of flourescent antibody titres against the local strains of E. histolytica in patients with amoebic infection and in the control bases.

Amoebic Infection: In intestinal amoebiasis, the titres ranged from 1:8 to 1:1024. 27 (90%) of the 30 cases gave a titre of 1:40 or above and 19 (63%) gave a titre of 1:80 or above. Proportionately higher titres ranging from 1:8 to 1:4096 were given by cases of extra-intestinal amoebiasis. In 10 of the 12 cases, the titre was 1:40 or above and in the remaining two cases, it was 1:80 or above.

In cases of non-amoebic infection the titre was less than 1:40. In a total number of 13 non-amoebic liver disease, the titre was 1:8 or less. In four cases of typhoid enterocolitis, the tirtre was less than 1:8.

Symptomless E.h. Passers:

In 12 (60%) of the 20 symptomless cyst passers the titre was 1:8 or less, but in 6(30%), it was 1:16 and in 2 cases, it was 1:40. Although the titre did not exceed 1:40 in any of the carriers, the titres were proportionately higher than those observed either in normal subjects or in patients with non-amoebic disorders.

It was also observed that the anti-IgG gave the most intense fluorescence with E. histolytica in contrast to the other classes of immunoglobulins.

7.6.5.3 Immunoglobulins:

The serum immunoglobulin concentrations for the various groups are summarized as geometrical mean values in Table 22.

Serum IgG: The mean serum IgG values are significantly elevated in all patients with infection with E. histolytica ($p < .001$) when compared with those of the symptomless controls, except the pregnant females with acute colitis. The latter groups had a mean value of 3077mg/100ml and the symptomless carriers, a mean value of 2729mg/100ml. About half of the pregnant women had IgG values above the upper limit of the normal range while the rest of the patients in this group had normal IgG values (McFarlane, 1966; Buckley, Dees and O'Fallon, 1968). The majority of the non-pregnant female patients with acute amoebic colitis had serum IgG values well above the upper limit of the normal range and 2 of these

patients had exceptionally high IgG values of 7700mg/100ml (the reason for this is unknown but a possible experimental error may be responsible). The adult male patients with acute amoebic colitis had moderately elevated IgG and the frequency distribution (Fig. 36) resembled that of the patients in the sick control groups. The patients with amoebic liver abscess had the highest serum IgG concentration, most of these having values between 4000 mg/100 ml and 5000 mg/100ml. Two patients had IgG values above 9000 mg/100ml, again this exceptionally high value may be due to an experimental error.

Serum IgA: As shown in Table 23 there is an appreciable difference in the mean IgA concentration between the non-pregnant female patients (183 mg/100 ml) with acute amoebic colitis and the pregnant females with acute amoebic colitis. There was one female patient with acute amoebic colitis with an IgA concentration of 19 mg/100 ml. The 15 male patients with amoebic colitis had a mean IgA level of 210 mg/100ml. Although the frequency distribution showed scatter, all the values were in the normal range. The same applies to the patients with amoebic liver abscess, as well as, those patients belonging to the sick control group.

Serum IgM: The mean values of the IgM in all the groups with acute amoebic colitis were similar and the frequency distribution appeared to be the same. On the other hand the patients with amoebic liver abscess

tended to have higher mean IgM concentration than the rest of the groups. The relatively high IgM values observed in the sick control group, is not surprising since other chronic infections may contribute to the higher IgM values (Houba and Allison, 1969). It may also be a non-specific globulin response (Humphrey, 1963).

7.6.5.4 Absorption Studies:

Table 24 shows the results of the quantitative immunoglobulin measurements obtained before and after the sera of various patients with amoebic infections were absorbed with E.h. antigens. Six of the sixteen sera from this group of patients produced strong precipitin lines in agar gel prior to absorption with specific amoebic antigens. After absorption no precipitin lines could be detected. Five of the six positive sera were obtained from patients with amoebic liver abscess.

Table 24 shows the percentage of IgA absorbed from all sera from patients with amoebiasis. The results show that the percentage IgA absorbed tended to be generally higher than the corresponding percentage of IgG or IgM absorbed. It is difficult to reconcile this with the immuno-electrophoretic or immunodiffusion findings. It is possible that IgA may also be playing some part in amoebiasis. This finding together with the identification of the various fractions after gel filtration on SG - 200 suggest that the antibody activity to E. histolytica antigens was of the low molecular weight (7S or 11S) variety.

7.6.5.5 Clinical Follow-Up Studies:

Figures 39 - 41 and Table 23 show that the immunoglobulin patterns of 15 patients with invasive amoebiasis compared with the picture in the symptomless controls. Of all the immunoglobulins studied the IgG pattern was most consistent in patients with symptomatic amoebiasis. As shown in Figure 39, it is rather striking to observe that all the amoebiasis patients studied showed a marked decrease of IgG concentrations two weeks after treatment had started and continued to fall throughout the entire period.

During treatment of patients with symptomatic amoebiasis neither the serum IgA nor the IgM showed consistent patterns. In 7 out of these patients with amoebiasis the serum IgM concentration showed a decrease in value after treatment had commenced, but in two of these the values increased again. With the exception of one, all the serum IgA showed a slight increase after about 20 days on treatment. The immunoglobulin patterns in the symptomless controls did not show definite change in concentrations over the period of study.

7.6.6 Comments:

The results of the studies presented here show that at least a portion of the immunoglobulin 'system' is active against E. histolytica infections. Thus the results show that although all the three major classes of immunoglobulin (G, A, and M) may be elevated in amoebiasis it is the IgG

that was more consistently elevated and significantly involved in the disease ($t = 3.14 - .3.61$; $p < .001$). Production of IgG class of antibodies may thus be regarded as one of the "immune" mechanisms mounted by the host in human amoebiasis. This is within the meaning that is appears in response to a specific stimulus, and reacts with a specific agent - E. histolytica or its products. This study thus confirms previous reports (Boonpucknavig and Nairn, 1967; Maddison, Kagan and Norman, 1968, and Savanat and Chaicumpa, 1969); that the antibody activity in amoebiasis is associated mainly with the IgG fraction of the serum.

The progressive fall of the raised IgG level during treatment is striking and highly suggestive of the specificity of this type of immunoglobulin in host response to amoebic infections. Similar trends were not observed in the level of the serum IgM and IgA. The present findings are in line with those of Ali Khan et. al. (1968) where IgG antibodies increased considerably with booster injections of E. histolytica antigens during immunization of rabbits. The demonstration in agar gel diffusion tests between whole immune sera or the gel filtration fractions and amoebic antigens, produces further evidence for IgG being the possible main reactive antibody in amoebiasis. In dddition, relatively large absolute amounts of IgG were shown to be absorbed by cultured trophozoites.

It is not however, certain whether the IgG antibodies are truly protective. It is conceivable that IgG antibody response to amoebic infection is very transitory, with consequent rapid decline in specific antibody level during convalescence, thereby, exposing the individual to the risk of re-infection.

A most striking observation is the differential reaction of pregnant amoebic patients to immunoglobulin production in contrast to other groups of amoebiasis patients. Thus the results show that all groups of amoebiasis patients except the pregnant ones were able to produce 6 Cyst passers increased levels of all the three major classes of immunoglobulins. The pregnant patients, on the other hand, were unable to produce the same immunoglobulins to give any significant difference in concentrations, even of the serum IgG when compared to the levels in asymptomatic amoebiasis ($t = 0.33 - 1.25$; $p > 0.1$). Thus the study gives an indication that there may be a derangement of the 'immune' mechanism under discussion in pregnancy.

The cause of the decrease of IgG following treatment in patients with amoebiasis is not clear but it is possible that it may be due either to the destruction of the antigen-amoebae and hence the removal of the stimulus to the production of the antibody. It may also be due to direct cytotoxicity of the drug used in the treatment or it may be due to both mechanisms combined.

Table 23

IMMUNOGLOBULIN IN AMOEBIASIS

(Follow-up Study)

*Figures in brackets indicate actual days.

	IgA mg/100ml			IgG mg/100ml			IgM mg/100ml		
	WEEK			WEEK			WEEK		
	0	1st-2	5th-6th	0	1st-2	5th-6th	0	1st-2	5th-6th
1 11H	235	165	200	(1)* 5300	* (15) 4700	(42) 3100	120	110	100
2 33H	400	400	385	(1) 4700	(12) 4000	(40) 3400	650	480	390
3 30H	400	380	340	7100	(16) 4700	(36) 3000	550	450	350
4 53H	400	255	290	7200	(14) 3400	(34) 2100	255	190	128
5 81H	961	85	90	3200	(15) 2600	(40) 1900	300	410	178
6 80H	165	165	168	4900	(15) 4400	(40) 3400	340	320	420
7 52H	400	165	178	6600	(16) 4800	(38) 2000	600	508	410
8 42H	180	165	175	6200	(13) 4700	(41) 4400	600	190	400
9 51H	440	230	250	5400	(15) 4100	(40) 3400	190	110	120
10 69H	120	112	170	7500	(15) 5000	(40) 3200	110	78	72
11 ADM2	400	290	190	4700	(15) 4300	(42) 3680	340	310	300
12 67H	320	240	300	6600	(15) 4600	(35) 4000	103	140	132
13 24H	90	110	125	4400	(14) 3600	(35) 3000	110	110	102
14 1MM1	(0) 200	150	152	(4) 3200	(15) 2300	(40) 2000	(0) 102	112	86
15	(1) 195			(7) 2600			(1) 110		

SYMPTOMLESS "CARRIERS"

	IgA mg/100ml			IgG mg/100ml			IgM mg/100ml		
	WEEK			WEEK			WEEK		
	0	1-2	5-6	0	1-2	5-6	0	1-2	5-6
1	72	88	76	1700	(15) 1800	(42) 1650	70	128	180
2	150	152	140	2100	(17) 2000	(40) 1900	72	90	84
3	90	110	102	2100	(16) 1900	(40) 1950	72	75	88
4	90	100	80	1900	(17) 1900	(38) 1700	78	90	72
5	165	162	104	3200	(15) 2800	(36) 2400	105	130	90
6	190	150	108	3000	(17) 3200	(40) 3000	76	78	92
7	150	154	148	3100	(15) 3400	(36) 3300	111	108	126
8	100	110	86	1900	(16) 2000	(40) 2500	170	100	188
9	85	90	90	2800	(15) 2500	(35) 2300	128	130	120
10	70	90	96	2500	(14) 2600	(35) 2100	129	120	198

TABLE 2

SERUM IMMUNOGLOBULIN LEVELS IN AMOEBIASIS

GROUP OF PATIENTS	No. of Subjects	Level of IgG mg/100ml				Level of IgA mg/100ml				Level of IgM mg/100ml			
		Geometric Means +SD	Median	t value	p	Geometric Means +SD	Median	t value	p	Geometric Means +SD	Median	t value	p
Acute Amoebic Colitis: Male	15	3727 + 713	4000	3.611	.001	210 +20	235	2	.05	108 +67	103	0.5	0.1
Acute Amoebic Colitis: Fem. Non. Preg (FNP)	13	4062 + 1674	4400	3.40	.001	183 +122	200	83	0.1	129 +58	111	1.375	0.1
Acute Amoebic Colitis: Female Preg (FP)	15	3077 + 893	3000	1.25	.1	145 +5	150	.33	0.1	107 +64	103	0.50	0.1
Amoebic Liver Abscess	11	4223 + 1145	4600	3.14	.001	184 +109	190	1.5	0.1	144 +103	111	2.299	0.01
Symptomless Carriers	17	2729 + 834	3000	-	-	152 +66	135	-	-	100 +68	105	-	-
Sick Controls	18	4147 + 1052	4150	-	-	210 +195	210	-	-	181 +71	119	-	-

Table 22

CHROMATOGRAPHIC SEPARATION OF THE SERUM OF A PATIENT (69H) WITH AMOEBIC LIVER ABSCESS.

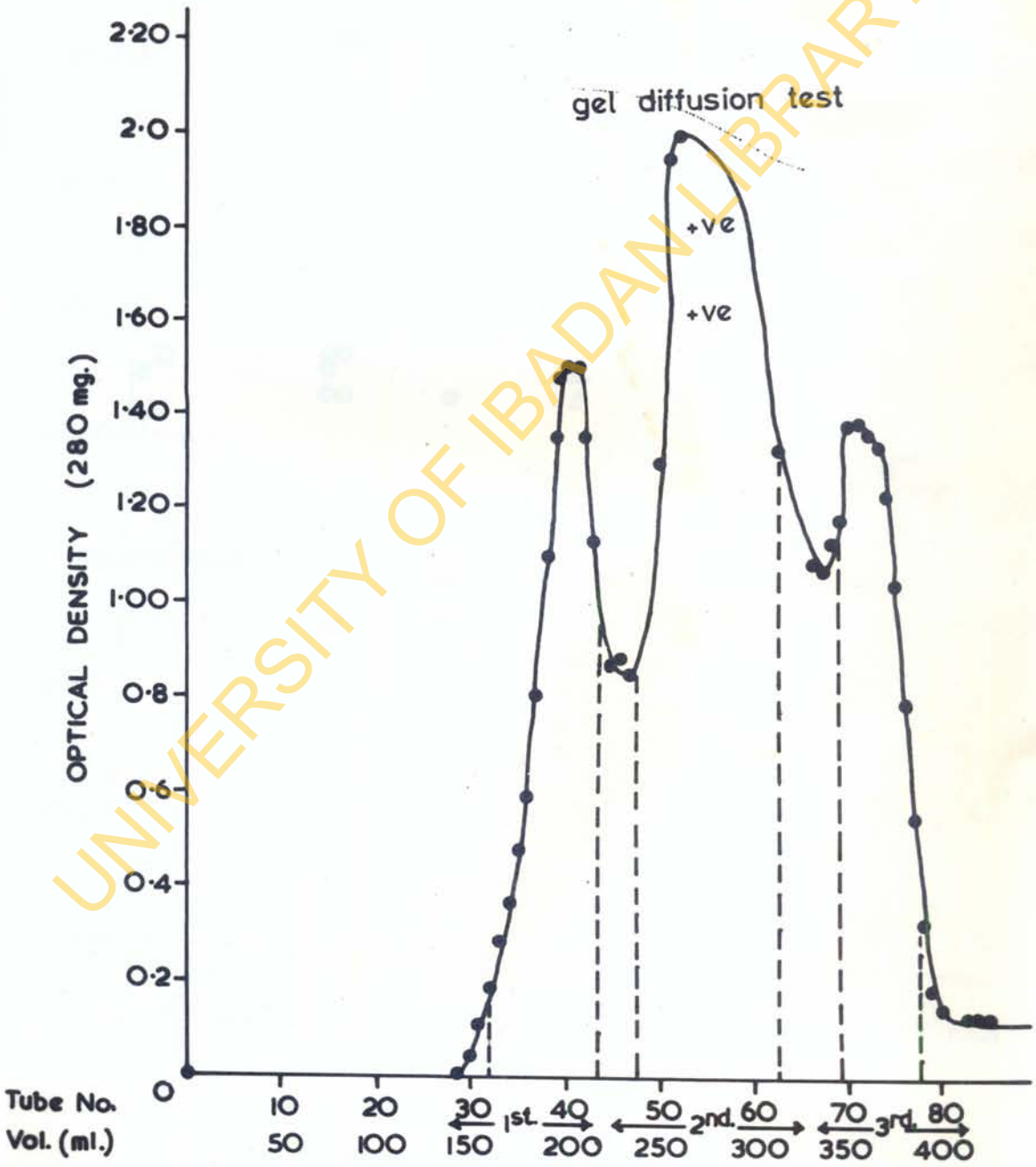


Fig. 25.

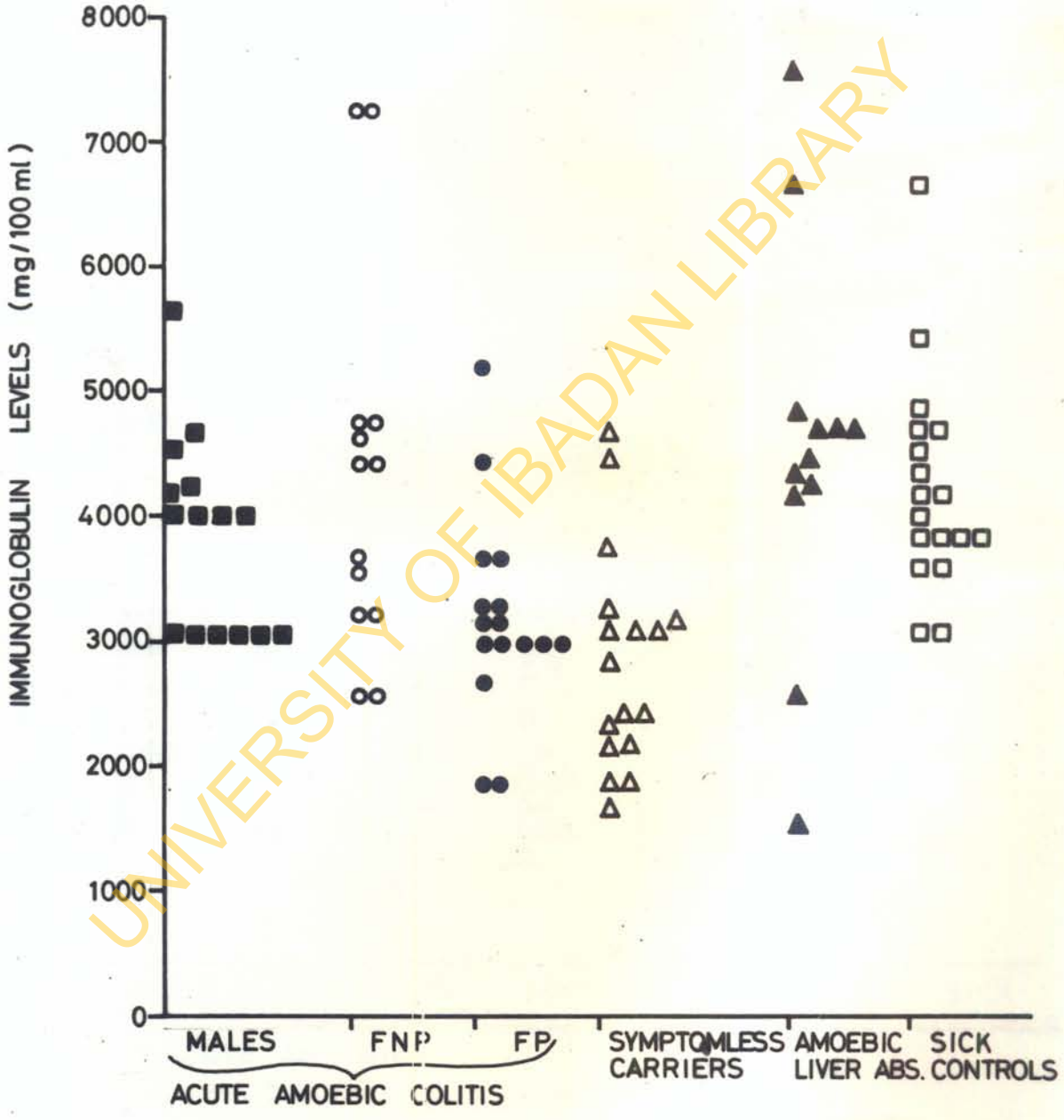


Fig. 36

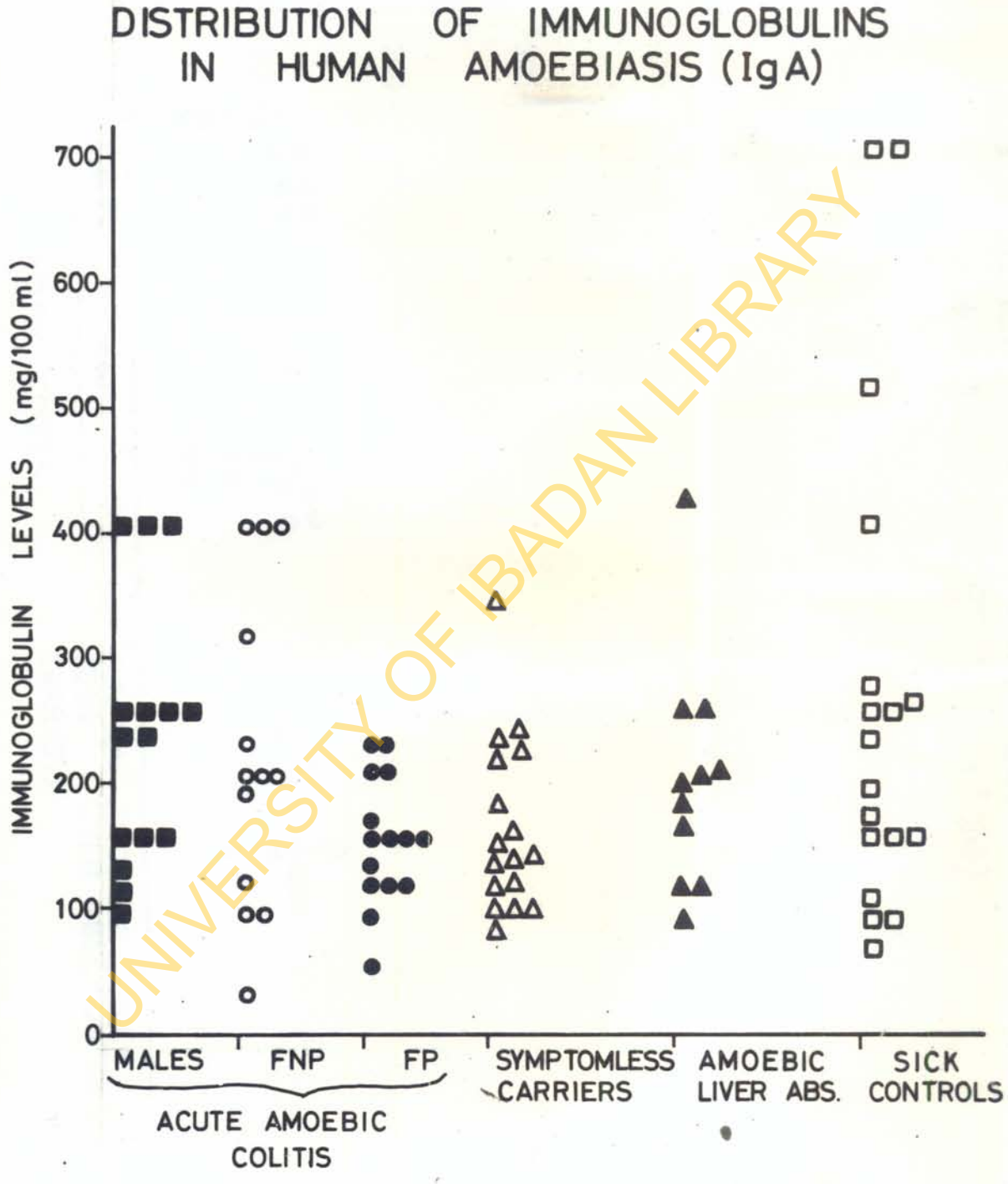
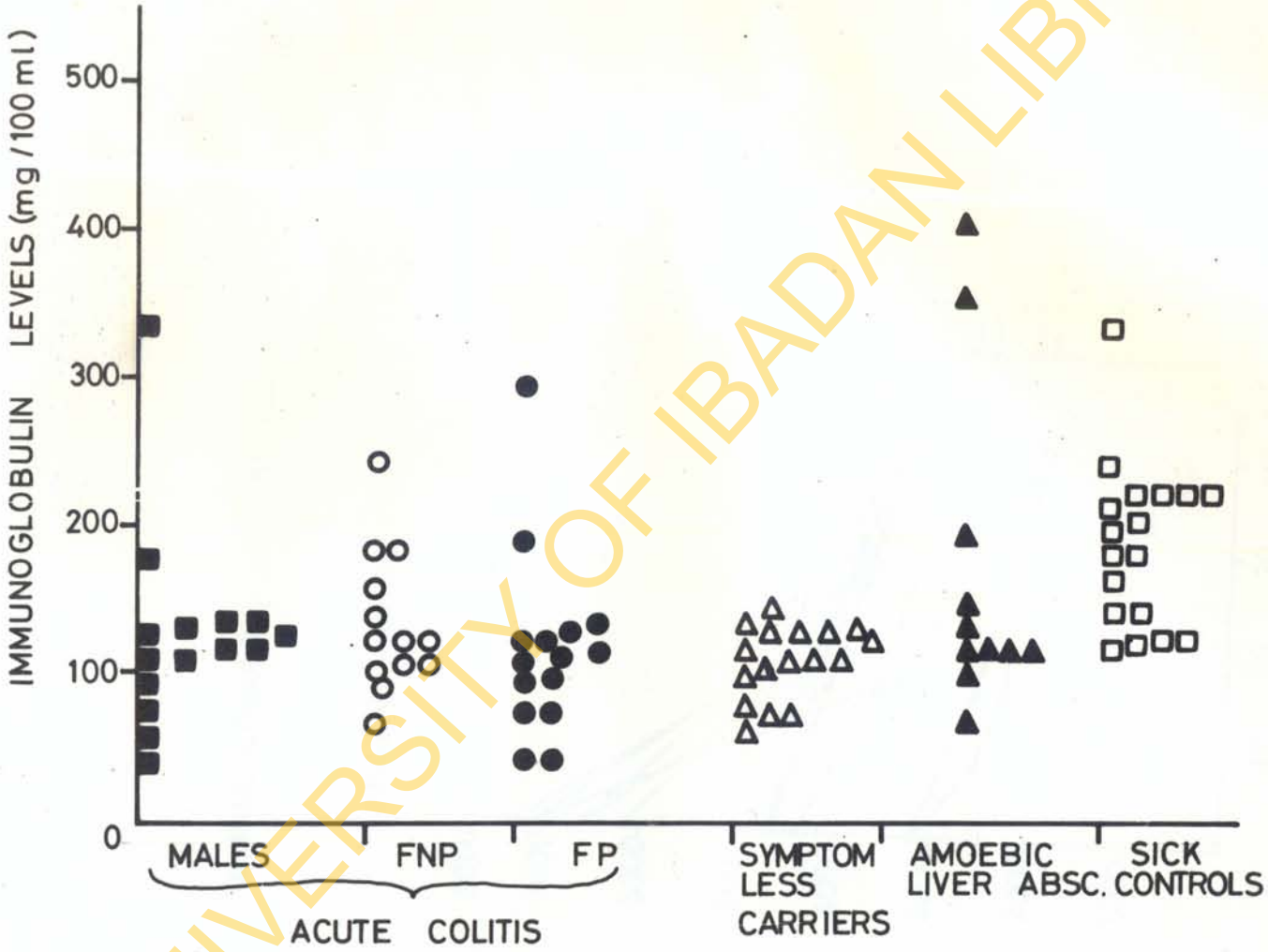


Fig. 37

Fig. 38.

DISTRIBUTION OF IMMUNOGLOBULINS IN HUMAN AMOEBIASIS (IgM)



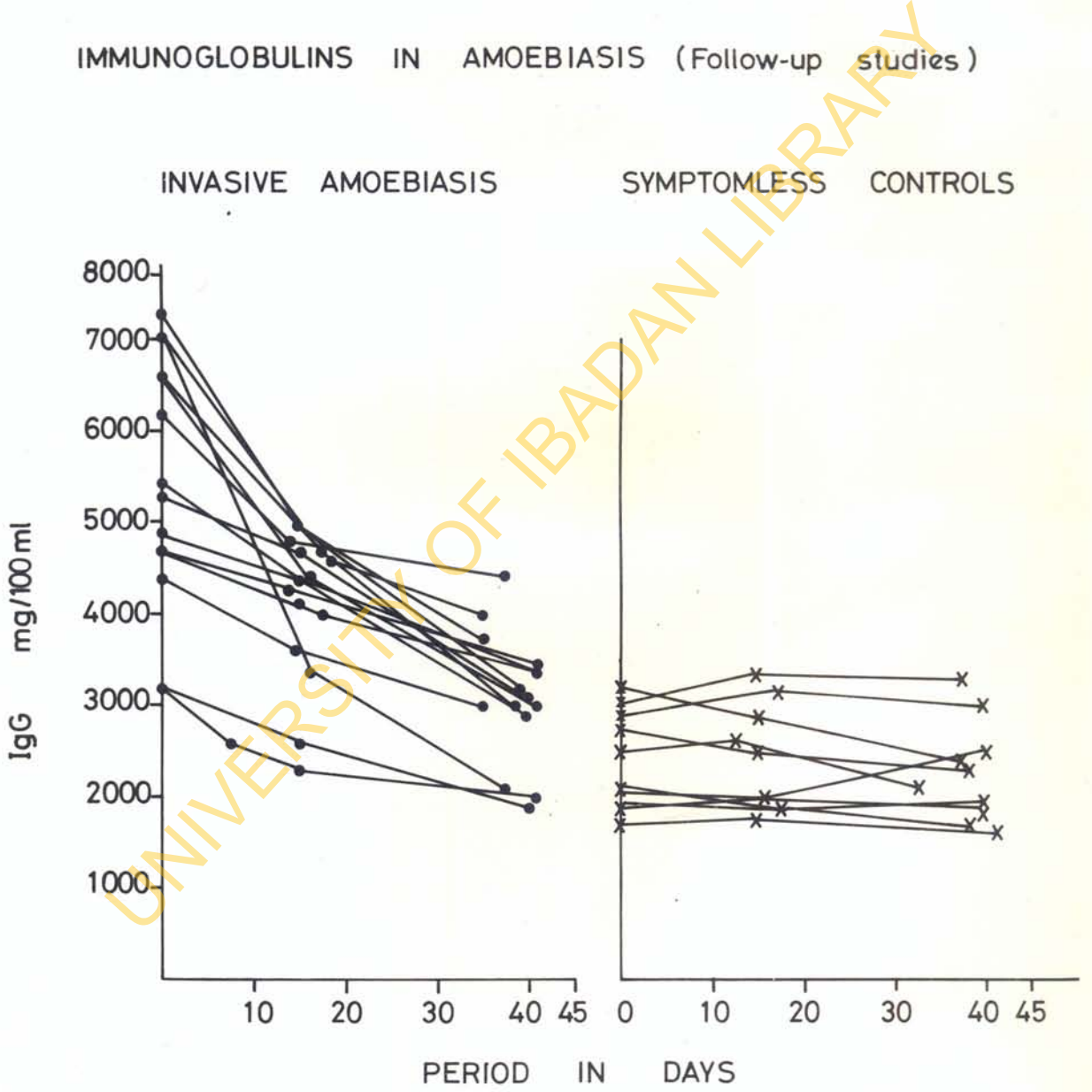


Fig. 39.

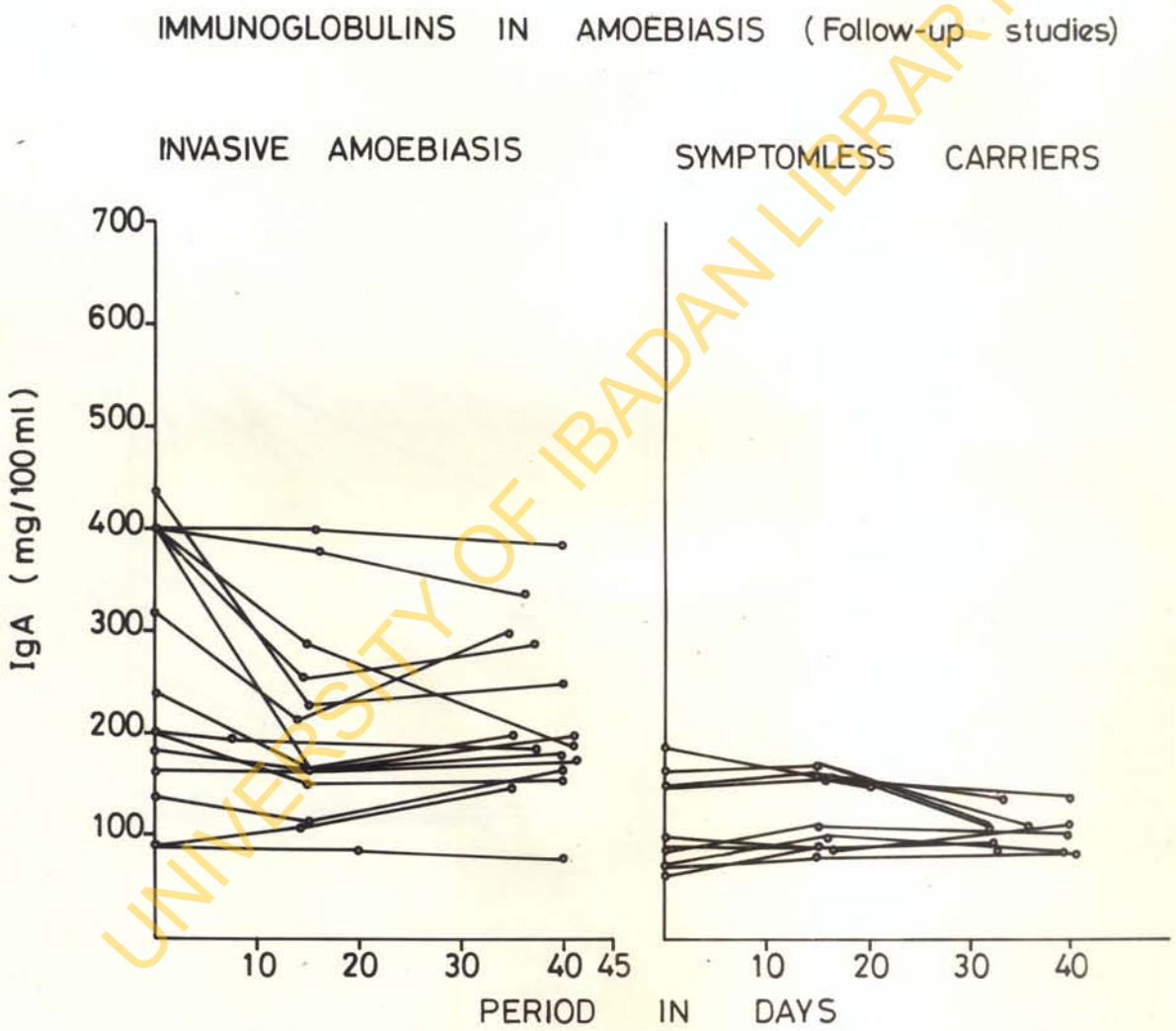


Fig. 40.

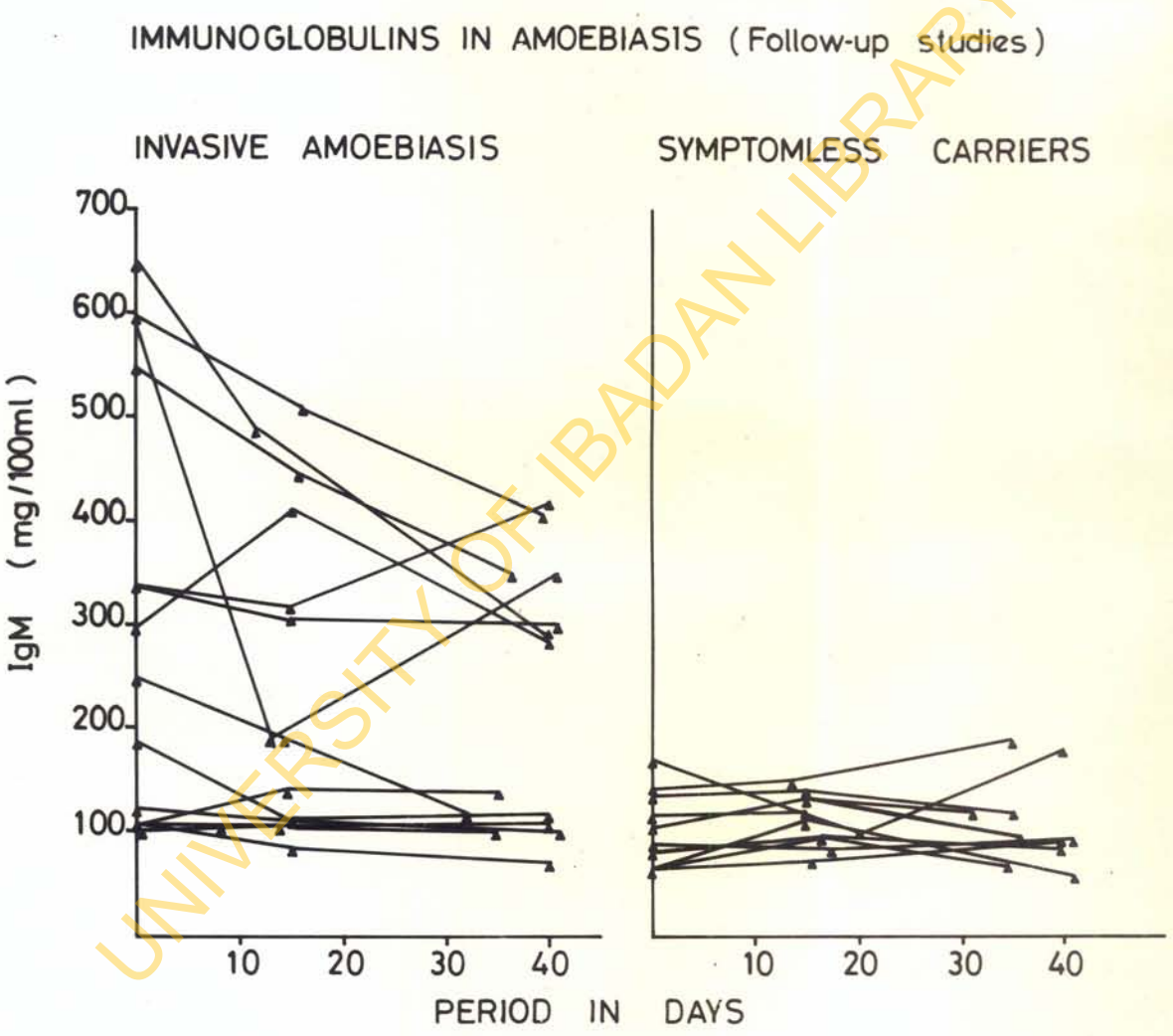


Fig. 41.

(To face Page 232)

Fig. 42. Serum Immunofluorescence In Amoebiasis.

Fig. 43 Immunoelectrophoresis In Human Amoebiasis.

Fig. 44 Immunoelectrophoresis of Pre- and Post- Absorbed Sera.

(A = Unabsorbed)
(B = Absorbed)

TABLE 21: SERUM FLUORESCENT ANTIBODY TITRES IN AMOEBIASIS/CONTROLS

Fluorescent Antibody titres	Amoebiasis		Carriers	Non-amoebic	Normal Subj.
	Intest.	Extra-Int.			
1:10240	0	11	0	0	0
1:5120	0	0	0	0	0
1:2560	0	2	0	0	0
1:1280	1	4	0	0	0
1:640	2	0	0	0	0
1:320	2	1	0	0	0
1:160	4	0	0	0	0
1:80	10	2	0	0	0
1:40	8	1	2	0	0
1:20	1	1	6	0	0
1:10	2	0	2	2	2
Less than 1:10	0	0	10	15	47
Total	30	12	20	17	49

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

8.1. Introduction:

The tests reported in this thesis have been briefly discussed and the results critically compared where applicable with findings from previous investigations. In this final chapter, attempt is made to draw some conclusions from the results and to stress the probable significance of the findings. The conclusions, no doubt, take the form of speculative submissions in part, and consequently, suggestions are also made as to the possibility of further research into poorly understood nature of the immunopathology of amoebiasis.

8.2. Associated Conditions And Complications Of Amoebiasis:

Before Faust and King (1933), and Homer and McNall (1961), called attention to the increased susceptibility of pregnant women to amoebic infections, amoebiasis has been singularly excluded from the array of clinical conditions which are known to complicate pregnancy. Even after the relevant clinical observation and remarks of these workers the notion still failed to gain a general acceptance. The non-acceptability of amoebiasis into the arena of clinical conditions which can complicate or be associated in its severest form with pregnancy, can be exemplified by 'Elsdon-Dew's later remarks on this topic. The latter, in his invitational paper presented as the 1963 Theobald Smith Memorial lecture to the

New York Society of Tropical Medicine, discussed the unfortunate sequelae and chain reactions set up by the false dictum that infection with Entamoeba histolytica always implies disease.

In doing this, he indirectly, probably unconsciously still detached amoebic infections from the list of conditions that may play a fatal role in pregnancy. He said, "excluding pregnancy, all conditions ranging from sore throat and dyspepsia to arthritis and iridocyclitis have all been attributed to the activities of E. histolytica". Although it is not being suggested in the present study that 'amoebiasis' can cause pregnancy!, but the quotation under reference connotes an apparent impression that there is no association whatsoever, between amoebiasis and pregnancy. While agreeing, therefore, with Elsdon-Dew that undue emphasis has been laid in the past on the significance of E. histolytica infections, it is sheer coincidence and an accident of history that the impression of amoebiasis being more severe in pregnancy started gaining ground soon afterwards. Thus Duque and several other independent workers already mentioned in the text realized that amoebiasis may be particularly severe when it occurs during pregnancy.

The relationship between pregnancy/puerperium and the general lowering of resistance to infections is undoubted. The fatality of amoebiasis occurring during pregnancy and the puerperium, compared with the same situation in other acute diarrhoeal diseases in Nigeria and

presumably in most other tropical African countries, has not hitherto been stressed. This is excusable because of the overlap in clinico-pathological features and lack of absolute criteria of clinical distinction between amoebiasis and the conventional acute diarrhoeal diseases. It is of interest, therefore, that some light was shed on the clinico-pathological problem during the post-mortem studies presented in this thesis. In the relevant section, it was confirmed by statistical analysis, that there is significant association between amoebiasis and death in pregnancy and the early puerperium, whilst similar analysis of cases of typhoid enterocolitis occurring in similar states shows no such significant association. Admittedly, the figures quoted in the relevant portion reflect the increased prevalence of amoebiasis in males, and do not necessarily indicate that the disease is apt to be more severe in males. As already noted, Gomez (1960) found that although males made up 64% of his clinical cases only 53% of the fatalities occurred in this same group of patients.

The present study shows that death due to amoebiasis during pregnancy contributed greatly to the high percentage of deaths due to amoebiasis recorded for the female patients of child-bearing age, thus showing more conclusively that amoebiasis carries a high mortality in pregnancy and the early puerperium.

It should however, be emphasized that several complications may occur in amoebiasis in Ibadan. Amoebic liver abscess and localized

peritonitis are by far the commonest complications. The remarkably high mortality due to amoebiasis occurring most commonly during the third and fourth decades of life has already been noted by Duque (1969). It was confirmed during the present review of deaths due to acute diarrhoeal diseases recorded in the post-mortem protocols of the Pathology Department of the University College Hospital, Ibadan. It may be inferred from the reports of Odunjo (1965); Lewis and Antia (1969); Duque (1969); and the present study that pregnancy/puerperium appear to be aggravating factors in amoebiasis. The overwhelming preponderence of deaths due to amoebiasis in pregnancy and the allied states, over deaths due to other acute diarrhoeal diseases in similar states as shown above, indicates the operation of some hitherto unknown factors, possibly, peculiar to amoebic infections.

The detection during in-vitro studies, of a serum factor known to enhance the growth of E. histolytica in high concentrations in the sera of pregnant women and of women in the early puerperium, is strong evidence in favour of the acceptance of the severity and usually fatal outcome of amoebiasis in pregnancy and the early puerperium as a real occurrence.

3. 3. Specific Diagnostic Serum Factors In Amoebiasis:

Apart from the clinico-pathological problems posed by the severity of amoebiasis during pregnancy and the early puerperium, the need for search for serum factors, becomes pressing as a diagnostic tool for

extra-intestinal amoebiasis.

During the studies on the complications of amoebiasis in the course of the present work, it was evident that amoebic liver abscess was encountered relatively frequently as a complication of intestinal amoebiasis in the materials available for study at the University College Hospital, Ibadan. Analysis of the results then showed that liver abscess occurred in 20.74% of cases of intestinal amoebiasis. It was also found that this condition was a significant complication of intestinal amoebiasis at 0.01 per cent level. As an example of extra-intestinal amoebiasis, the diagnosis of amoebic liver abscess in such an endemic area as Nigeria, may not be difficult when the condition presents with the cardinal symptoms and signs. Thus, it may be suspected in a patient presenting with pain in the right upper quadrant of the abdomen or in the lower right chest, associated with tender hepatomegaly and lower right intercostal tenderness.

However, not all patients show these features and the findings may suggest other intra-abdominal or pulmonary disease. The patient's history may be misleading, stool examination and haematological investigations are not diagnostic, and little assistance is provided by routine liver function tests (Powell, 1959). Liver biopsy has been found useful (Keeley, Schmaman and Scott, 1962), but in most clinicians' view it is rarely warranted especially considering the risk attached to such diagnostic procedure. Diagnostic aspiration is usually to be avoided in

the absence of signs from which the abscess can be located (Wilmot, 1962). Radiological investigations may show the liver to be the primary site of disease, but they cannot always establish amoebiasis as the cause and may be normal when the abscess is deeply situated or close to the inferior surface of the liver.

It is true that the microscopic detection of E. histolytica in the necrotic areas of the liver may be taken to be pathognomonic. However, since the absence of the organism does not exclude a diagnosis of amoebic liver abscess, the histological distinction between amoebic liver abscess and pyogenic liver abscess could be difficult and very frequently impossible. The establishment of a simple immuno-diffusion, technique for amoebiasis, using as antigens, extracts from local strains of E. histolytica, even if only as a negative evidence, seems to have overcome the difficulty in differentiating amoebic liver abscess from frank pyogenic abscess in the cases encountered in the University College Hospital, Ibadan.

8.4. Characterization Of E. histolytica Antigens:

The biological and immunological activities of parasite antigens have been under investigation since the turn of the century, and antigen-antibody interactions in helminthiasis, particularly, have been discovered to be many and complex (Soulsby, 1963). The use of parasitic antigens has not been limited to serology. They have also been used as vaccines to stimulate

host resistance. In the present study, attempts were made to characterize E. h. extracts and apply the result to the development of specific antigens for serological and immunological studies. In addition the characterization of the antigens was used to suggest the aetiogenesis of carrier states in amoebic infestation and the generally accepted 'weak' nature of amoebic antigens. All the theories postulated were based on Dineen's (1963) and Damian's (1964) provocative speculations on the host-parasite relationship. As already noted, these workers suggest that the immune response of the host may exert a selective pressure on the parasites that have less antigenic disparity with the host.

As a result of the characterization of E. h. antigens by various immunochemical methods, it has been possible to suggest that the possible weakness of E. h. antigens, if proven may be due to a successful host-parasite relationship of E. h. whereby the organism has possibly shared many antigenic determinants with its host. If this is true, it is being suggested that "molecular mimicry" between E. h. and its host may have resulted in a successful tissue graft that does not stimulate a rejection response on the part of the host, which may therefore give rise to the carrier state in amoebic infestation. This, in fact, is an attractive possible speculation for the E. h. carrier state, but the absence of conditions favourable for the invasion of host tissue by the organism, appears to be a more plausible explanation.

8.5. Characterization Of Antibodies To E. histolytica:

The characterization of antibodies is a vast area of research and includes the difinition of primary, secondary, tertiary, and quaternary structure; molecular and genetic variations; the determination of protein-bound carbohydrates, synthesis, distribution and turnover, serologic reactivity and specificity, and so forth. The work reported here concerns immunochemical and/or physicochemical characterization, and thus with the recognition of the three major immunoglobulin classes of antibody - IgA, IgG, and IgM. The techniques employed, therefore, include gel filtration on columns of Sephadex G-200; immunoelectrophoresis, serologic reactivity and specificity, with the aid of the immunodiffusion test and lastly the measurement of the serum immunoglobulin levels.

The application of these immunochemical techniques was based on some available data on the characterization of antibodies in parasitic infections. Thus Gilles and McGregor (1959), using parasitological and biochemical techniques, demonstrated that in the Gambian African children exposed to repeated and heavy malarial infections, develop serum gamma-globulin concentrations significantly in excess of concentrations of children living in identical circumstances but protected from birth against malaria by continuous chemoprophylaxis. The same workers again, in 1961 using similar techniques also found that malaria contributes considerably to the development and maintenance of hyper-gamma-

globulinaemia, in adult Africans. Similar results have been obtained by other workers in various parts of the world (Edozien, 1961; Deegan, Gilles, and McGregor, 1956; and Holmes, Stainier, and Thompson, (1955). More recently, Abele, Tobie, Hill, Contacos, and Evans (1965), published their findings on alterations in serum proteins and antibody production during the course of induced malarial infections in man. Sera were separated by gel filtration on columns of Sephadex G-200, and the fractions were tested for antibody activity by means of fluorescent - antibody test using a fluorescein - labelled rabbit anti-human immuno-globulin antiserum. In addition, reductive cleavage with mecaptoethanol was used to help differentiate IgG and IgM antibodies. In several of the volunteers antibody activity was confined to the IgM fractions of serum during the first few days of antibody production, but thereafter was found in both IgM and IgG. Approximately 40 days after infection antibody activity was present only in IgG. Again in 1966, Houba and Allison described their findings on M-antiglobulin (rheumatoid-factor-like globulins) and other globulins in relation to tropical parasitic infections.

The examples cited above illustrate that these techniques can be used in the field of parasitology in general. The findings in the present study confirm that similar methods can be applied to amoebic infections. Attempt was also made to use the results to explain the role of antibodies in a clinico-pathological problem posed by amoebiasis in pregnancy

and the allied states. The author has thus attempted to apply the characterization of antibody and antibody formation to the study of host response to E. histolytica. It should, however, be emphasized that to actually define an antibody as IgG, IgA, or IgM one must usually resort to studies employing antisera specific for the heavy chains of these three immunoglobulins. In the same breadth, to really confirm or otherwise, that an antigen such as those of E. h. used in this study as weak, it would be necessary to compare the immunogenicity of the antigens in rabbit both with an adjuvant and without. These are, therefore, being strongly advocated in future studies in the immunopathology of amoebiasis.

8.6. Influence of Various Serum Factors On Amoebiasis:

The immunological studies reported in Chapter Seven demonstrates the presence of antibodies both to the surface and intracellular antigens of E. histolytica, in the sera of patients with amoebic infections. The immunological technique employed for the detection of such antibodies did not differentiate either qualitatively of quantitatively between the severe and mild forms of the disease.

However, during the study of serum cholesterol - an agent known to enhance the in-vitro growth of E. h., it was found that the level of this serum component was unduly high in the sera of pregnant women and those in the early puerperium. The statistical correlational studies further give

an indication that serum cholesterol may be an aggravating factor in the severity of amoebiasis.

With the study of the immunoglobulin levels, it would appear that there is marked suppression of the synthesis of the various immunoglobulin ; classes by pregnant women and those in the early puerperium. The significance of this finding is not clear and the finding should be interpreted with caution. It is possible that they may have bearing on the changes in the immunoglobulins in parasitic diseases in general. It is known that raised immunoglobulin levels in parasitic diseases are common (Smithers, 1967). Unfortunately, most of the early studies on this subject were made before the recognition of the immunoglobulins as distinct types - IgG, IgM, IgA, IgD and IgE, and in most cases the relative increase in each immunoglobulin type is not known. To the best of the author's knowledge, no attempt has hitherto been made at the quantitation with statistical analysis of data of the immunoglobulins in amoebiasis. The generally raised levels of the immunoglobulins may be a reflection of similar event in other parasitic diseases.

In experimental and few instances of human parasitic diseases where attempts were made to study the immunoglobulin classes notably in trypnosomal and malarial infections the IgG, IgA, and IgM levels are all raised (Smithers and Terry, 1959; Tobie, Abele, Wolff, Contacos and Evans 1966; and Houba and Allison, 1969). Especially high IgM levels

are reached early in the infection, and it has been suggested that frequent antigenic variation of the malarial and trypanosomal parasite may contribute to the raised IgG levels (Brown and Brown, 1965).

On similar basis one can postulate that the raised immunoglobulin levels in amoebiasis may be part of the general ability of protozoal infections to provide a potent stimulus for immunoglobulin synthesis. The results of the absorption and follow-up studies in chapter seven give an indication that the fall in the levels of the immunoglobulins particularly of IgG class may be a reflection of the existence of 'specific' antibodies in amoebic infections. However, for a conclusive evidence, it would be necessary in future studies to show that the immunoglobulins produced are specific and have detectable affinity for the infecting Entamoeba histolytica. For this purpose it is being suggested that the fractional turnover rates of normal and immune IgG be compared by using IgG (labelled with 131_I and 125_I).

Granting that it is even possible to prove specificity, of immunoglobulins, it would still be necessary to correlate the antibodies in amoebiasis with protective role. It is then, perhaps, that one might be able to interprete the lowered immunoglobulin levels in pregnancy and the allied states with severe amoebiasis, as having been due to a derangement in the immune response to amoebic infections during these states.

8.7. Effects Of Immunosuppressive Agents On Immunity:

The manipulation of the immune response to amoebiasis by immunosuppressive drugs has been little studied. However, the adrenal steroids have been used in a variety of studies on immunity to parasites. It has been demonstrated for example, that the elimination of adult worms of Trichinella Spiralis in mice, probably an immune event, can be markedly inhibited by cortisone (Coker, 1956; Larsh, 1967).

Prednisolone has been used successfully to inhibit the immune elimination of N. braziliensis from the gut of rats (Ogilvie, 1965). Cortisone has been used to overcome "innate" resistance to such helminth parasites as Litomosoides carinii (Briggs, 1963) and Nematospiroides dubuis (Cross, 1960) in rats.

There are some cases of latent amoebiasis which flared up following treatment of cases of leukaemia and Hodgkin's lymphoma (seen in the course of this study) with cytotoxic agents such as cyclophosphamide (a known immunosuppressive agent). These cases may be regarded as the clinical evidence for the role of immunosuppressive drugs in amoebiasis. On the other hand, an entirely different mechanism may be operating in these cases of immunosuppressive therapy under reference.

Although interpretation of the effects of adrenal steroids and allied agents on immunity to parasites is difficult; the results of the studies reported in chapter six give an indication that the severity of amoebiasis in pregnancy and the puerperium may in part be due to increased hormones found in the serum during these states.

In conclusion, therefore, amoebiasis, has been found to be an acute fulminating and almost invariably a fatal disease when it occurs during pregnancy and the early puerperium. The demonstration of low serum immunoglobulin levels together with raised serum cholesterol levels during pregnancy and the early puerperium, raised the hope for search and discovery of specific agent(s) responsible for the undue severity of amoebiasis in pregnancy and early puerperium.

"The search for truth is in one way hard and
in another easy, For it is evident that no
one can master it fully nor miss it wholly.
But each adds a little to our knowledge
of Nature .....(Aristotle)"

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APPENDIX I

THE DEMONSTRATION OF ENDAMOEBAE IN TISSUE SECTIONS BY MEANS OF MODIFIED METHENAMINE-SILVER NITRATE TECHNIC

MATERIAL AND METHODS:

Paraffin-embedded sections of buffered, formalin-fixed human colon from patients with diagnosed amoebic colitis were used. Sections were stained with the Casella, Bauer, Hotchkiss-McManus, haematoxylin, azure A, eosin B methods. The modified methenamine-silve nitrate method follows.

Stock solutions: The reagents are identical with those previously described, except in the use of periodic acid (HIO_4) at 0.5 per cent concentration. Stock solutions for counterstaining with light green or eosin Y are 0.2 per cent solutions of dye in 0.2 per cent acetic acid. The working solutions are composed of 10 ml. of stock solution and 50 ml. of distilled water.

PROCEDURE:

1. Deparaffinize and take sections through graded alcohols to water.
2. Treat for 10 min. in an aqueous 0.5 per cent solution of periodic acid at room temperature (25 C.) Wash in running water for 5 min.
3. Treat with aqueous 5 per cent chromic acid for $1\frac{1}{2}$ hr. at room temperature. Wash for 10 min. in running water.

4. Place for 1 min in a solution of 1 per cent aqueous sodium bisulfite, in order to remove traces of chromic acid.

4. Wash for 5 min. in running water, and then rinse in distilled water.

6. Silver at 37 C. (a water-bath is used), using stock methenamine-silver nitrate solution, 25 ml, distilled water, 25 ml, and 5 per cent aqueous borax solution, 2 ml., for 2.5 to 3 hours.

7. Rinse in several changes of distilled water.

8. Tone for 5 min. in 0.1 per cent gold chloride solution.

9. Rinse thoroughly in 2 per cent sodium thiosulfate solution for 5 min., in order to remove unreduced silver, and wash for 10 min. in running water.

10. Counterstain with working solution of light green or eosin Y for 30 to 45 seconds. Dehydrate, clear, and mount in synthetic resin (Permount).

APPENDIX 2

Preparation of the Various Culture Media Assessed For the In-Vitro Cultivation Of Entamoeba histolytica

1. DOBELL & LAIDLAW'S NO. 1 MEDIUM:

(a) Wash 4 large eggs carefully with alcohol.

(b) Break into sterile flask containing glass beads or magnetic stirrer.

(c) Add 50 c. c. of Ringer's solution.

(d) Shake mixture thoroughly until a homogenous mixture is secured.

(*Ringer's solution consits of 9mg. NaCl; 0.2gm $CaCl_2$; 0.2gm KCl in 1 litre Distilled H_2O).

(e) Mix whites of the eggs with a litre of Ringer's solution.

(f) Distribute into tubes and allow to slant and solidy at 70°C.

(g) Autoclave tubes at 1 lb pressure for 20 minutes.

(h) Cover slants to a depty of about 1 cm. with a mixture of 1 part sterile inactivated human blood serum.

(i) Pass mixture through a filter and incubate at 37°C for at least 24 hours before used.

D. & L. MEDIUM NO. II

Undiluted serum is used for making the slants. Pass horse serum through a bacterial filter; place in tubes, slant and also solidify for 1 hr.

2. BOEK AND DRBOHLAV'S (Locke-egg-serum - L. E. S.) MEDIUM

Materials: (i) 4 large eggs; (ii) Ethyl Alcohol; (iii) Magnetic Stirrer/ glass beads; (iv) Locke's solution (i.e. 9gm NaCl,

0.2 gm $CaCl_2$; 0.4 gm. KCl, 0.2gm $NaHCO_3$, 2.5gm glucose dissolved in cold distilled water).

(iv) test tubes or bottles; (v) Inactivated Human serum

(vi) a bacterial filter.

Method: (a) Wash and brush the eggs with alcohol; (b) Break and mix eggs in sterile flask. (c) Fill test tubes or bottles to about 2" slants; (d) Insipate and heat at 70°C until mixture solidifies. (e) Autoclave at 15 lbs pressure for 20 minutes; (f) Cover tubes to a depth of about 1 cm with a mixture of equal part of sterile Locke's solution and 1 part of sterile human blood serum following passage of mixture through a bacterial filter; (g) Incubate at 37°C for at least 24 hours to confirm sterility.

**Modification of the original medium.

This medium was modified by adding 0.1 ml (i.e. 10,000 units streptomycin to 10 mls of the covering/or fluid overlay. Streptomycin was prepared by adding 10 mls sterilized distilled water to 1 gm ampoul of streptomycin.

3. CLEVELAND'S& COLLIER'S MEDIUM (Spingarn & Edelman - Am. J. Trop. Med. Hyg. 1: 412, 1952).

Endamoeba Medium (353)-

Dehydrated:

(i) Beef Liver, Infusion 27.2gm

(ii) Protease Peptone, Difco 5.5 gm.

(iii) Disodium phosphate 3 gm

(iv) Sodium Chloride 2.7 gm

(v) Bacto Agar. 11 gm.

To Rehydrate the medium:

Suspend 33 gm of Bacto-Endamoeba Medium in 1 litre of cold distilled water and heat to boiling to dissolve the medium completely.

Distribute in tubes/bottles and sterilize in the autoclave for 15 minutes at 15 lb pressure (121°C). Allow the medium to solidify with the container in the slanting position.

For use: Cover the slants with Bacto- or any other horse serum diluted 1:6 with sterile normal saline. Add sterile rice powder.

APPENDIX 3

ROBINSON'S MEDIUM: (Trans. roy. Soc. Trop. Med. Hyg. 62: 285, 1968).

This consists of: (i) Saline Slopes: Agar powder 1.5% with NaCl 0.7% in distilled water. Distribute in 5 or 10 ml (mod.) culture bottles, slope after autoclaving.

(ii) Erythromycin solution: Suspend base to about 20% in 70% ethanol in a sterile vessel. Further dilute to 0.5% after 2 hours by sterile water and store at 4°C. (N.B. Add 1.5ml of 70% ethanol to 300mg base and make up to 60ml by adding 58.5 mls sterile water. i.e. 6mls - 5%; 1.5ml - 20% or 60mls - 0.5%

(iii) Bactopeptone: Dissolve to 20% in water & autocl. OR 10% Neutralized Lablemco solution (10gm/100ml).

(iv) Sterile Rice Starch.

(v) PHTHLATE: (a) 20.4 gm Potassium (H) phthalate in 100ml 40% NaOH in water and make up to 2 litres. (b) Adjust to pH 6.8, distribute in bottles and autoclave. (c) For use dilute 1:10 in sterile water for use as 0.05M phthalate 6.8 diluent.

(vi) DEFINED MEDIUM "R" (SYNTHETIC) FOR GROWING Esh. coli

Concentrated Stock consists of: (a) 125gm NaCl; 50gm Citric Acid Monohydrate; (b) 12.5gm KH_2PO_4; (c) Ammonium Sulphate (25gm) (d) 1.25gm $MgSO_4 . 7H_2O$; (e) 100 ml Lactic Acid. Dissolved in 2.5 litres distilled water.

Working Solution:

(a) Stock Solution 100 ml

(b) 40% NaOH 7.5 ml

(c) 0.04% Bromothymol blue 2.5 ml.

(N.B. To prepare 0.04: Bromothymol Blue solution; Dissolve 4 gm in 100 ml of 50% Ethanol and dilute 1:100).

(d) Distilled water 1 litre.

Adjust to pH 7 by adding few drops of 40% NaOH, and autoclave.

Use Stock over 4 weeks to avoid change of pH on autoclaving.

vii. BASAL AMOEBIC MEDIUM "BR".

Grow Esch. coli strain B of K12 or any other strain for 2 days at 37°C in a hollow layers of B in sealed flat bottles and thereafter store at room temperature up to 2/12. Reaction should not exceed pH 7.3

viii. SHEEP SERUM "S".

Clear serum from the slaughter house by paper pulp on Buchner filter, Seitz-filter heat to 56°C on 3 successive days and store at 4°C.

ix. COMPLETE MEDIUM FOR AMOEBIC GROWTH

"BRS" (i) Mix equal volumes of sheep (or horse or human or Ox serum with BR incubate for 24-48 hours at 37°C. Store at room temperature up to one month.

TECHNIQUE FOR AMOEBIC CULTURE FROM FAECES:

(A) Fresh untreated faeces - 50 mg. (by wire loop) to culture bottle which receives at the time of inoculation

(ii)	0.5% Erythromycin	4 drops (0.12ml)
(iii)	Rice starch	10 mg (judge on blade).
(iv)	BR MEDIUM	(1.5ml) to 2/3 of the way up for 5ml culture bottle

Keep at 37°C.

(B) AFTER 24 hours at 37°C pipotte off the supernatant fluid and replace by equal volume of mixture of BRS and phthalate diluent (about 1.5ml) to 2/3 of the way up the slope with 2 drops (0.06ml) of 20% Bactopeptone (or Lablemco 10%); 2 drops (0.06ml) of 0.5% erythromycin and more rice starch.

On the second day (after a further 24 hours at 37°C) and again on the 4th day drops of culture sediments are examined microscopically in double strength Lugol's iodine.

SUB-CULTURE: Further subcultures are made if required in 3 ml volumes (to bottle neck) of phthalate dilutions of BRS, with starch, erythromycin and peptone. Sometimes faeces prove to fermentative to be restrained by erythromycin, replication with phenoxy-ethanol 0.01% may then succeed.

MODIFICATION OF ROBINSON'S MEDIUM:

1. Addition of 10% Lablemco, potassium phthalate solution, and Esch. coli cultures were omitted from subsequent subcultures once a culture is established.

2. Erythromycin has been replaced by 0.1% streptomycin/ 10ml culture and the original fluid overlay replaced by 18% Horse-saline (1:6).

APPENDIX 4

Ultra/Seitz-Filtration Using Pressure/Vaccuum Pump

"Speedivac" Model RB4 (BTL).

APPENDIX 5

"Warm-Environment" Microscope For Examining *E. histolytica* Cultures at 37°C.

APPENDIX 7

Freund s Adjuvant

1. Incomplete type (without mycobacteria), two formulae.

   (a) 3 parts light mineral oil U.S.P.; 1 part Aquaphore, Falba, or anhydrous lanolin; 4 parts physiologic phosphate buffer

   (b) 4 parts n-hexadecane; 1 part glycerol monooleate (Myverol, Distillation Products Inc.)

2. Complete type (with mycobacteria):

   Add mycobacteria (living avirulent tubercle bacilli, heat-killed virulent tubercle bacilli, Mycobacterium, or M. Smegmatis to make a final concentration by moist weight of 10 mg. per ml. of water -in-oil emulsion. The same final concentration of any particular antigen can be used. Bacilli and antigen should be suspended in the aqueous phase before emulsification.

3. Emulsification: Emulsifier (e.g. glycerol monooleate) is dissolved in oil, and oil is layered upon water. Water-in-oil emulsions are obtained mixing these two phases together vigorously with a syringe and large guage needle, or other such instrument. The stability of water-in-oil emulsion can be tested by dropping some on cold water; the drop should remain intact.

APPENDIX 8

ESTIMATION OF DIFFUSION COEFFICIENTS OF ANTIGENS

By Double Diffusion Technique.

Calculation. (For theoretical treatment Allison & Humphrey, 1960 refers).

The use of this arrangement for measuring diffusion coefficients has been described in chapter seven. The practical details have been given in the same chapter, and the ratio of the diffusion constant of antigen (D_g) and of antibody (D_b) is given by:

$$\tan\theta = (D_g/D_b)^{1/2}$$

where θ is the angle between the precipitation line and the antigen trough. Taking rabbit or human antibody diffusion coefficient as 3.8×10^{-7} (Kabat, 1964).

The coefficient of diffusion of the various components of E. histolytica antigens are as follows:

1st Component $\theta = 60 \pm 9.50$

$$\tan\theta = \sqrt{\frac{D_g}{D_b}}$$

$$\tan^2\theta = D_g/D_b$$

$$D_g = D_b \tan^2\theta$$

and $D_g = 3.8 \times 10^{-7} \tan^2 (60 \pm 9.5)$.
$= 6 \times 10^{-7}$ to 28×10^{-7}

Similarly for:

2nd component	D_g	$= 4.7 - 9.6 \times 10^{-7}$	$(\theta = 53 \pm 5^{\circ})$
3rd component	D_g	$= 4.1 - 5.4 \times 10^{-7}$	$(\theta = 46 \pm 4^{\circ})$
4th component	DD_g	$= 3.8 \times 10^{-7}$	$(\theta = 45^{\circ})$
5yh component	D_g	$= 3.1 \times 10^{-7}$	$(\theta = 42^{\circ})$.

APPENDIX 9

PRODUCTION OF RABBIT-ANTI-HUMAN SERUM

(Proom, H., J. Pathol. Bacteriol. 55:419(1943).

I. Mixture:

(i)	Human Serum	25 ml
(ii)	10% Pot aluminium sulphate ($KAI(SO_4)_2 . 12 H_2O$)	90 ml
(iii)	Distilled water	80 ml

Adjust pH to 6.5 with 5N. HaOH.

II. (a) Centrifuge the mixture; (b) Wash sediment twice with isotonic saline solution with merthiolate (1: 10,000). (c) Make sediment to 100ml with Isotonic saline.

Suspension can be kept for at least 14 days.

III. IMMUNIZATION OF RABBITS.

Inject Rabbits as follows→

1st day - Inject 5 ml intramuscularly in each buttock.

14th day - Inject 5 ml intramuscularly in each buttock.

24th day - Inject 1 ml untreated serum intra-peritoneally.

34th day - Collect approximately 50 ml blood from each animal.

Repeat the procedure 1 - 10 times after a rest period 2-3 weeks.