GUIDE TO POLIOVIRUS ISOLATION AND SEROLOGICAL TECHNIQUES FOR POLIOMYELITIS SURVEILLANCE

I. DÖMÖK

Chief, Division of Epidemiology and Microbiology,
National Institute of Hygiene,
Budapest, Hungary

and

D. I. MAGRATH

Division of Viral Products,
National Institute for Biological Standards and Control,
London, England

WORLD HEALTH ORGANIZATION
GENEVA
1979

WHO Offset Publication No. 46

CONTENTS

INTRODUCTION

Poliomyelitis is one of the six diseases whose control is being given special priority by WHO through inclusion in its Expanded Programme on Immunization. Experience in many countries shows that the incidence of paralytic cases drops quickly to low levels after the introduction of effective immunization programmes. This fall in incidence can be used as an indicator of the progress achieved in covering susceptible children.

1. POLIOMYELITIS CONTROL: THE ROLE OF PUBLIC HEALTH LABORATORIES

1.1 Planning

Before initiating any control effort, national authorities must assess the importance of the problem they are seeking to control. For poliomyelitis, this can be done through retrospective surveys of residual paralysis in children beyond the age of susceptibility. This technique was used in Ghana by Nicholas et al.[1] and is coming into increasing use in other countries. By determining the overall rate of paralysis and the age below which most children have acquired their paralysis, general recommendations concerning the inclusion of poliomyelitis in the national programme can be formulated.

To supplement the information gained from retrospective surveys, the public health laboratory can conduct serological surveys to define more accurately the age-specific infection rates for each of the three poliovirus types and to isolate the types of virus in circulation. It is important for the public health laboratory to be involved in the control programme at an early stage and to play a role in strengthening the diagnosis and reporting of currently occurring cases, because it is the ongoing reporting system that can provide the earliest indication of the successes and failures of the programme. Specimens from clinically

---

[1] Nicholas, D. D. et al. British medical journal, 1: 1009 (1977).

diagnosed cases should be routinely checked by the laboratory to confirm the diagnosis and, where discrepancies are encountered, laboratory and clinical personnel should collaborate in improving the criteria for diagnosis and the methods of specimen collection.

1.2 Quality control of vaccines

The quality control of vaccines within the country of use is being strongly promoted in the WHO Expanded Programme on Immunization. The first stage may simply entail reading and interpreting production protocols submitted by national or foreign manufacturers. It is, however, desirable to establish national laboratories to check the potency[1] and sterility[2] of the vaccines being used. This capacity also permits the laboratory to participate in a continuing evaluation of the efficacy of the cold chain for maintaining the potency of the vaccines throughout the distribution system. Samples of vaccines can be checked whenever it is suspected that a significant breakdown in the cold chain has occurred. Checks can also be carried out as a matter of routine at various points in the distribution system.

1.3 Impact of immunization

The impact of immunization is to be judged in terms of reduction of incidence rather than in terms of seroconversion and for this reason the main emphasis should be on disease surveillance, including lameness surveys.

---

[1] Tests for potency are given in Requirements for Biological Substances No. 7, revised 1971 (WHO Technical Report Series, No. 486, 1972, p. 20). However, WHO has recently organized a collaborative study of assay methods and may be able to offer, on request, more detailed advice.

[2] Tests for sterility are given in Requirements for Biological Substances No. 6, revised 1973 (WHO Technical Report Series, No. 530, 1973, p. 40). Twenty samples from the final containers of the vaccine should be tested as described in section 5 of these Requirements.

However, studies of poliovirus antibody levels in children known or presumed to have been vaccinated can provide early warnings of an epidemic and give national authorities time to prevent it.

1.4 Investigation of individual cases and small outbreaks

As poliomyelitis immunization programmes become more effective within a given country, increasing attention should be given to individual cases and small outbreaks of the disease. This would take the form of careful epidemiological analysis, supported by serological and virus isolation studies by the laboratory, to confirm that the disease in question is indeed poliomyelitis, to identify the most probable risk factors leading to the case or the outbreak, and to recommend actions to prevent further spread.

A poliomyelitis epidemic can be extinguished by the administration of monovalent vaccine of the appropriate type, but vaccination must not be delayed for the results of laboratory investigations. If this appears likely to be the case trivalent vaccine should be administered. The speed with which a country can control poliomyelitis will depend on the managerial and technical expertise available.

1.5 Serological surveys

In making serological surveys it is important to obtain sera from all social groups and both urban and rural commmunities.

Sera from all these groups must be collected quickly to avoid any bias that might be introduced by intercurrent infections. However, laboratory studies with the sera may be spread over several weeks. Two well trained technicians could titrate 50 sera a week for neutralizing antibodies to all three poliovirus types. If it is difficult to perform assays on all three types, then it is of the greatest importance to identify the proportion of children lacking antibody to the type 1 poliovirus and then to identify the proportion of children lacking antibody to all three types.

It is recommended that serum samples should be obtained from children up to 5 years of age, divided into the following age-groups.

Age	Number of samples required
6-11 months	50
12-23 months	100
24-35 months	100
36-59 months	50

Smaller numbers of samples cannot be relied on to produce useful information.

The results of such a serological survey, supported by the data from lameness surveys, will indicate the age-groups most in need of vaccination.

Further surveys of neutralizing antibody levels carried out after vaccination programmes have begun are not profitable unless there is reason to believe that the vaccine is failing to protect. In this case, the main emphasis should be on the complete investigation of all cases of paralysis and the surrounding epidemiological circumstances in order to confirm poliovirus as the causative agent.

It may appear that some cases of poliomyelitis are associated with the administration of vaccine, and in this instance WHO may be able to advise on further investigations.

1.6 Poliovirus isolation and the determination of neutralizing antibody in serum samples

Virus isolation and poliovirus antibody surveys are important components of poliomyelitis surveillance.

Poliovirus isolation is needed for: (1) the etiological confirmation of clinically suspected poliomyelitis cases; (2) the determination of the types of poliovirus playing a role in individual cases of paralysis; (3) the detection of the poliovirus type responsible for a localized outbreak or for an epidemic; (4) the measurement of the rate of poliovirus circulation among given

sections of a population or in a circumscribed area; and (5) the possible assessment of the origin of poliovirus strains from their specific intratypic characteristics.

Routine screening of the circulation of polioviruses in the community can be effected by isolating the virus from sewage samples. This may be especially valuable if samples can be drawn from the effluent from nursery schools and crèches.

Faecal specimens from clinical cases should be collected in the early stages of disease, preferably within a week of the onset of paralysis, when there is a 75% chance of isolating the virus.

The determination of poliovirus antibodies in sera is needed for: (1) the etiological confirmation of a clinically suspected case by the demonstration, during the course of the illness, of increasing antibody titre to a given poliovirus serotype; (2) the determination of the proportion of individuals with and without poliovirus antibodies in different age-groups; and (3) the determination, in special circumstances, of the seroconversion rates resulting from the use of vaccine.

Many methods have been described for isolating and identifying polioviruses and for determining antibodies; those described in sections 2 and 3 are recommended because of their comparative simplicity.

2. POLIOVIRUS ISOLATION

2.1 Collection of specimens

Stool samples. Stool samples are the most suitable material for poliovirus isolation. Though patients with poliomyelitis usually excrete poliovirus for weeks, the best isolation results are achieved from faecal samples collected in the acute phase of the illness. Since virus shedding may be intermittent, the isolation rate can be increased by collecting two stool samples with a 24-48 hour interval.

A stool specimen of 4-8 g is desirable; this is about the size of the thumb of an adult. It should be placed in a clean leak-proof glass or plastic receptacle specially designed for stool sample collection.

If stool samples cannot be obtained by the usual way, as may be the case with out-patients or those diagnosed under field conditions, it is possible to use the anal tubing technique or rectal swabbing.

Anal tubing. Open-ended glass tubes about 5 mm in diameter and 150 mm in length can be used for this purpose. The glass edges should be rounded by heating in a flame. The tube is sterilized and one end is plugged with cotton wool. The unplugged end is lubricated with liquid paraffin and inserted gently into the rectum. With slight movements, an appropriate amount of stool is usually obtainable. The tube containing the sample should be placed in a test-tube, which is securely stoppered.

Rectal swabbing. A sterile swab moistened with virus transport medium (VTM, see Annex 1) is inserted well into the rectum and the mucosa rubbed so that faecal material adheres to the swab, which is then withdrawn and placed in a test-tube containing 1-2 ml of VTM. Owing to the much smaller quantity of sample in comparison with a faecal specimen, the chances of isolating virus from a swab may be considerably less.

Autopsy specimens. In fatal cases, specimens from the central nervous system (cervical and lumbar spinal cord, medulla, and pons) and from the descending colon are needed. The materials should be excised as soon as possible after death, using sterile instruments for each excision. The size of the CNS samples should be about 1 cm^3; from the colon a segment about 5 cm long is needed, with the faecal content retained by ligation. Each specimen should be placed in a separate screw-capped sterile jar containing enough VTM to keep the specimen wet.

Labelling. Specimens taken for virus isolation should be labelled with the name of the patient, the type of specimen, and the date of collection. The label should remain legible even if it becomes wet. In addition the following information should be submitted to the laboratory for all the samples from each patient: name, address, age, sex, poliomyelitis vaccination history (including details of vaccine and dates of doses), clinical diagnosis, date of onset of symptoms, type of specimen collected, date of collection, and name and address of clinician.

2.2 Transport and storage

After collection, the samples are kept at 4-8°C and sent to the laboratory as soon as possible in an insulated box containing frozen pads or wet ice in a closed plastic bag. For longer storage the specimens should be placed in a freezer at -30°C or below and kept frozen during transport to the laboratory. Insulated boxes containing dry-ice are suitable for this purpose.

2.3 Preparation of materials

Stool and anal tubing samples. About 1-2 g of stool and 10-20 ml of cold Hank's balanced salt solution[1] are placed in a thick-walled 50-ml centrifuge tube containing 20-30 sterile glass beads. The tube is tightly closed with a rubber stopper and shaken until all the large particles of stool are broken up. The suspension is centrifuged for 30 minutes at 3000-8000 g in a refrigerated centrifuge. The supernatant fluid is transferred into an appropriate vial and antibiotic solution is added in an amount giving the following final concentrations per millilitre: penicillin, 600 µg; streptomycin, 1000 µg; neomycin, 500 µg; nystatin, 17 µg. The material is then stored in a freezer at -30°C or below until used.

---

[1] Hanks, J. H. & Wallace, R. E. Proceedings of the Society for Experimental Biology and Medicine, 71: 196 (1949).

Rectal swabs. The swab is expressed into the VTM medium by firmly pressing it along the side of the receptacle. The swab is then discarded. The material is transferred to a centrifuge tube containing sterile glass beads, and the processing is continued as described above for the stool extracts.

Autopsy specimens. A weighed fragment of central nervous tissue (1-2 g) is placed in a sterile mortar. The tissue is cut into small pieces with sterile scissors and a small amount of sterile quartz sand with about 2 ml of cold Hanks's balanced salt solution is added. The tissue fragments are ground with a pestle until a homogeneous paste is obtained. A further volume of fluid is added until a 100 g/l suspension has been made. The suspension is transferred to a centrifuge tube, and processed as described above for stool extracts.

2.4 Virus isolation

Polioviruses grow well in primary or secondary cultures of kidney cells of several monkey species (rhesus, cynomolgus, vervet, patas, baboon). If no monkey kidney cells are available in the laboratory the Hep-2 (Cincinnati) cell line is recommended.

For virus isolation, monolayer cell cultures prepared in test-tubes are used. The day before inoculation with processed sample material, the cultures are examined microscopically to ensure their normal development and the growth medium is replaced with maintenance medium in order to remove any viral inhibitors that may be present in the bovine serum used for the initial growth. Immediately before inoculation the maintenance medium is removed and the cultures drained. Four cultures are inoculated with the same sample by adding 0.2 ml to each test-tube. The test-tubes are placed in an almost horizontal position for 30 minutes at room temperature, allowing viral adsorption to take place. Maintenance medium (0.8 ml) is then added, and the tubes are incubated at 35°C ± 0.5°C for 14 days, with a change of maintenance medium on the seventh day. Uninoculated cultures derived from the same cell suspension are also incubated to serve as controls. Melnick's medium B

(see Annex 1) or medium 199 is recommended as the maintenance medium when monkey kidney cell cultures are used, but for Hep-2 cell cultures it is better to use basal medium Eagle with fetal or agammaglobulin calf serum (see Annex 1).

The cultures are examined under the microscope at least every other day, and those showing uncharacteristic degeneration during the period of incubation are subpassaged in four cell cultures. Cultures (including the subpassages) showing a viral cytopathic effect are harvested and kept in a freezer until the identification experiments are made.

2.5 Identification of poliovirus isolates

When isolation procedures are carried out in connexion with poliomyelitis surveillance, the aim is to differentiate the poliovirus from other virus isolates and to determine the type of poliovirus isolated. Thus only poliovirus typing sera are needed for these studies. Typing sera for use in laboratory work can be obtained through WHO.[1] It is recommended that mixtures of poliovirus typing sera are used instead of individual sera in order to avoid the mistake of placing isolates containing more than one poliovirus type among the non-poliovirus isolates. The following mixtures of antisera to the poliovirus types are needed: types 1+2 (first mixture); types 1+3 (second mixture); types 2+3 (third mixture); types 1+2+3 (fourth mixture).

In the mixtures, each serum type should be present in the amount of 20 international units of neutralizing antibody in a volume equal to that of the virus suspension to be used in the neutralization test - i.e., 0.025 ml using microtechniques and 0.1 ml using macrotechniques (see below).

---

[1] From International Laboratory for Biological Standards, State Serum Institute, 80 Amager boulevard, Copenhagen, Denmark.

The identification of isolates is carried out by observing the cytopathic effect and its neutralization in the same cell system as that used for the isolation procedure. Both micro- and macrotechniques can be used, the former being especially advantageous when there are a large number of strains for identification at any one time.

Microtechnique. The following equipment is needed: (1) sterile disposable microtitre plates suitable for cell culture containing 96 flat-bottomed wells each with a capacity of 0.3-0.4 ml; (2) pressure-sensitive adhesive film for sealing the plates; (3) sterile aluminium foil sheets measuring 14 x 10 cm (unless a cover plate is provided); (4) adjustable automatic pipetting device with sterile disposable plastic tips delivering volumes of 0.025-0.1 ml.

The titre of the virus isolates is determined by diluting them in dilution fluid (see Annex 5) in tenfold steps ranging from 1 : 10 to 1 : 1 000 000. A volume of 0.025 ml of each virus dilution is added to a row of four wells containing 0.025 ml of virus dilution fluid and 0.1 ml of a suspension of Hep-2 cells. The cells are prepared at a concentration of 5000-10 000 cells per ml in virus diluting medium. The wells are overlaid with 0.075 ml of liquid paraffin, and the microtitre plates are sealed with pressure-sensitive adhesive film. The plates are incubated at 35°C for 7 days and examined on the third or fourth day and again on the seventh day with an inverted microscope. Wells with degeneration indicative of virus growth are recorded as positive, and the virus titre is determined by the method of Reed and Muench. The dilution of the isolate that contains 100 CPD_{50} per 0.025 ml is calculated. This dilution is used in the virus typing procedure that follows.

One row (12 wells) of the microtitre plate is used for the test of each isolate - i.e., row A for the first isolate, row B for the second, etc. The diluted virus isolates are dispensed into the wells by means of an automatic pipetting device, which delivers volumes of 0.025 ml. Disposable pipette tips are used for measuring out the different isolates. The laboratory number of the virus isolate is marked on the left edge of the plate in line with the appropriate rows.

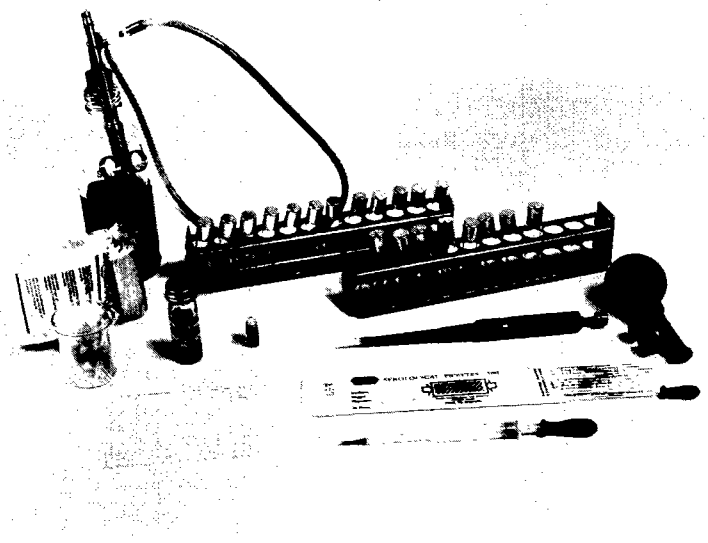

Fig. 1 The complete equipment required for serological examination by microtechniques

Fig. 2 The addition of a fixed volume of diluent to capped sterile test-tubes using an automatic syringe

Fig. 3 Making twofold dilutions of serum using an automatic pipette and sterile disposable tips

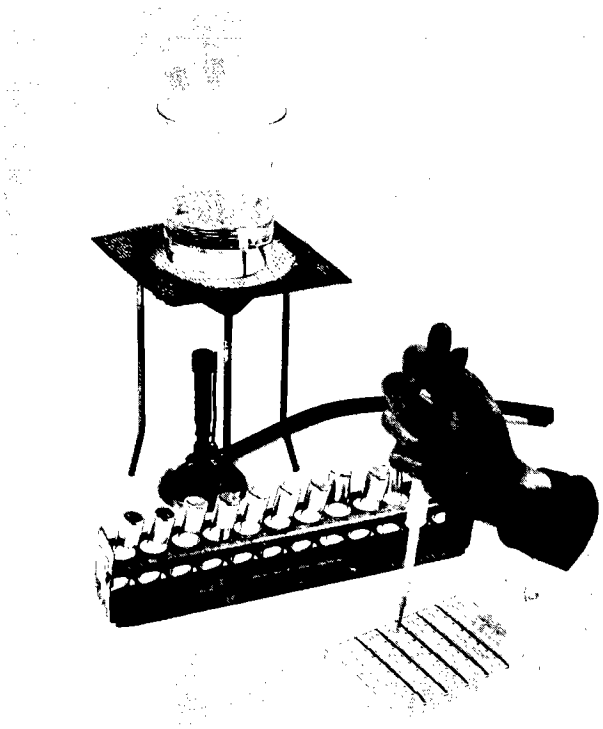

Fig. 4 The addition of serum or virus to the appropriate wells of a microtitre plate, using a pipette delivering a drop of 0.025-ml volume

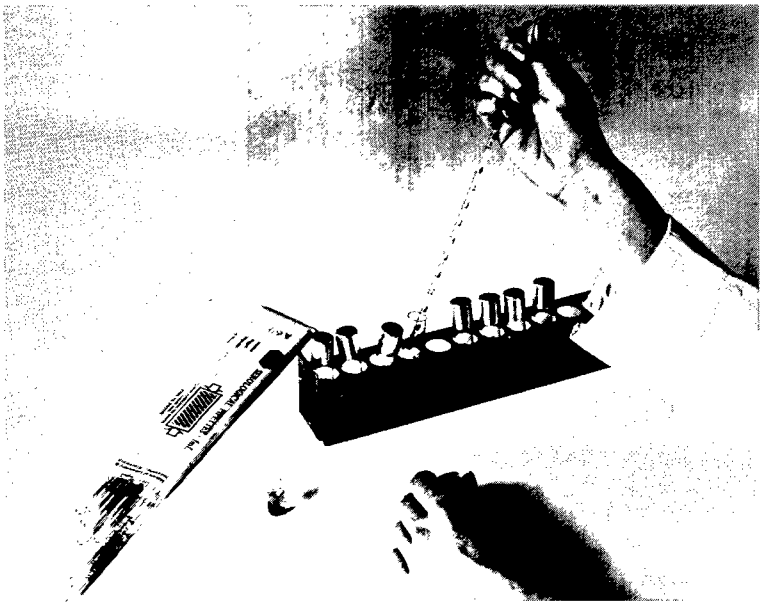

Fig. 5 Serial dilution of virus (a fresh pipette is used for each dilution)

Fig. 6 The sealing of the completed plate with adhesive film

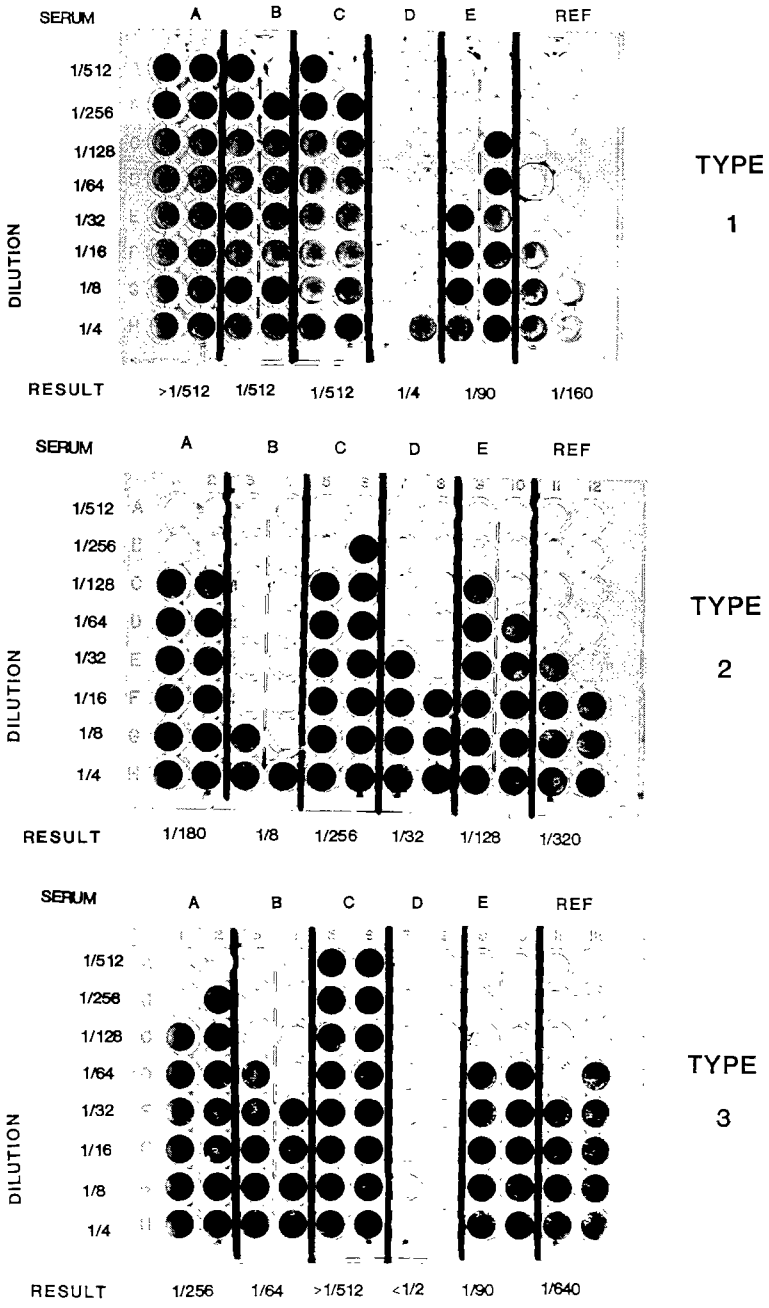

Fig. 7 The results of assays of five human sera for the presence of poliovirus neutralizing antibodies

After the isolates have been dispensed, 0.025-ml volumes of the mixtures of typing sera are added to the wells by an automatic pipetting device according to the following scheme. In each row the first mixture is added to wells 1 and 2, the second mixture to wells 3 and 4, the third mixture to wells 5 and 6, and the fourth mixture to wells 7 and 8. Wells 9-12 serve as controls and contain only 0.025 ml of maintenance medium. Separate pipette tips are used to measure each mixture and the maintenance medium. The typing serum mixtures are indicated by labelling the upper edge of the plate.

The plate is covered with sterile aluminium foil and incubated in a humidified atmosphere at 35°C ± 0.5°C for 60 minutes. After the incubation, a suspension of appropriate cells (section 2.4) containing 5-10 x 10^4 cells per ml is prepared in a medium consisting of basal medium Eagle with 1.3 g/l sodium bicarbonate and 40 g/l fetal or agamma-globulin calf serum, and 0.1 ml of this suspension is added to each well. The wells are overlaid with 0.075 ml of liquid paraffin, and the plate is sealed with pressure-sensitive adhesive film.

The plate is incubated at 35°C ± 0.5°C for 7 days and read daily using an inverted microscope. The results are evaluated as described below (section 2.6).

Macrotechnique. The neutralization tests are carried out in monolayer cultures of appropriate cells (section 2.4) prepared in test-tubes.

The titre of the virus isolate is determined as for the microtechnique, except that the virus dilutions are prepared in the appropriate maintenance medium (see section 2.4 and Annex 1), and 0.1 ml of virus dilution is added to each of four cultures containing 0.9 ml of maintenance medium. The cultures are read and the dilution of isolate that contains 100 CPD_{50} per 0.1 ml is calculated. A volume of 0.3 ml of this virus dilution is dispensed into each of five test-tubes. Identical volumes (0.3 ml) of the four poliovirus antiserum mixtures are then added to the test-tubes as follows: tube No. 1, first mixture; tube No. 2, second mixture; tube No. 3, third mixture; tube No. 4, fourth

mixture. The fifth test-tube serves as a control, and 0.3 ml of maintenance medium only is added to it. The test-tubes are stoppered and incubated at 35°C ± 0.5°C for 60 minutes, after which 0.2 ml of each virus-antiserum mixture is added to each of two cell cultures, and two further cultures are inoculated with 0.2 ml (each) of the control virus suspension. To each tube 0.8 ml of maintenance medium is added and the cultures are incubated at 35°C ± 0.5°C for 7 days. The results become apparent on the third or fourth day but the final readings are made on the seventh.

2.6 Evaluation of results

The virus control cell cultures must be positive. The evaluation of the results is then as follows.

(1) If the tubes inoculated with the first, second, and fourth serum-virus mixtures remain negative, the isolate is type 1 poliovirus.

(2) If the tubes inoculated with the first, third, and fourth serum-virus mixtures remain negative, the isolate is type 2 poliovirus.

(3) If the tubes inoculated with the second, third, and fourth serum-virus mixtures remain negative, the isolate is type 3 poliovirus.

(4) If the tubes inoculated with the first and fourth serum-virus mixtures remain negative, the isolate contains types 1 and 2 poliovirus.

(5) If the tubes inoculated with the second and fourth serum-virus mixtures remain negative, the isolate contains types 1 and 3 poliovirus.

(6) If the tubes inoculated with the third and fourth serum-virus mixtures remain negative, the isolate contains types 2 and 3 poliovirus.

(7) If the tube inoculated with the fourth serum-virus mixture remains negative, the isolate contains types 1, 2, and 3 poliovirus.

(8) If none of the tubes inoculated with serum-virus mixtures remains negative, the isolate contains either poliovirus and another enterovirus simultaneously, or a non-polio enterovirus, or a virus strain belonging to another virus group (reovirus, herpesvirus, adenovirus, etc.). In this case the isolate is recorded as unidentified.

3. DETERMINATION OF NEUTRALIZING ANTIBODY

3.1 Serum samples

The minimum amount of serum needed for an assay is 0.1 ml. A finger prick will yield 0.2-0.3 ml of blood, which may be collected in a small stoppered sterile glass or plastic tube. The blood is allowed to clot, and the serum is separated as soon as possible and transferred to plastic vials for storage at -20°C or lower. It is recommended that before storage the serum samples be inactivated at 56°C for 30 minutes, because this may reduce the problems caused by bacterial contamination of the specimens.

3.2 Reagents and cell cultures

WHO international reference antisera. These provide a continuing check on the sensitivity of the assays and permit the antibody levels to be quoted in international units. Ampoules of these preparations are available through WHO.[1]

Working reference antisera. These should be prepared in the laboratory and their potency determined in international units by assay in comparison with the international reference antisera.

Stock virus preparations of proven purity and known titre prepared in the laboratory. WHO can provide suitable samples of polioviruses of each type, from which stock viruses for use in the tests can be prepared (see Annex 2).

---

[1] From Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.

Cell cultures. Any cell cultures sufficiently sensitive to poliovirus may be used (see section 2.4). If the Hep-2 (Cincinatti) cell line is being used, a cell bank must be established as a continually available source in order to avoid the use of cells at high passage levels, which may result in a loss of viral sensitivity. Annex 3 describes the procedures for preparing cells for serum assays and Annex 4 gives a method of preparing a cell bank for storage in liquid nitrogen.

Cell culture media. The cell culture media may affect the sensitivity of cell cultures to poliovirus but usually not critically. Basal medium Eagle is recommended and may be obtained in powder form from a number of commercial sources. It should be prepared according to the manufacturer's instructions.

3.3 Special equipment

Glass or plastic bottles that provide a flat surface for cell growth of 25-35 cm^2 (or of up to 250 cm^2) are useful as culture vessels and for the preparation of virus pools. It is assumed that the antibody assays will be performed in disposable plastic plates containing small flat-bottomed wells. The microtitre plate with 96 wells, in 12 rows of eight, each of approximately 0.3 ml volume, is ideal. The plates are sealed with pressure-sensitive adhesive film.

Rapid and accurate work with a microtitre plate requires pipettes or droppers that deliver volumes of 0.025 ml. If large serological surveys are undertaken, automatic pipetting devices designed to speed the use of microtitre plates are an advantage.

3.4 Procedure

For each serum to be tested prepare a series of eight test-tubes of which the first contains 0.3 ml of diluent (see Annex 5) and the rest 0.2 ml of diluent (Figure 2). With the aid of an automatic pipetting device, add 0.1 ml of neat serum to the first tube and mix well by stirring. With the aid of another pipette transfer 0.2 ml of the

diluted serum to the second tube and mix well. Transfer 0.2 ml from the second tube to the third (Figure 3). Repeat the operation successively until all eight tubes have been used. This provides serum dilutions from 1:4 to 1:512 in twofold steps. Take three microtitre plates and label them type 1, type 2, and type 3. Using a 0.025-ml dropper pipette (Figure 4), add one drop of the most dilute serum (1:512) to wells 1 and 2 in row A of each of the three microtitre plates, expel the remaining fluid from the pipette and refill with the next dilution (1:256). Add one drop each to wells 1 and 2 in row B of each of the three plates. Continue until the 1:4 dilution is placed similarly in row H. The operation may be repeated until the three plates contain the dilutions from six sera. This may be done for as many groups of three plates as can be conveniently handled, but in one group of three plates leave at least one section of two columns of eight wells for the reference sera. Every test must include assays of reference sera types 1, 2 and 3, which should be diluted appropriately and each added to a plate labelled with its own type number.

Prepare suspensions of challenge virus types 1, 2, and 3 such that 0.025 ml contains an estimated 100 CPD_{50}. The dilutions are prepared in a manner similar to that used for diluting the serum, but using tenfold steps (Figure 5). Add 0.025-ml volumes of the type 1 challenge virus to all plates labelled type 1 and carry out a similar operation for types 2 and 3. Cover the plates and incubate at 35°C ± 0.5°C for 3 hours.

To determine the titre of the challenge virus, prepare from it a series of three tenfold dilutions (0.5 ml of virus plus 4.5 ml of diluent) and transfer 0.025-ml volumes of these dilutions, together with 0.025-ml volumes of the stock challenge virus suspension, to each of four wells containing 0.025 ml of diluent instead of serum. The plate is incubated together with the plates containing the serum virus mixtures.

On completion of the incubation period, add to each well in all the plates 0.1 ml of cell suspension prepared as described in Annex 3, sections 2-4, but suspended in diluent (Annex 5) instead of growth medium. The cells may

be added by any method that delivers a volume of 0.1 ml consistently. There are then about 5×10^3 cells per well. Seal the plates with pressure-sensitive adhesive film (Figure 6), and incubate at $35^{\circ}C \pm 0.5^{\circ}C$ for 7 days.

3.5 Evaluation of results

Observe the cytopathic effect on the third or fourth day and again on the seventh day. Record as positive all wells with a complete or almost complete destruction of the cell sheet. When examining the cell monolayers in plates during the incubation period it is preferable to use an inverted microscope. The test is valid if the estimate of the titre of the challenge virus is between 30 and 200 TCD_{50} per well. The serum titre is expressed as the highest dilution at which at least one well shows no viral cytopathic effect and is calculated in international units, i.e., as a ratio of the potencies of the international reference sera.

The results obtained in the assay of five human sera and the reference sera are given in Figure 7. To demonstrate more clearly the wells with and without a cytopathic effect, the surviving cells have been stained.

Annex 1

COMPOSITION OF MEDIA

Virus transport medium

Hanks's balanced salt solution (sterile)	86.0 ml
bovine albumin solution, 100 g/l	10.0 ml
$NaHCO_3$, 56 g/l	1.5-2.0 ml*
penicillin (6 mg/ml) streptomycin (10 mg/ml) solution in phosphate-buffered saline solution A	1.0 ml
nystatin (0.83 mg/ml in phosphate-buffered saline solution A)	1.0 ml
phenol red, 4 g/l, in distilled water	0.5 ml

* as necessary to adjust pH to neutrality.

Melnick's medium B

Earle's balanced salt solution containing 0.02 g/l phenol red	86.0 ml
lactalbumin hydrolysate 50 g/l in Hanks's balanced salt solution without bicarbonate or phenol red	10.0 ml
$NaHCO_3$, 75 g/l	3.0 ml
antibiotic solution (penicillin 6 mg/ml, streptomycin 10 mg/ml, nystatin 0.83 mg/ml)	1.0 ml

Maintenance medium for Hep-2 (Cincinnati) cell line

basal medium Eagle in Earle's balanced salt solution	95.5 ml
$NaHCO_3$, 75 g/l	1.5 ml
fetal calf serum or agammaglobulin calf serum	2.0 ml
antibiotic solution (penicillin 6 mg/ml, streptomycin 10 mg/ml, neomycin 3.2 mg/ml)	1.0 ml

* * *

Annex 2

PREPARATION OF VIRUS STOCKS

The seed virus strains received from WHO should be stored at -30°C or below. When required for use, dilute them 1:10 in virus diluent.

Select some Hep-2 cell cultures that have confluent cell sheets and wash them free of growth medium by rinsing them with two changes of medium (virus dilution fluid or Hanks's balanced salt solution), using a volume equivalent to about 1 ml per 5 cm^2 of cell sheet. Drain the cultures and add a similar volume of virus dilution fluid but adjusted to ensure that when the virus seed is added it will be diluted a further hundredfold in the final volume (i.e. the original seed will then have been diluted a thousandfold).

* * *

Annex 3

CULTIVATION OF Hep-2 (CINCINNATI) CELLS IN BOTTLES

Reagents

Trypsin

final concentration 2.5 g/l in balanced salt solution (Hanks's, Earle's, or phosphate-buffered saline, pH about 7.8)

Medium

basal medium Eagle in Earle's balanced salt solution	95 ml
fetal calf serum	5 ml
penicillin	6 mg
streptomycin	10 mg

To buffer, add sodium bicarbonate to a final concentration of 0.88 g/l. Warm the medium and the trypsin solution to 37°C before use.

Subculturing of cells

(1) Select a culture with a confluent cell sheet, pour off the medium, and rinse twice with 5 ml of trypsin solution. Pour off the trypsin and allow the bottle to remain at room temperature (or incubate at 37°C) until the cells begin to detach from the glass.

(2) Add about 5 ml of warm medium (37°C). Agitate the bottle so that the medium washes back and forth and releases the cells completely from the bottle wall. Alternatively aspirate and expel with a 5-ml pipette and bulb, in order to effect maximum disaggregation of cells.

(3) Add a further 55 ml of medium (making a total of 60 ml) and distribute a quarter of the total volume of cell suspension (i.e., 15 ml) to each of three additional bottles having the same surface area as the first. This is a 1:4 "split".

(4) Incubate the cultures at 37°C.

(5) Repeat the 1:4 "split" at intervals of 3-4 days, when the cells will have formed confluent sheets.

(6) Keep a careful record of the number of passages through which the cells have been propagated.

* * *

Annex 4

PRESERVATION OF A Hep-2 CELL BANK IN LIQUID NITROGEN

Reagents

Trypsin

Trypsin in Hanks's balanced salt solution, 2.5 g/l

Medium 1

basal medium Eagle in Earle's balanced salt solution (sodium bicarbonate 0.55 g/l)	85 ml
fetal calf serum	15 ml

Medium 2

basal medium Eagle in Earle's balanced salt solution (sodium bicarbonate 0.55 g/l)	65 ml
fetal calf serum	15 ml
dimethyl sulfoxide or glycerol	20 ml

Freezing of cells

(1) Use bottles containing confluent sheets of cells.

(2) Remove the cell sheets by trypsinizing in the usual manner (see Annex 3).

(3) Effect disaggregation of cells in 0.5 ml of medium 1 by alternately aspirating and expelling through a pipette to give a concentration of about 6×10^6 cells per ml.

(4) Add 0.5 ml of medium 2 to the cell suspension about 30 minutes before sealing the cells in ampoules. Thus the final concentration of dimethyl sulfoxide or glycerol is 100 µl/ml and that of the cells about 3×10^6 per ml. This is roughly the yield obtained from a confluent monolayer of cells in a 150-ml bow bottle with an area of 50 cm^2.

(5) To an ampoule of about 1.5 ml capacity add about 1 ml of cell suspension and seal.

(6) Freeze cells by reducing the temperature at a rate of less than 5°C per minute until a temperature of about -50°C is reached. The rate of cooling may be controlled by placing the ampoules in a container that is gradually lowered into the vapour phase above liquid nitrogen. The temperature of the ampoules is observed by means of an alcohol thermometer and the rate of lowering adjusted to give the desired rate of cooling.

(7) Immerse and store ampoules in liquid nitrogen.

Reconstitution of cells

(1) Remove the ampoules to be thawed, and thaw the cells rapidly by immersing in water at about 40°C.

(2) Wipe the outside of the ampoule with 700 ml/l alcohol.

(3) Using a pipette with a fine capillary tip, withdraw the contents of the ampoule and seed each 1-ml suspension of cells into a prewarmed 150-ml bow bottle.

(4) Aspirate and expel cells alternately, using a pipette with a fine capillary tip.

(5) Add, slowly, about 15 ml of prewarmed antibiotic medium (composed as in Annex 3 but with 10 ml of serum).

(6) Incubate for 2-4 hours at 37°C, then remove the medium and replace it with 15 ml of fresh medium.

(7) Cells should reach confluency in 4-7 days.

* * *

Annex 5

DILUTION FLUID FOR SERUM AND VIRUS

Dilution fluid for serum and virus consists of basal medium Eagle containing 1.3 g/l of sodium bicarbonate and 40 ml/l of fetal calf serum plus penicillin and streptomycin at 120 µg and 100 µg respectively.

WHO publications may be obtained, direct or through booksellers, from:

ALGERIA	Société Nationale d'Edition et de Diffusion, 3 bd Zirout Youcef, ALGIERS
ARGENTINA	Carlos Hirsch SRL, Florida 165, Galerías Güemes, Escritorio 453/465, BUENOS AIRES
AUSTRALIA	*Mail Order Sales :* Australian Government Publishing Service Bookshops, P.O. Box 84, CANBERRA A.C.T. 2600 ; *or over the counter from :* Australian Government Publications and Inquiry Centres at : 113–115 London Circuit, CANBERRA CITY A.C.T. 2600 ; Shop 42, The Valley Centre, BRISBANE, Queensland 4000 ; 347 Swanston Street, MELBOURNE, VIC 3000 ; 309 Pitt Street, SYDNEY, N.S.W. 2000 ; Mt Newman House, 200 St. George's Terrace, PERTH, WA 6000 ; Industry House, 12 Pirie Street, ADELAIDE, SA 5000 ; 156-162 Macquarie Street, HOBART, TAS 7000 — Hunter Publications, 58A Gipps Street, COLLINGWOOD, VIC 3066
AUSTRIA	Gerold & Co., Graben 31, 1011 VIENNA I
BANGLADESH	The WHO Programme Coordinator, G.P.O. Box 250, DACCA 5 — The Association of Voluntary Agencies, P.O. Box 5045, DACCA 5
BELGIUM	Office international de Librairie, 30 avenue Marnix, 1050 BRUSSELS — *Subscriptions to World Health only :* Jean de Lannoy, 202 avenue du Roi, 1060 BRUSSELS
BRAZIL	Biblioteca Regional de Medicina OMS/OPS, Unidade de Venda de Publicações, Caixa Postal 20.381, Vila Clementino, 04023 SÃO PAULO, S.P.
BURMA	*see* India, WHO Regional Office
CANADA	*Single and bulk copies of individual publications (not subscriptions) :* Canadian Public Health Association, 1335 Carling Avenue, Suite 210, OTTAWA, Ont. K1Z 8N8. *Subscriptions : Subscription orders, accompanied by cheque made out to the* Royal Bank of Canada, Ottawa, Account World Health Organization, *should be sent to the* World Health Organization, P.O. Box 1800, Postal Station B, OTTAWA, Ont. K1P 5R5. *Correspondence concerning subscriptions should be addressed to the* World Health Organization, Distribution and Sales, 1211 GENEVA 27, Switzerland
CHINA	China National Publications Import Corporation, P.O. Box 88, PEKING
COLOMBIA	Distrilibros Ltd., Pío Alfonso García, Carrera 4a, Nos 36–119, CARTAGENA
CZECHOSLOVAKIA	Artia, Ve Smeckach 30, 111 27 PRAGUE 1
DENMARK	Ejnar Munksgaard Ltd., Nørregade 6, 1164 COPENHAGEN K
ECUADOR	Librería Científica S.A., P.O. Box 362, Luque 223, GUAYAQUIL
EGYPT	Nabaa El Fikr Bookshop, 55 Saad Zaghloul Street, ALEXANDRIA
EL SALVADOR	Librería Estudiantil, Edificio Comercial B No 3, Avenida Libertad, SAN SALVADOR
FIJI	The WHO Programme Coordinator, P.O. Box 113, SUVA
FINLAND	Akateeminen Kirjakauppa, Keskuskatu 2, 00101 HELSINKI 10
FRANCE	Librairie Arnette, 2 rue Casimir-Delavigne, 75006 PARIS
GERMAN DEMOCRATIC REPUBLIC	Buchhaus Leipzig, Postfach 140, 701 LEIPZIG
GERMANY, FEDERAL REPUBLIC OF	Govi-Verlag GmbH, Ginnheimerstrasse 20, Postfach 5360, 6236 ESCHBORN — W. E. Saarbach, Postfach 101 610, Follerstrasse 2, 5 COLOGNE 1 — Alex. Horn, Spiegelgasse 9, Postfach 3340, 6200 WIESBADEN
GREECE	G. C. Eleftheroudakis S.A., Librairie internationale, rue Nikis 4, ATHENS (T. 126)
HAITI	Max Bouchereau, Librairie " A la Caravelle ", Boîte postale 111-B, PORT-AU-PRINCE
HONG KONG	Hong Kong Government Information Services, Beaconsfield House, 6th Floor, Queen's Road, Central, VICTORIA
HUNGARY	Kultura, P.O.B. 149, BUDAPEST 62 — Akadémiai Könyvesbolt, Váci utca 22, BUDAPEST V
ICELAND	Snaebjørn Jonsson & Co., P.O. Box 1131, Hafnarstraeti 9, REYKJAVIK
INDIA	WHO Regional Office for South-East Asia, World Health House, Indraprastha Estate, Ring Road, NEW DELHI 110002 — Oxford Book & Stationery Co., Scindia House, NEW DELHI 110000 ; 17 Park Street, CALCUTTA 700016 (*Sub-agent*)
INDONESIA	M/s Kalman Book Service Ltd., Jln. Cikini Raya No. 63, P.O. Box 3105/Jkt, JAKARTA
IRAN	Iranian Amalgamated Distribution Agency, 151 Khiaban Soraya, TEHERAN
IRAQ	Ministry of Information, National House for Publishing, Distributing and Advertising, BAGHDAD
IRELAND	The Stationery Office, DUBLIN 4
ISRAEL	Heiliger & Co., 3 Nathan Strauss Street, JERUSALEM
ITALY	Edizioni Minerva Medica, Corso Bramante 83–85, 10126 TURIN ; Via Lamarmora 3, 20100 MILAN
JAPAN	Maruzen Co. Ltd., P.O. Box 5050, TOKYO International, 100–31
KOREA, REPUBLIC OF	The WHO Programme Coordinator, Central P.O. Box 540, SEOUL
KUWAIT	The Kuwait Bookshops Co. Ltd., Thunayan Al-Ghanem Bldg, P.O. Box 2942, KUWAIT
LAO PEOPLE'S DEMOCRATIC REPUBLIC	The WHO Programme Coordinator, P.O. Box 343, VIENTIANE

A/2/78

WHO publications may be obtained, direct or through booksellers, from:

LEBANON	The Levant Distributors Co. S.A.R.L., Box 1181, Makdessi Street, Hanna Bldg, BEIRUT
LUXEMBOURG	Librairie du Centre, 49 bd Royal, LUXEMBOURG
MALAYSIA	The WHO Programme Coordinator, Room 1004, Fitzpatrick Building, Jalan Raja Chulan, KUALA LUMPUR 05–02 — Jubilee (Book) Store Ltd, 97 Jalan Tuanku Abdul Rahman, P.O. Box 629, KUALA LUMPUR 01–08 — Parry's Book Center, K. L. Hilton Hotel, Jln. Treacher, P.O. Box 960, KUALA LUMPUR
MEXICO	La Prensa Médica Mexicana, Ediciones Científicas, Paseo de las Facultades 26, Apt. Postal 20–413, MEXICO CITY 20, D.F.
MONGOLIA	*see* India, WHO Regional Office
MOROCCO	Editions La Porte, 281 avenue Mohammed V, RABAT
MOZAMBIQUE	INLD, Caixa Postal 4030, MAPUTO
NEPAL	*see* India, WHO Regional Office
NETHERLANDS	N.V. Martinus Nijhoff's Boekhandel en Uitgevers Maatschappij, Lange Voorhout 9, THE HAGUE 2000
NEW ZEALAND	Government Printing Office, Mulgrave Street, Private Bag, WELLINGTON 1. *Government Bookshops at :* Rutland Street, P.O. 5344, AUCKLAND ; 130 Oxford Terrace, P.O. Box 1721, CHRISTCHURCH ; Alma Street, P.O. Box 857, HAMILTON ; Princes Street, P.O. Box 1104, DUNEDIN — R. Hill & Son Ltd, Ideal House, Cnr Gillies Avenue & Eden Street, Newmarket, AUCKLAND 1
NIGERIA	University Bookshop Nigeria Ltd, University of Ibadan, IBADAN — G. O. Odatuwa Publishers & Booksellers Co., 9 Hausa Road, SAPELE, BENDEL STATE
NORWAY	Johan Grundt Tanum Bokhandel, Karl Johansgt. 43, 1010 OSLO 1
PAKISTAN	Mirza Book Agency, 65 Shahrah-E-Quaid-E-Azam, P.O. Box 729, LAHORE 3
PAPUA NEW GUINEA	The WHO Programme Coordinator, P.O. Box 5896, BOROKO
PHILIPPINES	World Health Organization, Regional Office for the Western Pacific, P.O. Box 2932, MANILA — The Modern Book Company Inc., P.O. Box 632, 926 Rizal Avenue, MANILA
POLAND	Składnica Księgarska, ul Mazowiecka 9, 00052 WARSAW *(except periodicals)* — BKWZ Ruch, ul Wronia 23, 00840 WARSAW *(periodicals only)*
PORTUGAL	Livraria Rodrigues, 186 Rua do Ouro, LISBON 2
SIERRA LEONE	Njala University College Bookshop (University of Sierra Leone), Private Mail Bag, FREETOWN
SINGAPORE, REPUBLIC OF	The WHO Programme Coordinator, 144 Moulmein Road, G.P.O. Box 3457, SINGAPORE 1 — Select Books (Pte) Ltd, 215 Tanglin Shopping Centre, 2/F, 19 Tanglin Road, SINGAPORE 10
SOUTH AFRICA	Van Schaik's Bookstore (Pty) Ltd, P.O. Box 724, 268 Church Street, PRETORIA 0001
SPAIN	Comercial Atheneum S.A., Consejo de Ciento 130–136, BARCELONA 15 ; General Moscardó 29, MADRID 20 — Librería Díaz de Santos, Lagasca 95, MADRID 6 ; Balmes 417 y 419, BARCELONA 6
SRI LANKA	*see* India, WHO Regional Office
SWEDEN	Aktiebolaget C.E. Fritzes Kungl. Hovbokhandel, Regeringsgatan 12, 10327 STOCKHOLM
SWITZERLAND	Medizinischer Verlag Hans Huber, Länggass Strasse 76, 3012 BERNE 9
SYRIAN ARAB REPUBLIC	M. Farras Kekhia, P.O. Box No. 5221, ALEPPO
THAILAND	*see* India, WHO Regional Office
TUNISIA	Société Tunisienne de Diffusion, 5 avenue de Carthage, TUNIS
TURKEY	Haset Kitapevi, 469 Istiklal Caddesi, Beyoglu, ISTANBUL
UNITED KINGDOM	H.M. Stationery Office : 49 High Holborn, LONDON WC1V 6HB ; 13a Castle Street, EDINBURGH EH2 3AR ; 41 The Hayes, CARDIFF CF1 1JW ; 80 Chichester Street, BELFAST BT1 4JY ; Brazennose Street, MANCHESTER M60 8AS ; 258 Broad Street, BIRMINGHAM B1 2HE ; Southey House, Wine Street, BRISTOL BS1 2BQ. *All mail orders should be sent to* P.O. Box 569, LONDON SE1 9NH
UNITED STATES OF AMERICA	*Single and bulk copies of individual publications (not subscriptions) :* WHO Publications Centre USA, 49 Sheridan Avenue, ALBANY, N.Y. 12210. *Subscriptions : Subscription orders, accompanied by check made out to the* Chemical Bank, New York, Account World Health Organization, *should be sent to the* World Health Organization, P.O. Box 5284, Church Street Station, NEW YORK, N.Y. 10249; *Correspondence concerning subscriptions should be addressed to the* World Health Organization, Distribution and Sales, 1211 GENEVA 27, Switzerland. *Publications are also available from the* United Nations Bookshop, NEW YORK, N.Y. 10017 *(retail only)*
USSR	*For readers in the USSR requiring Russian editions :* Komsomolskij prospekt 18, Medicinskaja Kniga, MOSCOW — *For readers outside the USSR requiring Russian editions :* Kuzneckij most 18, Meždunarodnaja Kniga, MOSCOW G-200
VENEZUELA	Editorial Interamericana de Venezuela C.A., Apartado 50785, CARACAS 105 — Librería del Este, Apartado 60337, CARACAS 106
YUGOSLAVIA	Jugoslovenska Knjiga, Terazije 27/II, 11000 BELGRADE
ZAIRE	Librairie universitaire, avenue de la Paix N° 167, B.P. 1682, KINSHASA I

Special terms for developing countries are obtainable on application to the WHO Programme Coordinators or WHO Regional Offices listed above or to the World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland. Orders from countries where sales agents have not yet been appointed may also be sent to the Geneva address, but must be paid for in pounds sterling, US dollars, or Swiss francs.

Price : Sw. fr. 4.—

Prices are subject to change without notice.