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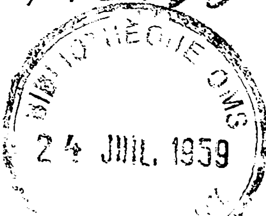

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TECHNIQUE OF COLLECTION AND EXAMINATION OF THICK BLOOD FILMS FOR MALARIA PARASITES
(With a modification of the J.S.B. Staining Method)

by

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The present paper incorporates the results of a few years' experience in carrying out advisory malaria surveys in the field and might be of use to those who find themselves for the first time entirely on their own in out-of-the-way places. There is a great difference between the special training that can be given in a well-equipped laboratory and practice in the field in an under-developed country with frequent shortages of supplies and equipment.

Techniques of Blood Collections in the Field

Thick and thin blood films on the same slide are normally taken from each person examined. If time and trouble are not to be wasted it is most important that these films should be free from artefacts and the thick film should be properly taken.

Freedom from artefacts can only be ensured if blood is drawn from a clean dry skin on to a clean slide, allowed to dry free from dust and flies, well wrapped up and stained.

These are conditions that are easily achieved in a laboratory but less easily in a dusty village with swarming flies and with the operator perhaps taking his blood films while seated on a mat on the ground.

The most common causes of a poor film in our experience are the use of dirty slides, the presence of alcohol on the skin and the touching of the sweaty skin with the slide.

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Slides should always be cleaned at a central laboratory before issue for field work. Ordinary household detergents (not soaps) give excellent results. A strong solution of detergent will remove immersion oil from slides that do not have to be kept. We use a good-sized basin, half full of water containing a tablespoon of detergent. After examination all slides not required for demonstration or checking are put in this basin. Next day the slides are transferred one by one to a second basin also containing detergent where they remain for a day. They are then rubbed lightly and rinsed in clean water. They are dried with a clean soft hand towel, wrapped in thin paper in batches of ten or twenty and returned to their boxes. We have found non-absorbent toilet paper very suitable for wrapping slides. All new slides should be washed in detergent before use, even the slides said to be "pre-cleaned".

If it is necessary to keep slides for demonstration purposes the oil can be removed with xylene. However, we have several times been supplied with stocks of xylene which removed stain from blood smears. In one place we found filtered petrol to be an excellent substitute as it did not remove the stain.

When taking blood films it is very important that frightened children should be properly handled. Older children should be dealt with first in the presence of the younger ones. Any child up to the age of six years is liable to be frightened by blood taking and should be placed in the arms of an adult, preferably the mother, with its back to the operator and its arms round the neck of the adult. The left arm is then drawn down behind the back and twisted so that the thumb is uppermost. This causes no discomfort to the child and if the arm is held straight it cannot easily be moved if the child struggles.

To clean the skin we use two cotton-wool swabs damped with 75% spirit. One swab is used first to get the majority of the dirt off and the second completes the cleaning process. Two dry cotton-wool swabs are then used to dry the skin, and these also are used in the same order each time, because the first soon gets damp with spirit from the skin. Dirt which is ingrained in the skin is not all removed but this seems to give no trouble in practice.

Sometimes it is convenient to have an assistant to clean the skin of a number of children awaiting their turn. In this case keep a swab of dry cotton-wool to wipe off sweat which soon appears on the skin in hot weather. It is essential that the sweaty skin should not be touched by the slide when the blood film is being taken.

It is most important that in pricking the skin needless pain and fright should be avoided, otherwise the children will hide or run away.

We have often found field staff using ordinary sewing needles or pins or old hypodermic needles with bent tips. Every effort should be made to have these replaced by proper surgical needles such as the 2-inch straight Hagedorn with cutting edge or the straight 2-inch surgical needle with a triangular point. With these a shallow prick can produce a good drop of blood with little pain and it is possible to take blood from a sleeping infant under the age of nine months without awakening it. Round pointed needles have to be plunged deeply and it is often difficult to obtain an adequate amount of blood when they are used.

The taking of thick blood films is presumably familiar to anyone to whom this article is of interest. Our main concern is that these should be of adequate thickness and this can only be achieved with experience so that the majority of fields contain at least 20 leucocytes. We take three to four small drops of blood and spread them with the corner of the next clean slide. A thick blood film should be about 1 cm diameter. Too small a film, if thick, will not haemolyse well and is readily detached from the slide during staining.

It is very important that the laboratory register should show from whom any batch of slides has been received and should have a column to show any films in which artefacts made examination difficult or impossible. If this is not done much time will be wasted both in taking blood films in the field and also in the laboratory. An inspector who persistently sends in many blood films full of artefacts should be re-trained in the technique of blood taking and his kit should be examined to ensure that he has the necessary equipment for taking good blood smears. He should be shown the difference between a dirty and a clean film under the microscope.

When the blood film has been taken it must be allowed to dry free from dust and from the attentions of flies, while lying flat so that the blood of the thick film does not run to one side. For this it is necessary to have a tray on which the slides can be set face downwards while the blood is drying. A suitable tray that can be made by any carpenter is shown in Figure 1. Always leave a gap between adjacent slides so that the air beneath them does not become saturated with moisture, which spoils the thin films. A number of trays are carried. If it is necessary to move on while some blood films are still wet, stack the trays with an empty tray on top and bind them with a rubber band. The strips on the underside of each tray will press on the slides of the tray underneath and prevent them moving over each other. The trays should not be painted or polished. The advantage of such trays over slotted types of slide boxes is that they are easier to make, to handle, to place flat, and cannot be knocked over.

Thick films should not be left out in the sun, exposed to high temperatures or stored for a long time otherwise there may be difficulty in removing haemoglobin. If this trouble occurs try soaking in 0.5% solution of acetic acid for a few minutes. Rinse well before staining.

In hot, damp climates moulds grow readily in thick films. This can be prevented by dipping them for a few minutes in filtered water in order to remove the haemoglobin a day or two after taking. The thin film must not get wet and it is best to fix it first with methyl alcohol. The de-haemoglobinized thick film can then be fixed with methyl alcohol when dry, though this is not essential.

When the blood films are dry, it is important to wipe the backs of the slides with a clean cloth to free them from dust before stacking them in order of their number, and wrapping in thin paper on which should be written the date and name of the place where the blood films were taken.

For fixing thin films use a small jar nearly full of absolute methyl alcohol (Methanol), (specify as "acetone free" when ordering). If a deep jar is used, the concentrated fumes of alcohol in the upper part may tend to fix the thick film. Dip quickly and set in the slide rack to dry.

Keep a working bottle into which the methyl alcohol is poured after use. Never pour it back into the stock bottle. The methyl alcohol takes up moisture from the air and so should not be left exposed too long.

The labelling of blood films can be done best with an ordinary lead pencil on the thin film if both a thin and a thick film are taken from each subject on the same slide. When only thick films are taken, labelling cannot be done on the film in the same way; in this case a wax-pencil should be used for marking on the glass slide, although it must be remembered that wax-pencil markings often peel off when the slide is being stained. A diamond pencil marks the slide securely but interferes with its future re-use. The best method is undoubtedly that of using slides with a frosted edge, available from some makers, but more expensive than ordinary glass slides. It is easy to write in pencil on the frosted edge and to erase the marking when necessary. Indian ink can be used for marking glass slides; it does not peel off when the slide is stained but dries too quickly on the pen. Among common errors in labelling glass slides the following should be noted: writing in ordinary washable ink, sticking paper labels, using bits of sticking plaster and (last but not least!) not labelling the slide at all.

Simple field apparatus. Our own blood taking kit is designed to be as simple as possible and comprises the following:

A light, easily portable bag to hold the equipment. We have found the overnight bags issued by air lines quite suitable but these have the disadvantage of not standing up to rough field conditions for more than a few months. We have therefore designed a special bag made of leather which is shown in Figure 2. It was designed to the following specifications: (a) to be light but strong and weather proof; (b) to be easy to carry by hand or strapped to the carrier or handlebar of a bicycle; (c) to have a special compartment to carry the slide trays and some drugs; (d) to be equally suitable for use by a surveillance inspector or by a doctor making a survey.

If the bag is likely to be carried long in heavy rain, the leather should be well treated with a silicone polish.

The contents of the bag are as follows:

1. Two containers for cotton wool. One of these is filled with dry cotton wool torn into suitable sized pieces. The other is used for the disposal of dirty swabs. We have found cigarette tins (50 size) suitable for this purpose.

2. Two wide mouth polythene specimen bottles about 50 cc size. These are filled with swabs of dry cotton wool and then 70% spirit is poured into them to soak the swabs. In the field the swabs used to clean the skin are taken from one of these and the other is used for cleaning the pricking needle. This is done by jabbing the needle once or twice into the spirit-soaked swabs. Glass jars of the hair cream type could be used, but we prefer polythene jars as they are light and unbreakable, tight, and in particular do not turn the point of a needle if it is accidently struck against the side of the jar.

3. Pricking needles. We prefer the Hagedorn straight with cutting edge but triangular pointed surgical needles are also very suitable. For ease of handling the needle can be passed through a cork or mounted in a pencil of wood. In this case a small piece of wire should be passed through the eye of the needle to prevent it turning inside the hollow pencil.

4. Slide trays as shown in Figure 1. The number depends on the amount of work to be done. A surveillance inspector taking only occasional blood smears will need two or three trays. A doctor making surveys will need about five trays.

5. Microscope slides, well wrapped in paper to protect them from dust.

6. A clean cloth for wiping dust off slides. This is very important as while the blood films are drying face downwards a great deal of dust may be deposited on the backs of the slides. Before the dry slides are stacked and wrapped in paper this dust should be wiped off.

7. A soft lead pencil. We use this to number and date our slides by writing directly on the thin blood smear or alternatively a glass-writing (wax) pencil may be used.

8. Record sheets or a record book.

9. Appropriate drugs which at present usually include chloroquine and pyrimethamine.

10. A small (50-100 cc) polythene bottle and a dessert spoon. This gives a simple way of treating small children and any others that have difficulty in swallowing tablets. A number of spoonfuls of water are put into the bottle and the same number of tablets added. When the tablets have disintegrated, one spoonful can be regarded as containing one tablet. Of course, the same spoon must be used. The bottle should be shaken before the dose is poured out. Standard size measures are difficult to find in under-developed countries and this method has the advantage that none is required except the continued use of the same spoon.

Technique of Blood Staining

As the thick films used by us are too thick to stain satisfactorily using the standard rapid J.S.B.[1] technique, one of us (L.L.) introduced the following modification which involves staining the blood film first in a very dilute solution of eosin (1 in 7500) for 5 to 8 minutes and then staining in the routine manner with J.S.B.I (polychrome methylene blue). This has the advantage that most of the haemoglobin and blood proteins come out into the eosin which is discarded after staining about 50 slides. This greatly prolongs the life of the J.S.B.I stain which is more expensive and takes longer to prepare.

Two solutions are used: eosin solution and J.S.B. stain.

1. Eosin, a stock solution of 1 g of eosin Y in 100 cc water is made up and two drops (not more) of 40% formaldehyde added to prevent moulds growing.

On the day of use, dilute 1 cc of this stock solution with 74 cc of clean water (= 1 : 7500) and filter before using.

This dilute solution can be used to stain and simultaneously dehaemoglobinize 50 thick blood films, after which it is thrown away. The used solution is in any case not kept overnight.

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[1] For full description of the original technique see: Jaswant Singh & Misra (1956) Indian J. malar. 10, 115

If the laboratory is issued with 0.2% eosin (J.S.B.II for the original rapid method), 5 cc of this can be diluted with 70 cc water to make 1/7500 solution.

2. The preparation of J.S.B.I stain is as follows:

Medicinal methylene blue	0.5 grams
Potassium dichromate	0.5 grams
Disodium hydrogen phosphate, dihydrate	3.5 grams

Add the methylene blue to 500 cc water in a flask.
Add 3.0 cc 1% sulfuric acid slowly, while stirring to mix.
Add the potassium dichromate. Mix.
Boil for one hour or longer until the solution is blue and remains blue on cooling.
When cool make up volume to 500 cc with more water.

This stain can be used immediately and keeps well. It can be used repeatedly but must be filtered before using each day.

The water used for rinsing and for diluting the eosin stock solution does not have to be distilled, but must be clean, without any sediment.

Very precise adjustment of pH is not required. The optimum is between 5.5 and 6.5 The Universal Indicator is a sufficiently accurate guide; the colour should be between yellow and orange. If the water is alkaline, adjust by adding a few drops of 5% acetic acid or a small quantity of potassium dihydrogen phosphate. Do not attempt to keep a large quantity of buffered water, as moulds, ciliates and flagellates grow quickly in the tropics.

Staining technique. The following steps are used in staining:

1. The thin film having been fixed and the methyl alcohol allowed to dry, the whole slide, thick film downwards, is immersed into the 1 : 7500 eosin for 5-8 minutes.

2. Let the slide drain well. Proceed immediately or within an hour to stain with J.S.B.I stain.

3. Stain in J.S.B.I for 30-80 seconds. The optimum time for a batch of stain must be found by experiment. It is usually 45 seconds, but longer staining may be needed when the stain has been used many times.

4. Rinse in water of pH 5.5-6.5 (see above). If the platelets are too darkly stained by the blue J.S.B.I then wash longer or make the water more acid. When rinsing, the thin film requires a rapid dip only, the thick film may need 5-10 seconds; plunge the whole slide in and withdraw rapidly until only the thick film is under water.

Appearance of the stained film. A well stained film resembles a film stained by Giemsa, though cytoplasm, particularly in young rings, tends to be pale.

A red fibrous background results from staining the film too soon after it has been taken, in which case there is also unnecessary distortion of parasites and white cells. For survey work blood films should be left for one or two days before staining. Other reasons for too red a film are: leaving too long in eosin or using too strong a solution of eosin. Excess of eosin can be washed out by leaving for a time in slightly alkaline water (pH 8-10). Excess of blue shows particularly in deep staining of platelets which distracts the eye when searching for parasites. It can be removed by further washing in the acid rinsing water.

It is emphasized that every new batch of stain varies to some extent and different types of water also tend to affect staining. Therefore when using a new batch of stain or when using a new source of water a complete batch of blood films should never be stained all at once. A few slides should be stained singly using different timings until good results are obtained.

Filtration. If good and consistent results are to be obtained all stain and rinsing water (unless the source is very pure) should be filtered daily before use. Filter funnels need not be of glass. Most tinsmiths sell small metal funnels that are used to prevent spilling when filling lamps with kerosene and plastic funnels are also sold for this purpose. Such funnels are quite suitable for laboratory work. Use separate ones for eosin, water and J.S.B.I.

We prefer Whatman No. 4 filter paper of 12.5 cm diameter, which is faster than No. 1. If no filter paper is available the following can be tried as substitutes: unglazed local paper, paper handkerchiefs, blotting paper, absorbent toilet paper, etc. When using an improvised filter paper do some test staining to ensure that there are no chemicals in the paper that may interfere with staining.

It is not necessary to change the filter paper daily but only when filtration becomes slow or if the paper becomes torn.

If grossly dirty water from streams or wells has to be used, filter it through cotton wool before filtering through filter paper.

Simple laboratory apparatus. One difficulty that can arise is that glass staining troughs may arrive broken, in inadequate numbers or are too small to deal with the number of slides that may have to be handled.

We have devised a type of staining trough that can be made very cheaply in any place that has tinsmiths; and wooden clips that can be made cheaply and easily by anybody with a saw and some three-ply wood.

The staining trough is shown in Figure 3. It is made of thin galvanized iron sheeting and measures 1 1/4 inches (3 cm) wide by 4 inches (10 cm) long and 3 inches (7.5 cm) deep. Care should be taken to see that the tinsmith makes it watertight and he should be told to make a loose fitting lid. The lid is only to keep out dust and flies when the trough is not in use and if the tight fitting lid (beloved by tinsmiths) is allowed, stain will be spilt when putting the lid on and taking it off.

The clips are shown in Figure 4. Their function is to hold batches of slides for staining. They are a modification of the well-known method of putting a square of cardboard between each slide and binding batches in this way with rubber bands. We find our clips an improvement as they are permanent apart from being easy to make and to handle. Eleven clips are held together by rubber bands and a five-inch nail is passed through the holes in the clips. The five-inch (12.5 cm) nail can take two sets of 11 clips and these can hold 20 slides, a number that fits into the staining trough described above.

Each wooden clip is made of three-ply wood and measures 1 3/4 inches (4.3 cm) by 1 inch (2.5 cm). At the central point where diagonal lines drawn from corner to corner cross each other a hole is bored of a size to allow the passage of a five-inch (12.5 cm) nail. Notches are cut in the short sides to hold the rubber bands that keep a set of clips together. The long side between the notches is bevelled on both aspects by a file to allow easy insertion of slides which should be pressed down to the level of the five-inch nail.

For small batches of slides three staining troughs are used, one for J.S.B. blue stain, one for eosin and one for washing water. However, if many slides have to be dealt with the number of troughs of eosin can be increased, e.g. for 100 slides, if five troughs of eosin are used, the staining, with the modified technique described above, will still only take about ten minutes.

When slides have to drain or dry they do not have to be removed from the clips as they stand well and can just be placed upright on the bench, preferably on a piece of clean rag laid flat to absorb any liquid that drains off.

Sometimes we have seen slides spread out to dry leaning against pieces of wood scattered all over a laboratory bench. This is quite unnecessary and we wish to draw attention to a well-known type of rack that is easy and cheap to make. This is a piece of board 1/2 inch (1.25 cm) thick and of any convenient size with parallel saw cuts about 1/2 inch (1.25 cm) apart cut across the grain of the wood. The saw cuts should be made at an angle of about 60 degrees to the base and should be about 1/4 inch (0.6 cm) deep. They should be wide enough to take the thickest slide without difficulty. Figure 5 shows such a rack. This rack is used for holding slides after they have been dipped in methyl alcohol to fix the thin film.

Technique of blood examination

It is of great importance, particularly in the surveillance stage of malaria eradication, that many low density infections should be found. This is not possible if the thick films are too thin and in most of the countries we have visited we found that field staff take films that are very thin indeed and are quite inadequate for

the detection of very low density infections. Furthermore, we have noted that there is very great variation in the thickness of blood films taken by different field workers; they may be as little as five times the thickness of a plain film, and it is common experience to find some "thick" films that when stained are difficult to locate on the slide.

Since great variations occur in the thickness of thick films, such statements as "100 fields examined" or "blood films examined for five minutes" have little meaning for the reader of a report as he has no idea as to the volume of blood examined. Some degree of uniformity is obviously necessary for comparing parasite rates found by different workers.

Any standard procedure adopted should ensure the examination of as much blood as is technically possible without taking too much time; and it should be sufficiently simple for relatively inexperienced workers to use.

Obviously the first need is to have a blood film of adequate thickness. Perhaps the best definition of this is "a thick film in which, except at the edges, the majority of fields contain 20 or more leucocytes when examined with a hinocular microscope using a 1/12-inch oil immersion objective and X5 oculars". Such a thick film we have found in practice to be about 50 times the thickness of a thin film; i.e. if there is about one parasite per field of the thick film it will be necessary on the average to examine about 50 fields of the thin film to find one parasite. Films of this thickness can be stained with Giemsa's stain and are similar to those described in the standard books on this subject, though possibly slightly thicker than those preferred by some workers.

We suggest that a good standard procedure which allows the detection of low parasite densities is to examine 100 fields of the type of thick smear described above, but that 'a field' to be counted should be defined as: "containing not less than 20 leucocytes, well stained, with a pale but not colourless background". The film is examined systematically from edge to edge but fields containing less than 20 leucocytes are not included in the count of 100. The thinner fields are examined because gametocytes of *P. falciparum* and sometimes *P. vivax* parasites tend to be found near the edge.

Such a standard blood examination might be called a "2000 plus" count, as the minimum amount of blood examined must have contained not less than 2000 leucocytes. If an arbitrary count of 8000 leucocytes per cm is assumed, the volume of blood

examined can be estimated as 0.25 cm and a rough Parasite Density Index calculated similar to that used in West Africa.[1] In this case the average leucocyte count for field examined should be as close to 20 as possible.

We find that the time taken for our counts of 100 fields seldom exceeds four minutes, so if it is thought advisable to establish a standard time of five minutes for each slide, an adequate volume of blood will probably be examined if the blood films are of the thickness described. Microscopists should be instructed to examine systematically from edge to edge but to ignore the poorly stained areas.

Technical hints

Light sources for microscopes

A very common error is to take light from a blue sky. In this case the blueness of the light is so strong that the value of the staining is largely nullified. The light intensity is inadequate for the use of a x 10 ocular for checking doubtful objects and is quite inadequate for the use of a binocular microscope.

The best light is electric, using a 100 watt frosted bulb. A desk lamp with a flexible "goose neck" stem is quite adequate and special lamps requiring transformers are unnecessary for this type of work.

Where electricity is not available the next best light can be obtained from a pressure lamp which is adequate for all oculars and for binocular microscopes. Some form of screen should be arranged so that the face of the microscope is shielded from the light and heat radiated.

In hot climates a pressure lamp may cause too much discomfort owing to the heat produced. In such cases light may be taken from the reflection of sunlight shining on a white piece of paper or on a whitewashed wall. Alternatively, direct sunlight shining through frosted glass may be used.

Air bubbles

When re-examining slides kept for demonstration purposes a highly refractile film may be seen obscuring the blood. This is due to minute air bubbles trapped on the rough surface. Remove the oil with xylene or petrol and re-apply oil while still wet with the solvent. If this does not cure the condition, remove the oil again and let dry. Then dip quickly into neutral water, let dry and examine.

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[1] Bruce-Chwatt, L. J. (1958) Trans. roy. Soc. trop. Med. Hyg. 52, 389

Fig. 1 a

TRAY VIEWED FROM ABOVE
PLATEAU: PLAN SUPÉRIEUR

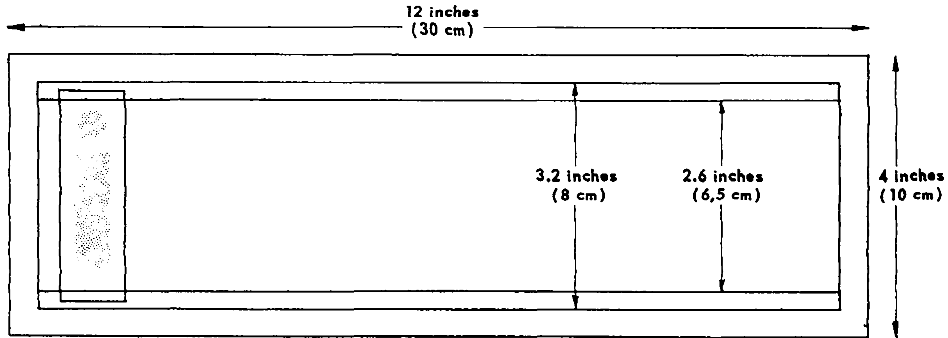

Fig. 1 b

UNDERSIDE OF TRAY - PLATEAU: PLAN INFÉRIEUR

THE RUNNERS PRESS ON THE SLIDES IN THE TRAY BENEATH TO PREVENT MOVEMENT – LES GLISSIÈRES APPUIENT SUR LES LAMES DU PLATEAU INFÉRIEUR ET LES EMPÊCHENT DE BOUGER

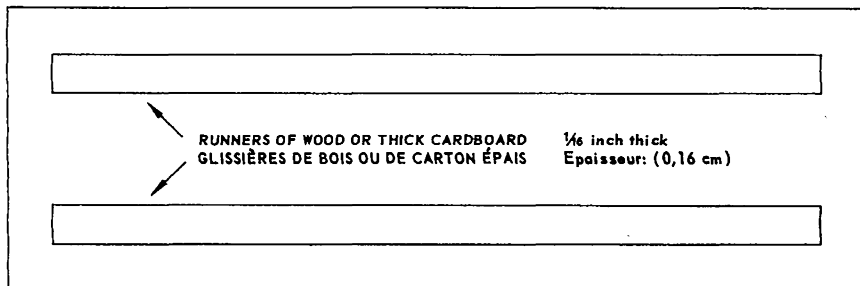

Fig. 1 c

DETAIL OF TRAY
PLATEAU: DÉTAIL

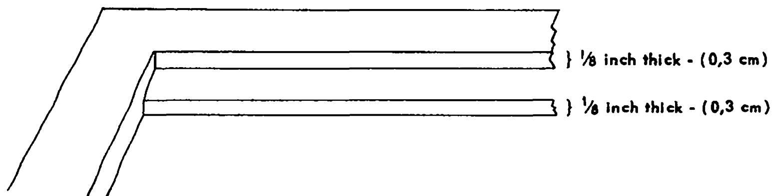

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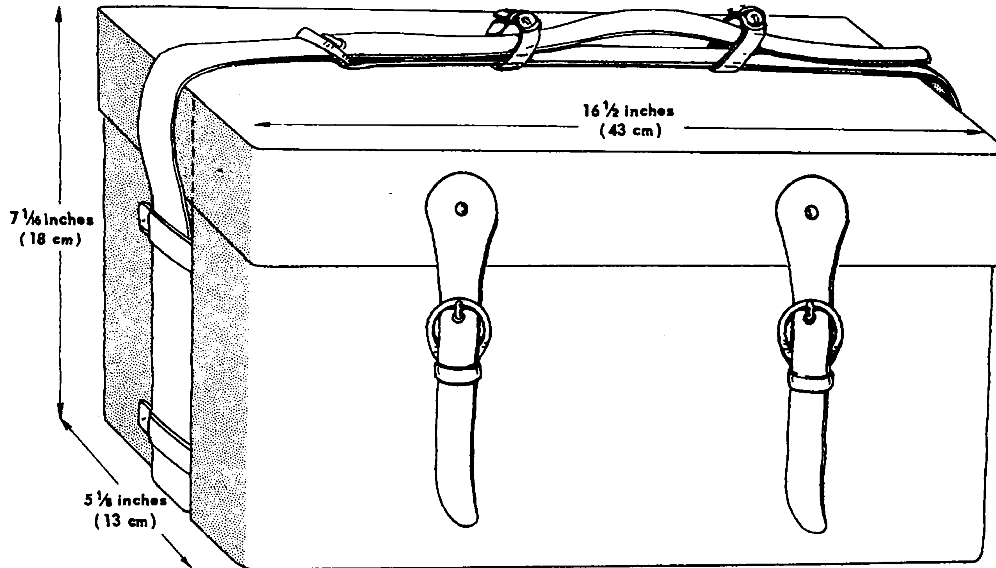
Fig. 2 a

CLOSED - SAC FERMÉ



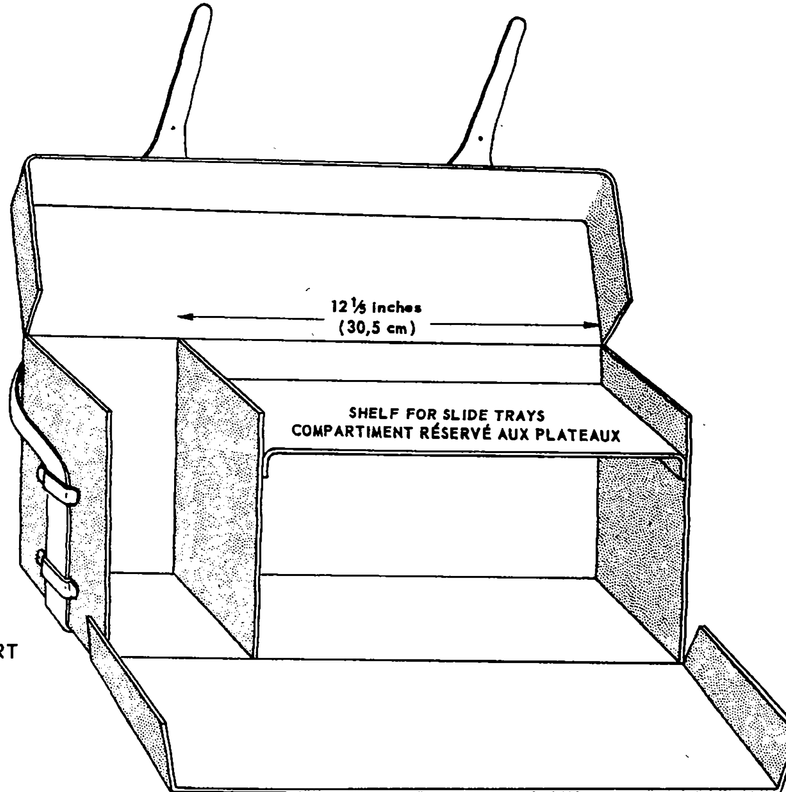


Fig. 2 b

OPEN - SAC OUVERT

WHO 9304

Fig. 3

STAINING TROUGH MADE OF THIN GALVANISED IRON SHEET
BAC DE COLORATION EN TÔLE GALVANISÉE MINCE

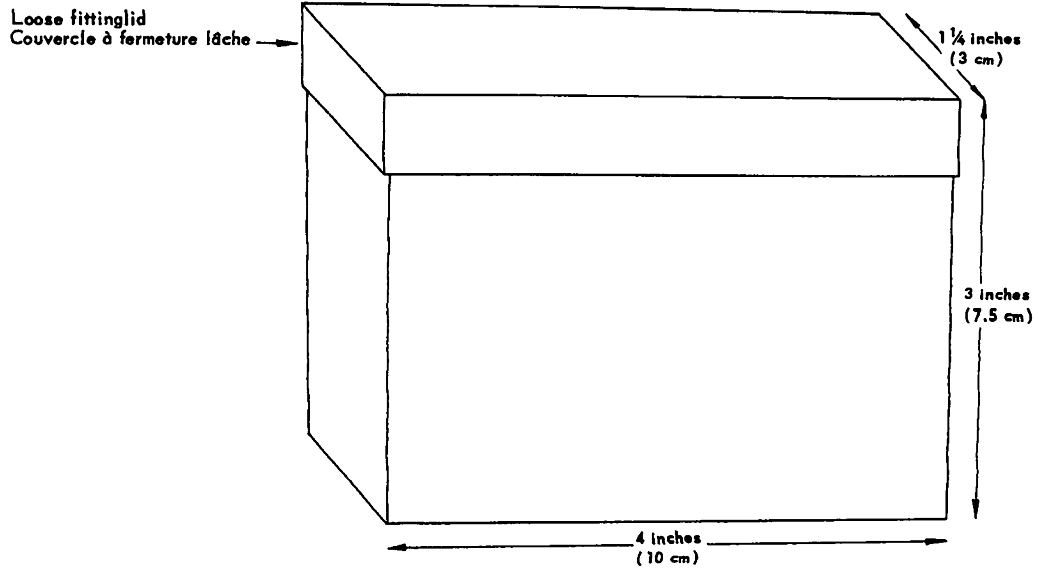


Fig. 4

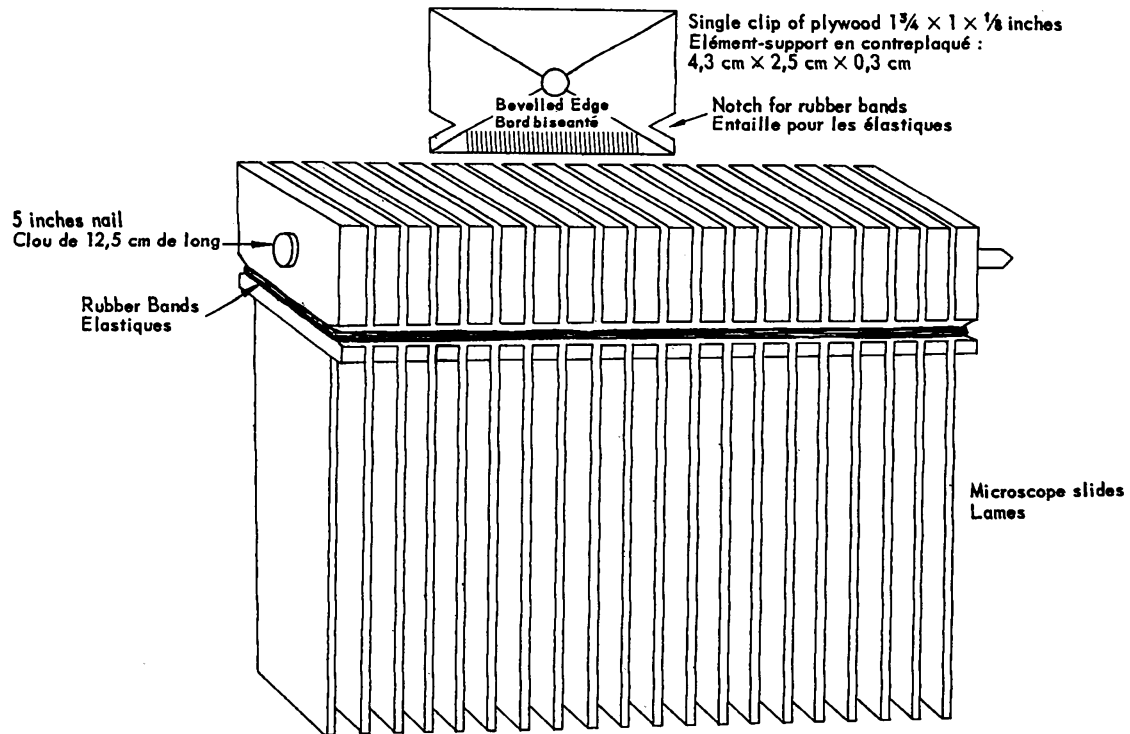


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Fig. 5

SLIDE RACK - PORTE-LAMES