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A Comparative Study of Thick and Thin Blood Films in the Diagnosis of Scanty Malaria Parasitaemia

M. A. C. DOWLING [1] & G. T. SHUTE [2]

In an attempt to explain the shortcomings of the routine thick-film examination in the diagnosis of scanty malaria parasitaemias, a direct comparison, in terms of positivity and parasite counts, was made between the results of routine thick-film study and long-term examination of thin films taken at the same time from the same individuals. Calculation of the average thickness of the thick and thin films prepared allowed these comparative results to be corrected according to the actual volume of blood examined. From these corrected figures it was observed that both parasite counts and positivity were significantly higher in the thin-film series, and it has been deduced that heavy losses in parasites, varying from 60% to 90%, occurred during the dehaemoglobinization and staining of thick films.

The epidemiological implications of this finding in malaria practice are discussed. Emphasis is laid on the importance of further research in order to improve the sensitivity of the routine thick film in the diagnosis of the scanty parasitaemias met with in the later stages of malaria-eradication programmes.

In a previous unpublished study, it was noted that an increase in malaria positivity in a group of semi-immune adults resulted from prolonging the time for which blood slides were examined. It was shown that the observed parasite rate—38% in routine 100-field thick-film examination—was doubled when this examination time was extended, owing to the prevalence of very scanty parasitaemias in this group of adults which escaped detection in routine study. These findings underline the shortcomings of the thick-film examination currently employed in malaria practice and point to the need for an improvement in sensitivity in the diagnosis of low-grade parasitaemia. It was also noted during this investigation that the results obtained from the examination of thin films taken from the same individuals at the same time were much better than would have been expected from the smaller quantity of blood examined.

The present work was directed towards trying to find the reasons for this apparent inferiority of the thick film and the relatively greater efficiency of the thin film. Before a precise comparison of the results of thick- and thin-film examination could be made, a quantitative study of the mean areas and volumes of each type of film was required. Such a study could perhaps permit of a direct conversion of counts per 100 fields to counts per mm³ on a volumetric basis; and this, in its turn, would enable the results of a large series of paired thick and thin films to be directly compared in terms of relative efficiency.

EXPERIMENTAL PROCEDURE

Films were prepared of blood taken from 233 individuals in different age-groups in Nigeria. These individuals were selected at random in clinics and in routine parasite surveys, and on each occasion paired thick and thin films were prepared at the same time. The normal field procedure used by the authors was followed in each case. Thick films were spread with the Hagedorn needle used for finger pricking and thin films were spread with the edge of a selected microscope slide. In all cases the films were stained some 24 hours later with Giemsa stain by the normal laboratory technique.

[1] Formerly, Director, WHO Malaria Eradication Training Centre, Lagos, Nigeria. Present address: Division of Malaria Eradication, World Health Organization, Geneva, Switzerland.

[2] WHO Malaria Eradication Training Centre, Lagos, Nigeria.

In order to obtain as many data as possible, several methods of examination were used. The thick films were examined by one of us (GTS) and the thin films by the other (MACD), although frequent cross-reference was made throughout the work. The thick films were subjected to three separate studies:

(*a*) A routine three-minute examination of all films, using a × 100 oil-immersion objective. Positivity and species diagnosis were established as a result of this examination. Parasite counts were carried out on all positive films, based on a further examination of 100 microscopic fields. In the case of very heavy infections, the number of fields examined to determine the parasite count was reduced.

(*b*) Scanning of a larger area of film for a further three-minute period, using a × 50 oil-immersion objective. This was for diagnosis only, primarily for locating gametocytes of *Plasmodium falciparum*, and no routine parasite counts were carried out with this scanning objective.

(*c*) Calculation of the average leucocyte count per field in that part of the film used for the routine 100-field study.

The thin films were examined on the following basis, using entirely the × 100 oil-immersion objective:

(*a*) Thirty-minute examination of the thin film. Both authors adjusted their speed of examination so that a regular rate was maintained of 100 fields in three minutes. Thus, the 30-minute examination was equivalent to 1000 fields of the thin film. Parasite counts, as with the thick films, were based on random counts of a smaller number of fields (usually 100) when heavy parasitaemia was present. Where only a few parasites were present, the counts over the entire 30-minute period were recorded.

(*b*) In order to give some indication of the effects of a shorter examination of the thin films, positivity and counts at the end of the first 10 minutes of examination were recorded in each case.

(*c*) For a number of thin films the average number of erythrocytes per field in the part of the film normally examined was recorded.

Every attempt was made throughout the study to ensure that identical procedures were used for the examination of both types of film. Any doubtful parasites were checked by both authors, and were excluded from the series if the uncertainty persisted.

RESULTS OF BLOOD-FILM EXAMINATIONS

Comparative positivity and species rates

Table 1 shows the breakdown of the results of the various types of thick- and thin-film examinations by species and by age-group. For each individual examined the maximum information obtained from all the different types of examination was recorded as " over-all results ". In this way the best results obtained from the combination of methods could be taken as the maximum efficiency, within the limitations of the series. The results of each individual examination method could therefore be compared directly with one another in terms of maximum possible efficiency. These comparative efficiencies are represented graphically in Fig. 1.

It can be seen, from a study of the table and the graph, that the prolonged examination of a thin film produces a considerably better result than the routine thick-film examination. Even the relatively short 10-minute examination of the thin films gave results comparable to those obtained from the three-minute thick-film studies, in spite of the considerably smaller quantity of blood examined.

Parasite counts

There is little to be gained from detailing the individual results; in 126 positive cases, the mean parasite counts per individual in thick and thin films were 618 and 440 per 1000 fields, respectively. The distribution of counts in terms of parasite density and age-group is discussed later in the paper (see Table 14).

Leucocyte count in thick films

Study of the results of the trial indicated a normal distribution of leucocyte count per field in the thick films. Table 2 gives details of the distribution, which has a mean of 16.7 leucocytes per field.

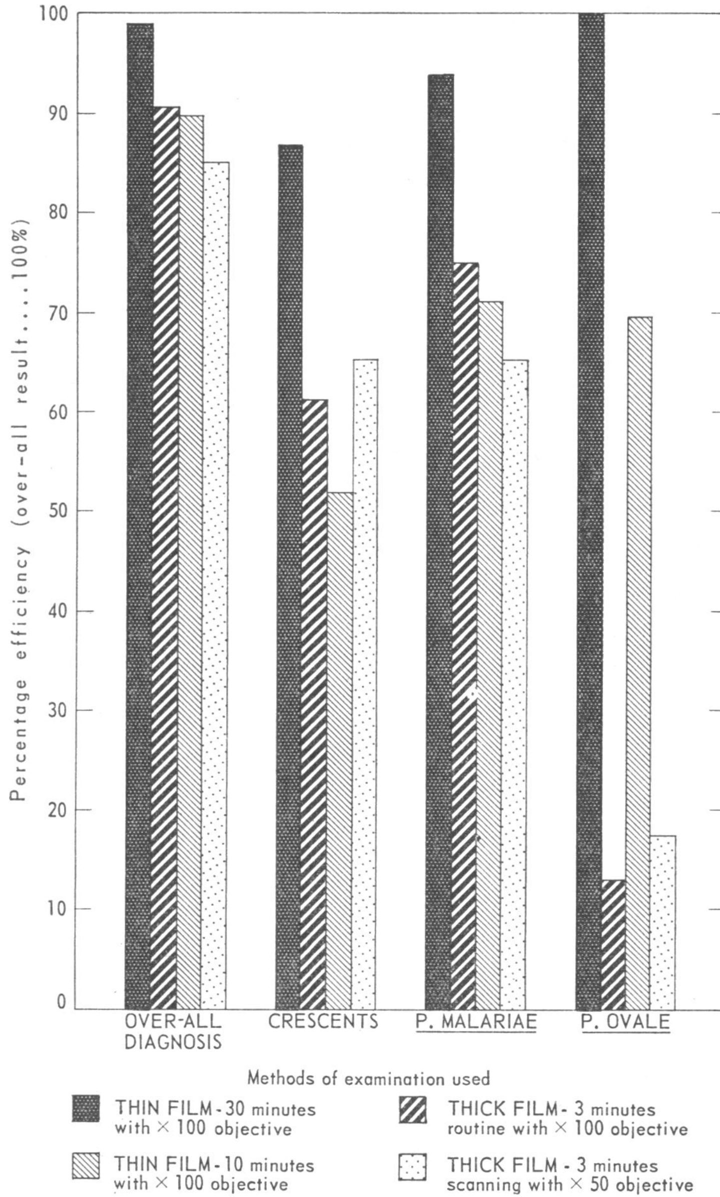
These cell counts were carried out in the " best part " of each film—usually the thickest part—normally used for the routine three-minute parasite examinations and counts. There was little variation in count in the different age-groups, as shown in Table 3.

As parasite count per 100 fields and leucocyte count per field are recorded for each positive specimen, individual parasite counts for calculation of the parasite density index (Bruce-Chwatt, 1958) could be easily determined, and use is made of this later in the paper (see Table 14).

TABLE 1. COMPARATIVE RESULTS OF SERIES EXAMINATIONS OF THICK AND THIN FILMS IN TERMS OF PARASITE AND SPECIES RATES

	Age-group (years)				Total (all ages)	Parasite and species rates (%)	Maximum efficiency of series (%)
	0-11/12	1-4	5-14	15 and over			
No. examined	19	96	73	45	233		
			Over-all results				
No. positive	15	91	64	25	195	83.7	100
Total *P. falciparum*	13	91	61	24	189	81.1	100
P. falciparum trophozoites	11	85	57	23	176	75.5	100
P. falciparum gametocytes	8	64	22	4	98	42.1	100
P. malariae	5	56	21	1	83	35.6	100
P. ovale	0	15	8	0	23	9.9	100
			Thick × 100 × 3 min				
No. positive	12	88	58	19	177	76.0	90.8
Total *P. falciparum*	11	86	56	18	171	73.4	90.5
P. falciparum trophozoites	9	83	55	18	165	70.8	93.8
P. falciparum gametocytes	8	41	10	1	60	25.8	61.2
P. malariae	2	46	15	0	63	27.0	75.9
P. ovale	0	2	1	0	3	1.3	13.0
			Thick × 50 × 3 min				
No. positive	12	88	56	10	166	71.2	85.1
Total *P. falciparum*	11	85	55	10	161	69.1	85.2
P. falciparum trophozoites	9	82	53	7	151	64.8	85.8
P. falciparum gametocytes	8	40	13	3	64	27.5	65.3
P. malariae	2	40	12	0	54	23.2	65.1
P. ovale	0	3	1	0	4	1.7	17.4
			Thin × 100 × 30 min				
No. positive	15	91	63	24	193	82.8	99.0
Total *P. falciparum*	12	91	60	23	186	79.8	98.4
P. falciparum trophozoites	10	85	57	22	174	74.7	98.9
P. falciparum gametocytes	8	56	18	3	85	36.5	86.7
P. malariae	3	54	20	1	78	33.5	94.0
P. ovale	0	15	8	0	23	9.9	100
			Thin × 100 × 10 min				
No. positive	14	88	57	16	175	75.1	89.7
Total *P. falciparum*	12	86	54	15	167	71.7	88.4
P. falciparum trophozoites	10	84	52	15	161	69.1	91.5
P. falciparum gametocytes	7	30	13	1	51	21.9	52.0
P. malariae	3	41	14	1	59	25.3	71.1
P. ovale	0	10	6	0	16	6.9	69.6

FIG. 1
GROUPED HISTOGRAMS SHOWING COMPARATIVE EFFICIENCY AT OVER-ALL AND SPECIES DIAGNOSIS OF THICK- AND THIN-FILM EXAMINATIONS [a]



[a] For details see Table 1.

TABLE 2
DISTRIBUTION OF LEUCOCYTES IN 231 THICK FILMS [a]

No. of leucocytes per field	No. of films
5-9	2
10-14	48
15-19	155
20-24	20
25-29	3
30-34	1
35-39	0
40-44	2
Total	231 [b]

[a] Mean, 16.7 leucocytes per field; range 7-43 per field.
[b] Two films from the series were discarded as, in error, leucocyte counts per field were not made.

TABLE 3
LEUCOCYTE COUNTS PER FIELD ACCORDING TO AGE-GROUP

Age-group (years)	No. examined	Average leucocyte count per field	Range of leucocytes per field
-11/12	19	16.1	10-23
1-4	94	17.6	8-43
5-14	73	16.4	10-40
15 and over	45	15.8	7-19
Total	231	16.7	7-43

MEASUREMENTS OF THICK AND THIN FILMS

In order to understand better the results of the series described above, it was necessary to have a clear idea of the relative quantities of blood examined in 100 fields of a thick film and 1000 fields of a thin film. It must be stressed that all these measurements refer to films prepared by the authors. There is no doubt that, although thin films are relatively consistent, the size and thickness of thick films vary according to the practice of individual operators. With this proviso, the measurements that follow can be safely interpreted. In order to obtain a figure for the average thickness of thick and thin films, calculations of mean areas and volumes were made as described below. Although the blood dries after spreading, this in no way affects the fact that the solid constituents corresponding to that original thickness will be present in the dried film. It is only in the solid constituents that we interest ourselves in routine malaria microscopy.

Areas of blood films

Thick films. A method was devised for the calculation of the areas of thick and thin films in which error was reduced to a minimum. A sheet of drawing paper 1.5 m × 2 m in size was marked off into small squares with sides of 3 cm. This sheet was used as a projection screen. By means of a Leitz slide projector, a machined cover-glass with a side of 0.25 in (6.35 mm) was projected on to the screen. The projector distance was adjusted until the image covered an area of exactly 13 × 13 squares on the screen. This was further checked for accuracy by projecting a slide with micrometer graduations. Thus, it could be calculated that the length of the side of one square on the screen was equivalent to 0.4885 mm on the objective in the projector. The equivalent area, therefore, of one square on the screen was 0.2386 mm^2 on the object projected.

One hundred thick films prepared successively in a normal manner were projected one after the other on to the screen. The number of squares covered by the image of each successive film was recorded; the results are shown in Table 4. As each screen square represents 0.2386 mm^2, the average can be calculated to be *70.1* $\pm$ *11.3* mm^2 *per thick film.* Fig. 2 shows a typical thick film projected on to the screen.

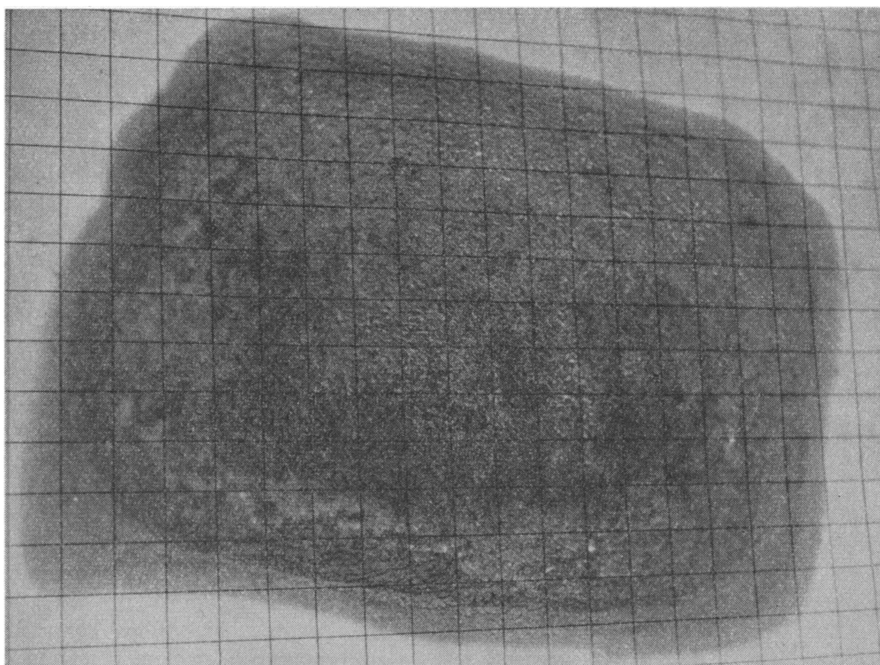
TABLE 4
FREQUENCY DISTRIBUTION OF SCREEN AREAS OF 100 CONSECUTIVE THICK FILMS

No. of screen squares per thick film [a]	No. of films
200-249	16
250-299	41
300-349	34
350-399	6
400-449	1
450-499	2
Total	100

[a] Mean, 294 $\pm$ 47.5 squares.

FIG. 2

PHOTOGRAPH OF TYPICAL UNSTAINED THICK DROP PROJECTED ON TO MEASURING SCREEN: RELATIVELY THICKER AREA IN CENTRE OF FILM IS CLEARLY VISIBLE



Thin films. A similar technique was used for calculating the areas of thin films, but a different magnification was needed, owing to the much larger size of the average thin film. The projector was moved closer to the screen until each side of the 0.25-in-square (6.35 mm × 6.35 mm) cover-slip corresponded with nine squares on the screen. Thus the length of one side of a square on the screen was then equivalent to 0.706 mm on the projected object and one square on the screen to an area of 0.498 mm^2. Fifty thin films prepared in the normal manner were projected one by one on the screen and the numbers of squares covered by the image recorded. Table 5 shows the result. As each screen square represents 0.498 mm^2, the average area can be calculated to be *373.5 ± 44.8 mm^2 per thin film.*

TABLE 5

FREQUENCY DISTRIBUTION OF SCREEN AREAS OF 50 CONSECUTIVE THIN FILMS

No. of screen squares per thin film [a]	No. of films
600-699	13
700-799	29
800-899	5
900-999	1
1 000-1 099	2
Total	50

[a] Mean, 750 ± 90 squares.

It will be noted that there is a relatively low coefficient of variation in each of these two sets of measurements—16% in the thick films and 12% in the thin films. The area varies directly with the size of the original drop, as the thickness is fairly constant. A larger drop is automatically spread by the operator over a correspondingly bigger area than a smaller drop. This is particularly important in the preparation of thick films, in which the visual control of successive films is based on thickness rather than on size.

Volumes of blood films

Thin films. With a fine-bore graduated tuberculin syringe, 0.1 ml of Sequestrene-treated blood was dispensed in normal-size drops on successive blood slides. Each drop, visually considered as normal for the preparation of thin films, was spread in a regular manner with the edge of a selected microscope slide. A single slide was used as a spreader for making all the films and thus the amount of blood lost in spreading was negligible.

In two successive replicates, 0.1 ml of blood produced 107 and 106 thin films, respectively; i.e., 200 mm^3 of blood produced 213 thin films. From this it can be calculated that the average volume was *0.939* mm^3 *per thin film.*

In order to verify the volume of the average thin film as calculated by the above volumetric method, a torsion balance, accurate to 0.05 mg within a quoted 3% error, was used. The quarter-inch-square cover-slips were weighed separately, and then reweighed, each with a drop of fresh blood corresponding in size to that normally used in the preparation of a thin film. As measurements were carried out very rapidly in a humid atmosphere, loss of blood by evaporation was likely to be slight. The distribution in weight of a series of 30 replicates was 0.65-1.55 mg, with a mean weight of 0.99 mg per drop.

The specific gravity of blood is quoted as varying between 1.048 and 1.066. The mean figure (1.057) was therefore taken for conversion from blood weight to volume. As the mean weight of each thin-film drop was 0.99 mm, the mean volume would be expected to be 0.99/1.057 mm^3 or 0.937 mm^3 per thin film. This figure corresponds very closely indeed to the figure obtained by the volumetric method.

Thick films. The simple volumetric method used to estimate the volume of the average thin film could not be used with thick films. Large quantities of blood must be treated with an anticoagulant if this method is to be used and both Sequestrene and heparin lower the surface tension of the blood. For the very small drop used in thin-film preparation, this lowering of surface tension did not affect the visual estimation. With the much bigger drop used for thick films, however, the Sequestrenized blood drops spread out and gave the impression of being far larger than their actual volume. Weighing was also difficult because the size of the drop was too much for convenient handling on the quarter-inch cover-slip. A glass slide with a larger surface area was too heavy for the torsion balance available.

It has, however, been noted above that there is a very close relation between the size of drop and the area over which it is spread in the preparation of thick films. A further series of 20 thick films prepared for another reason (see Table 10) showed an average area varying from that of the bigger series of 100 by less than 0.6%. In view of these consistent measurements, a method was devised for the accurate estimation of the volume of thick films. A 20-mm^3 pipette was treated with silicone to ensure that it would empty completely. From each successive 20 mm^3 of fresh blood taken from a finger prick, five drops were dispensed on to microscope slides and spread in the normal manner. It was apparent to the naked eye that the average size of the spread smears was less than that normally observed in field practice. As the average volume of each of the five drops prepared from the 20 mm^3 must have been 4 mm^3, it was therefore evident that the normal thick film is prepared from a rather larger quantity of blood. Eleven replicates (a total of 220 mm^3 of blood dispensed and spread on 55 consecutive microscope slides) were projected and measured on the screen for estimation of the area, using the same technique as that employed previously and described above. Table 6 shows the frequency distribution of areas on the screen covered by these 55 consecutive thick films.

Comparison with Table 4 indicates the shift to the left of all measurements, demonstrating the

TABLE 6

FREQUENCY DISTRIBUTION OF SCREEN AREAS OF 55 THICK FILMS PREPARED FROM 220 mm^3 OF BLOOD

No. of screen squares per thick film [a]	No. of films
151-199	1
200-249	25
250-299	20
300-349	6
350-399	2
400-449	1
Total	55

[a] Mean, 267 ± 39 squares.

smaller size and range of these films, each prepared from an average of 4 mm³ of blood. A total of 14 765 squares on the screen represented the area covered by 220 mm³ of blood. The size on the screen of the average thick film has already been demonstrated to be 294 squares (see Table 4). The volume, therefore, of an average drop can be calculated to be (294×220/14 675) mm³ or *4.4 mm³ per thick film.*

Thickness of blood films

The average areas and volumes of thin and thick films having been estimated, it was possible to obtain a reasonable estimate of the average thickness.

Thin films. The thickness of an average thin film varies considerably from the spreading edge to the fimbriated extremities. It is considered, however, that the part normally examined represents an average thickness of the film. This can be calculated from the area and volume to be 0.939/373.5 mm or *0.0025 mm (2.5 μ) per thin film.* It must be remembered, when considering these measurements, that the thickness quoted refers to original whole blood and not to the dried film. However, as the final microscopic material remaining on the dried film represents all the solid constituents of blood, these calculations are entirely valid.

Thick films. A different concept must be used when estimating the average thickness of the thick film. It is our normal procedure to examine the " best part " of the thick film in order to give the greatest possible chance of finding parasites. This part is considerably thicker than the average, as can be seen clearly in Fig. 2. It was therefore necessary to obtain a measurement for applying to the average thickness of the film that would take into account this selective study of the most favourable area. The average thickness itself can be calculated easily from the volume and area to be 4.4/70.07 mm or *0.063 mm (63 μ) per thick film.*

The distribution of leucocytes in the thick film, unlike that in the thin, is even and therefore varies directly with the thickness of the part of the film examined. Three small thick films were therefore prepared from three different individuals of known leucocyte count and examined in their entirety, leucocyte counts being made in successive rows of a squared eyepiece. These counts were carried out along the long axis of each thick film and were recorded for convenience in groups of six rows (each six being equivalent to a width of approximately 0.6 mm). The results are shown in Table 7.

TABLE 7
DISTRIBUTION OF LEUCOCYTES COUNTED IN THE ENTIRETY OF THREE SMALL THICK FILMS

No. of rows of squared eyepiece examined (groups of 6)	No. of leucocytes counted in each group of 6 rows [a]		
	Film 1	Film 2	Film 3
6	79	239	92
12	360	917	492
18	558	756	702
24	741	950	785
30	(799)	1 001	799
36	(882)	1 088	806
42	(928)	1 263	1 104
48	(892)	(1 421)	(1 615)
54	(762)	(1 589)	(1 325)
60	862	(1 590)	(1 320)
66	791	(1 478)	(1 293)
72	753	(1 470)	(1 282)
78	714	1 417	1 194
84	674	1 361	984
90	551	978	378
96	326	847	—
102	308	661	—
108	130	458	—
Total	11 110	19 538 [b]	14 171

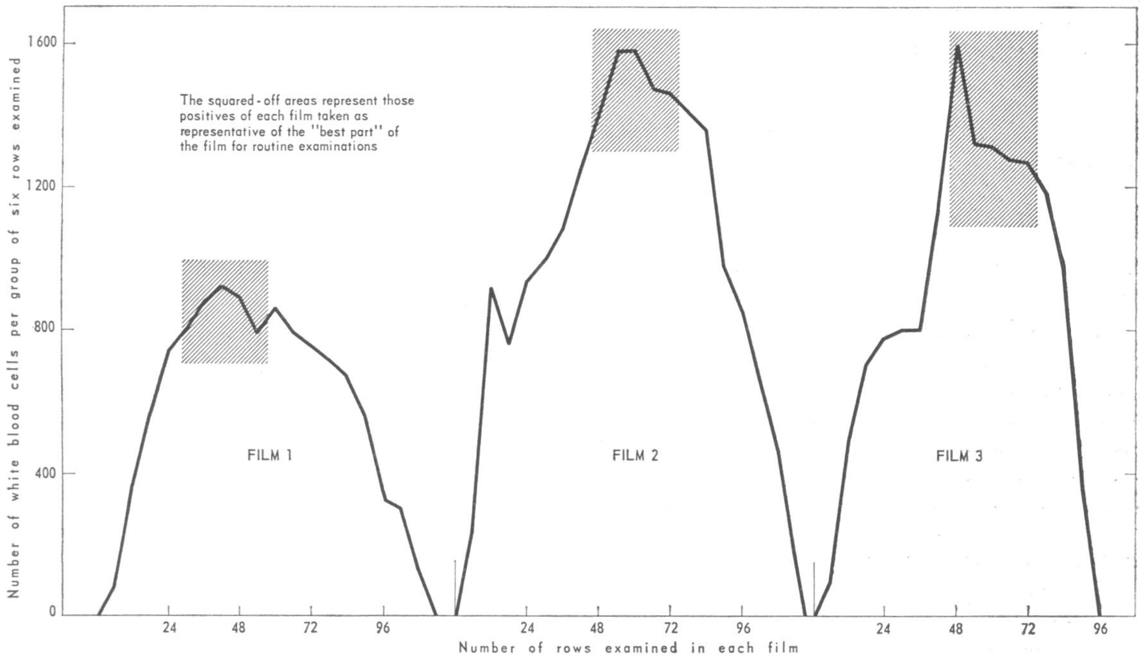
[a] Values in parentheses are representative of the " best part " of the film (see text).

[b] Includes four additional rows (109-112) totalling 54 leucocytes.

A glance at this sequence and at the corresponding graphs in Fig. 3 shows how closely these distributions approximate to the normal curve. In addition, the leucocyte counts of the three individuals from whom the films were made showed the same proportional relationships to each other as did the total film counts (see Table 8).

It is therefore reasonable to assume that these three films are similar in volume. If, in general, the thicker part of each film is examined in the routine parasite examination, it may be that some reasonably constant relationship exists between the thickness of this part of the film and the average for the whole film. In Table 9, for each film the average leucocyte

FIG. 3
DISTRIBUTION OF LEUCOCYTES IN THREE THICK FILMS EXAMINED IN THEIR ENTIRETY[a]



[a] See Table 9.

TABLE 8
COMPARISON OF RATIO OF TOTAL COUNTS IN THREE THICK FILMS WITH RATIO OF ORIGINAL LEUCOCYTE COUNTS IN THE THREE INDIVIDUALS

Film no.	Leucocyte count per mm³	Ratio of leucocyte counts	Total leucocyte count in films	Ratio of leucocyte counts in films
1	5 300	1.0	11 110	1.0
2	9 600	1.8	19 538	1.8
3	6 900	1.3	14 171	1.3

TABLE 9
RELATIONSHIP BETWEEN LEUCOCYTE COUNT IN " BEST PART " OF THICK FILM AND OVER-ALL AVERAGE COUNT FOR WHOLE FILM

	Film 1	Film 2	Film 3
Average leucocyte count per group of 6 rows in whole film	617	1 046	945
Average of highest consecutive 5 groups of 6 rows	853	1 510	1 367
Ratio between " best part " of film and over-all average	1.38	1.44	1.45

count for each group of six rows is compared with the average count for the highest consecutive 30 rows, chosen arbitrarily to represent the " best part " of the film and indicated in Table 7 and Fig. 3. The relationship is remarkably constant, in spite of the different shapes of curve for each film, and, although this is a very small sample, the mean factor of 1.42 has been taken in subsequent calculations to represent the relationship between the thickness of the " best part " of the film examined and the average thickness of the whole film.

It is interesting in this context to note the observed distribution of leucocytes in a normal thin film as compared with the even distribution described above for the thick film. Owing to the different system of spreading and the thinness of the film, the leucocytes are not evenly distributed. There is a heavy concentration, especially of polymorphonuclears, in the fimbriated edges of the spread film, and a count per unit area gives neither a regular result nor an accurate differential distribution of different types of leucocyte. This is not true of the parasite distribution, at least in the series described. Careful examination of the fimbriated tails and the free border of the films certainly revealed many parasites, but not apparently in greater concentration than in the body of the film. In order to take this possible variant into account, however, the 100-field examination of each thin film was always carried out so as to include one of the free borders and thereafter succeeding rows of the main body of the film.

Even with the most careful staining procedure, a certain amount of blood is lost in the preparation of the average thick film. This is a visual loss, which can be readily seen on careful scrutiny of the stained film. Although this factor does not affect the calculations that follow, it was considered of interest to record the degree of loss that normally would be expected to occur in films stained 24 hours after collection. Table 10 shows the paired measurements of a series of 20 consecutive thick blood films before and after normal staining. In all cases there was a small loss, usually on the fringes of the film. In several cases (especially in films 8 and 10), however, a considerable amount of the film was washed away in staining; this is to be expected in all thick-film procedures. As can be seen, there is an average loss of just over 9% as a direct result of the staining procedure.

TABLE 10
COMPARISON OF AREAS OF 20 THICK FILMS BEFORE AND AFTER STAINING

Film no.[a]	Area in screen squares	
	Before staining	After staining
1	251	240
2	255	247
3	310	298
4	251	243
5	279	268
6	334	326
7	392	382
8	271	124
9	247	217
10	271	102
11	275	275
12	293	276
13	281	284
14	234	225
15	278	272
16	334	324
17	330	284
18	293	255
19	357	345
20	374	371
Total	5 910	5 358 [b]

[a] Films 1-3 are not those referred to in Fig. 3 and Tables 7-9.
[b] Average loss in staining, 9.3%.

Loss of leucocytes during staining of thick films

In addition to the obvious visual loss in part of the area of thick films, there is a universal loss of leucocytes during the process of staining that needs to be taken into account in later calculations. Some time ago one of us (GTS, unpublished work) observed that individual blood elements became detached from thick blood films during dehaemoglobinization; this observation was made during a study carried out to demonstrate the occurrence of parasite transfer during routine thick-film staining. It must be appreciated, however, that, for each element of blood that is transferred to a film on another slide, there must be large numbers that are merely deposited elsewhere on the slides, in the staining fluid and on the surface scum, and are permanently lost from individual films.

In order to obtain a measure of this loss a simple device was resorted to which gave a direct measure for comparison. On a number of microscope slides nine circles were engraved with a diamond ringer in three rows of three, care being taken to ensure that all nine rings were sited within the normal area of a thick film. Normal thick films were then prepared from different individuals and spread in the usual fashion so that they covered the ringed zone. The following day a 1% Giemsa stain solution (filtered to remove the precipitate resulting from the mixture of Giemsa stain and water) was poured on to each thick film. The leucocytes took up sufficient stain for examination to be carried out almost at once but, during the course of the ensuing examination, did not become over-stained. Leucocytes within the rings were counted by using the low-power (× 10) objective of the microscope. Immediately after this procedure the films were stained with Giemsa stain by a standard thick-film technique. Successive films were dealt with individually so that the process of dehaemoglobinization involved in the preliminary Giemsa staining did not unduly prolong the treatment of each film. After staining, counts were again carried out within all the rings and direct comparison was made between the two counts. Table 11 shows that there is a regular loss during staining, amounting, on average, to 8%. This proportion appears to be constant and forms part of later calculations.

TABLE 11

LOSS OF LEUCOCYTES IN STAINING BASED ON COUNTING OF NINE RINGED AREAS ON EACH OF 10 FILMS

Film no.[a]	No. of leucocytes counted in ringed areas		
	Before staining	After staining	Loss during staining
1	291	274	17
2	443	372	71
3	894	831	63
4	664	601	63
5	895	819	76
6	494	453	41
7	521	483	38
8	740	685	55
9	718	672	46
10	600	560	40
Total	6 260	5 750	510 [b]

[a] Films 1-10 are not identical with the films of the same numbers in Table 10.

[b] Average loss in routine staining process, 8%.

Thick- and thin-film conversion factors

Enough information had now been gathered to permit factors to be calculated for direct conversion of counts per unit area of film to counts per mm³. The area of each film examined has been based throughout on a specified number of fields. It is important for an observer to ascertain the area of the microscopic fields with which he is working. Where there is no additional magnification incorporated in the body of the microscope, the diameter in millimetres of a microscopic field is obtained from the formula:

$$D = \frac{N}{M}$$

where D is the field diameter in millimetres, N the field number for the eyepiece used (based on the manufacturer's calculation) and M the inscribed magnification of the objective.

In the current trial, compensating eyepieces × 6 were used with a field number of 18, and the objective was × 100. Thus the field diameter was 18/100 or 0.18 mm. This was further checked by means of a micrometer slide with graduations of 0.01 mm. When the scale was viewed through the × 100 objective, using × 6 compensating eyepieces, there were 18 graduations exactly across the diameter of the field, the equivalent of 0.18 mm.

From this measurement it is easy to calculate the area of a single microscopic field as 0.02545 mm².

Conversion factor for thin films. In the series under discussion, counts of parasites in the thin films have been expressed in terms of 1000-field examination. The area of 1000 microscopic fields is 1000 × 0.02545 or 25.45 mm². The average thickness of the thin film is 0.0025 mm (see p. 256). Thus the volume of 1000 fields of a thin film is 25.45 × 0.0025 mm³, or 0.0636 mm³. Thus, to convert an area count per 1000 fields of a thin film to a volumetric count per mm³, one should multiply the count by 1/0.0636, i.e., by 15.7. This figure is the *thin-film factor* for converting counts per 1000 fields to counts per mm³.

Conversion factor for thick films. Parasite counts in the series were recorded per 100 fields of thick film. The area of 100 fields is 100 × 0.02545 mm,

or 2.545 mm². The average thickness of the thick film is 0.063 mm (see p. 256). Thus, the volume of 100 fields of average thickness of a thick film would be 2.545 × 0.063 mm, or 0.16 mm³. It has already been pointed out that routine examination of the thick film is carried out in the "best part" of the film, the thickness of which is 1.42 times the average thickness of the whole film. Thus the volume of 100 fields in the best part of a thick film would be equivalent to 0.16 × 1.42 mm³, or 0.227 mm³. Therefore, to convert a count per 100 fields of a thick film to a count per mm³, one should multiply the count by 1/0.227, i.e., by 4.4. This figure is the *thick-film factor* for converting counts per 100 fields to counts per mm³.

Verification of conversion factors. Before proceeding to apply these factors to the results obtained, it was considered essential to test their accuracy by cross-checking.

The average thin film in the series consisted of 0.939 mm³ of blood spread over an area of 373.5 mm². As the area of a microscopic field is 0.02545 mm², the number of theoretical field areas in an average thin film would be about 373.5/0.02545, or 14 676.

The average erythrocyte count in all age-groups is likely to be in the region of 4 500 000 per mm³. The mean number of erythrocytes per microscopic field would therefore be expected to be (4 500 000 × 0.939/14 676) or 228. This figure agrees very closely with the approximate averages observed during random counts in the series, in which figures between 250 and 300 erythrocytes per field were usual.

Applying the thin-film factor directly to these figures, erythrocyte counts of 250-300 per field must be multiplied by 1000 × 15.7 in order to convert them to a count per mm³. This gives a range of 3 925 000 to 4 710 000 per mm³, which is a realistic range for local conditions.

As a direct check on the accuracy of the thick-film factor, thick films were prepared from blood of an individual with a leucocyte count of 5925 per mm³. The average of several counts of leucocytes per field in the "best part" of the film was 12.6. Thus, the average number of leucocytes per 100 fields would be 1260. Applying the thick-film conversion factor, the average leucocyte count in the thick film should be in the region of 1260 × 4.4 or 5544 leucocytes per mm³ of stained film. However, it has already been demonstrated that 8% of the leucocytes are lost from the body of the film during staining. The original leucocyte count, therefore, of the blood before staining would have been (5544 × 108/100) or 5987 per mm³. This is only 1% different from the actual count of 5925 per mm³, determined by means of a haemocytometer.

These calculations are based on the assumption that the leucocytes are evenly distributed throughout the thick film and that the average count per field bears a constant relationship to the total initial count of the individual. In Table 8 it is demonstrated that the ratios of leucocyte counts per mm³ and of the total film counts are the same for three thick films, illustrating that the films were similar in volume. The drops of blood from which these films were prepared were about half the normal size, in order to diminish the work-load of total leucocyte counts. However, the average numbers of leucocytes per field in the "best part" of each of these three films were in exactly the same ratio (1.0:1.8:1.3) as the initial counts.

As can be seen, when these factors are applied to the series the resultant conclusions are reasonable. No claim is made for complete accuracy of such factors, since they are based on a human procedure which, although constant on average, allows considerable individual variation. Such figures can therefore only be safely applied to groups of observations, in the same manner as the parasite density index, and should not be relied upon for an individual measurement.

Application of conversion factors to results of series examination

Average leucocyte count of series. It has already been shown (Table 2) that the average leucocyte count per field in the whole series was 16.7, i.e. 1670 per 100 fields of thick-film examination. Applying the thick-film factor for volumetric conversion, this is equivalent to a count of 1670 × 4.4 or 7348 leucocytes per mm³ of stained film. Correcting for the observed loss of 8% of leucocytes during staining, the expected original average count of individuals in the series would have been (7348 × 108/100), or 7936 leucocytes per mm³.

This figure corresponds very closely to the figure quoted by Bruce-Chwatt (1958), and supported by haematologists working in Nigeria, of 8000 leucocytes per mm³ as a reliable average for the community. This difference of only 0.8% between observed and expected figures also forms a useful additional check on the accuracy of the thick-film factor.

The figures in Table 3 of average and range of leucocytes per field in the different age-groups can,

TABLE 12

CONVERSION OF AVERAGE LEUCOCYTE COUNTS PER FIELD IN 231 INDIVIDUALS TO COUNTS PER mm³ BY APPLICATION OF THICK-FILM FACTOR

Age-group (years)	Leucocyte count per field (observed)		Leucocyte count per mm³ (calculated)	
	Average	Range	Average	Range
0-11/12	16.1	10 - 23	7 651	4 750 - 10 930
1-4	17.6	8 - 43	8 364	3 800 - 20 430
5-14	16.4	10 - 40	7 793	4 750 - 19 010
15 and over	15.8	7 - 19	7 508	3 330 - 9 030
Total	16.7	7 - 43	7 936	3 330 - 20 430

by application of the thick-film factor, be converted into counts per mm³. The results are shown in Table 12 and it can be seen that both averages and ranges are within reasonable limits.

Comparison of parasite density index (Bruce-Chwatt) with index calculated on volumetric basis. The parasite density index (PDI), now widely employed, is that put forward by Bruce-Chwatt in 1958 and calculated on the basis of the parasite/leucocyte ratio. The conversion to count per mm³ is then carried out, using an assumed mean leucocyte count of 8000 per mm³. Such an index, to be reliable, must depend upon two factors:

(*a*) the correctness of the assumed average leucocyte count of 8000 per mm³

(*b*) a constant relationship between parasites and leucocytes throughout at least those parts of a thick film that are normally examined.

The accuracy of the assumed mean leucocyte count has been demonstrated in the previous paragraph. In order to check the second variable, a normal thick film was prepared from a proven infected individual and was examined in its entirety. using a squared eyepiece. Once again, only a small film was prepared, in order to reduce the work of counting. The numbers of leucocytes and parasites in each series of two rows examined with the squared eyepiece were recorded, so that a ratio of parasites to leucocytes could be easily calculated. It can be seen, from the results in Table 13, that there is a constant relationship throughout the whole film between leucocyte and parasite count, a feature that confirms the appropriateness of basing the parasite density index on the parasite/leucocyte count ratio.

TABLE 13

RESPECTIVE NUMBERS OF PARASITES AND LEUCOCYTES COUNTED IN SUCCESSIVE DOUBLE ROWS IN THE ENTIRETY OF ONE SMALL THICK FILM OF BLOOD

No. of rows examined	No. of leucocytes (L)	No. of parasites (P)	$\frac{P}{L}$
2 [a]	130	645	5.0
4	309	1 968	6.4
6	285	1 675	5.9
8	381	2 291	6.0
10	305	1 784	5.8
12	315	2 179	6.9
14	377	2 361	6.3
16	387	2 360	6.1
18	404	2 361	5.8
20	376	2 452	6.5
22	401	2 407	6.0
24	430	2 584	6.0
26	408	2 282	5.6
28	355	2 245	6.3
30	400	2 088	5.2
32	352	1 903	5.4
34	296	1 685	5.7
36	325	2 023	6.2
38 [a]	312	1 674	5.4
Total	6 548	38 967	6.0 ± 0.44

[a] The first and last groups contain an unspecified number of rows in the irregular tails of the film.

Throughout the series, careful parasite counts per 100 fields of thick film examined, together with individual average leucocyte counts per field, were recorded. By simple proportion, the parasite counts per 8000 leucocytes can be calculated from these figures for 126 positive cases, and thus the PDI computed for each age-group. Similarly, by applying the thick-film conversion factor, the parasite counts per 100 fields can be converted to a count per mm³, and therefore a PDI can be calculated on a volumetric basis. The results of a comparison of the two types of index are shown in Table 14.

TABLE 14
COMPARISON OF PARASITE DENSITY INDEX (PDI) WITH SIMILAR INDEX CALCULATED ON VOLUMETRIC BASIS

Age-group (years)	No. examined	PDI	PDI (volumetric)
0-11/12	8	6.4	6.0
1-4	70	5.5	5.4
5-14	30	4.3	4.3
15 and over	18	1.0	1.0
Total (all ages)	126	4.6	4.5

It will be observed that there is no significant difference between the indices, and all the indications in this study suggest that the currently used method of PDI calculation is completely adequate for computing the density of parasites in a community, within the limitations of thick-film examination.

Parasite loss during thick-film staining. Investigation has already shown that there is a regular loss of leucocytes during staining of the thick film, amounting in the present study to 8%. Attention has previously been drawn to the relatively poor efficiency of thick films in the diagnosis of scanty parasitaemia, and Muirhead-Thomson (1954) and others have described the phenomenon of infectivity of asymptomatic carriers to mosquitos even though the routine 100-field examination of thick films (and even longer-term examination in some cases) was negative for gametocytes of *P. falciparum*. Confirmatory evidence on this point was obtained by Dowling & Shute (unpublished). This poor performance of the routine thick film can only be explained by loss or obscuring of a significant proportion of parasites in the staining process. Such an occurrence would have no effect on the diagnosis of heavy parasitaemia but would affect the efficiency of the method where parasites were scanty. It would hardly be surprising if a proportion of the parasites was lost during staining, when one considers the upheaval

TABLE 15
COMPARISON OF CORRECTED TOTAL PARASITE COUNTS IN 126 POSITIVE THICK AND THIN FILMS, SHOWING VARIATION WITH DEGREE OF PARASITAEMIA

Degree of parasitaemia	Type of film	No. examined	Observed no. of parasites per film [a]	Corrected parasite counts per mm³	Ratio of average counts (thin : thick)
Low (less than 20 per 100 fields of thick film)	Thick	26	4.6	20	5.74
	Thin	26	7.4	115	
Medium (20-199 per 100 fields of thick film)	Thick	30	89	391	3.56
	Thin	30	89	1 390	
Heavy (200-999 per 100 fields of thick film)	Thick	37	526	2 314	2.63
	Thin	37	387	6 080	
Very heavy (1 000 and over per 100 fields of thick film)	Thick	33	1 686	7 417	2.45
	Thin	33	1 160	18 205	
Total (all classes of parasitaemia)	Thick	126	618	2 717	2.54
	Thin	126	440	6 911	

[a] Per 100 fields for thick films; per 1 000 fields for thin films.

that must be caused in a thick film by dehaemoglobinization.

In the current series, careful parasite counts per 100 fields in the thick film and per 1000 fields in the thin film were carried out in paired films from 126 positive cases. Application of the thick-film and thin-film conversion factors to the average counts would be expected to give corrected parasite counts per mm³ that were similar in both types of film. Table 15 shows, however, that the corrected parasite count for the thin-film series was, on the average, two-and-a-half times that for the thick films, with even greater differences for the low-grade parasitaemia.

As the thin films are fixed from the outset, it is reasonable to assume that there is no significant loss during staining. Study of the figures in Table 15 shows that, by comparison with the thin film, more than 60% of the total parasites are either obscured or are missing from the thick film. A loss of this magnitude during staining would account for the low sensitivity of the thick film in the diagnosis of scanty parasitaemia. It was considered possible that parasites might have been missed during the routine examinations of thick films in the series. As a further check, therefore, a series of 19 paired thick and thin films was taken at random from schoolchildren and subjected to very careful study. Whereas the thin films were examined for 1000 fields (a time factor was no longer applied), the thick films were examined for 300 fields, instead of the 100 employed during routine testing in the series. Table 16 shows the results of this comparative study. It should be stressed that examinations of the thick films were carried out with very great care, so that parasites would not be missed. In spite of this, it can be seen that the corrected counts show a 65% loss of parasites in the thick film as compared with the corresponding thin films. It is interesting to note that, in spite of the prolonged examination of the thick films, the positivity of the thin-film series was still the higher of the two.

Further evidence is provided by a study of *P. falciparum* gametocyte counts in the present series. Not only was the crescent rate over 41% higher in the thin films (1000 fields) than in the routine thick-film examinations (actual figures 36.5% and 25.8%), but individual crescent counts were also considerably higher in the thin films than would have been expected. For a series of 46 individuals for whom corresponding crescent counts were recorded in both thick- and thin-film examination, the distribution of crescent counts per film has been tabulated in Table 17.

TABLE 16

RESULTS OF COMPARATIVE STUDY OF LONG-TERM THICK- AND THIN-FILM EXAMINATIONS

No. of subject	Thick films: Parasite counts in 300 fields	Thin films: Parasite counts in 1 000 fields
1	18	4
2	117	17
3	39	8
4	55	21
5	92	23
6	Negative	Negative
7	Negative	1
8	68	30
9	Negative	Negative
10	197	48
11	Negative	Negative
12	27	5
13	9	6
14	127	36
15	Negative	Negative
16	2	7
17	Negative	1
18	19	3
19	27	5
Parasite rate	68.4 %	78.9 %
Average parasite count per positive case	20.4 per 100 fields	16.4 per 1 000 fields[a]
Average parasite count (applying conversion factors)	89.8 per mm³ (65 % loss)	257.5 per mm³
No. of films with:		
P. malariae	3	5
P. ovale	0	1

[a] Excluding No. 7 and 17, for which comparison is impossible.

These figures, when corrected by means of the conversion factors, point to a very considerable loss of crescents during staining, amounting to 86%. This would mean that an actual count of 40 crescents per mm³ of blood would only be expected to yield,

TABLE 17
DISTRIBUTION OF CRESCENT COUNTS IN 46 INDIVIDUALS POSITIVE BOTH IN THICK- AND THIN-FILM EXAMINATIONS

No. of crescents counted	No. of films containing specified no. of crescents	
	Thick films (per 100 fields)	Thin films (per 1 000 fields)
1	21	15
2	10	7
3	6	2
4	3	4
5	4	2
6	1	5
7	1	2
8	0	3
9	0	1
10	0	1
13	0	1
14	0	2
20	0	1
Total crescents counted	104	209
Mean crescents per film	2.26	4.54
Corrected count per mm³	9.9	71.3

on average, a single crescent in 100 fields of the stained thick film. This would help to explain the phenomenon of infective feeds from " negative " carriers, since an average mosquito feed is about 1 mm³ of blood.

In view of the importance in epidemiological practice of this loss of crescents, two further counter-checks were made. In the first, six pairs of thick and thin films were taken from a proven heavy gametocyte carrier. In all, 1800 fields of thick-film examination were compared with 3000 fields of thin-film examination divided equally between the 12 films. The corrected figures (3851 and 909 gametocytes per mm³, respectively) showed a rather smaller loss (77%) than that demonstrated in Table 17.

The second countercheck produced an even more striking result. Six paired films were selected from the original series in which a single *P. falciparum* gametocyte had been found in the 1000-field examination of thin films and none in the corresponding thick films. The thick films were then re-examined for a 30-minute period each and crescent counts were recorded. Table 18 shows the result of this investigation.

As examination of the thick film over 30 minutes covered the greater part of the film, the average thickness, and not the " best part " thickness, has been taken in calculating the quantity of blood examined. It can be seen that in the thin films six crescents were found in a total of 0.38 mm³ of blood, whereas in the thick films only eight crescents were found in 9.6 mm³ of blood. The loss in crescents as demonstrated by this very careful search is of an even higher order than before, and is in the region of 94%.

From these various checks it is evident that many crescents are either lost or obscured during staining. It is difficult to see how they could be obscured or insufficiently stained, as at least the characteristic pigment would show and the two counterchecks

FIG. 4
RELATIONSHIP BETWEEN RATIOS OF CORRECTED PARASITE COUNTS (THIN FILM/THICK FILM) AT DIFFERENT PARASITE DENSITIES

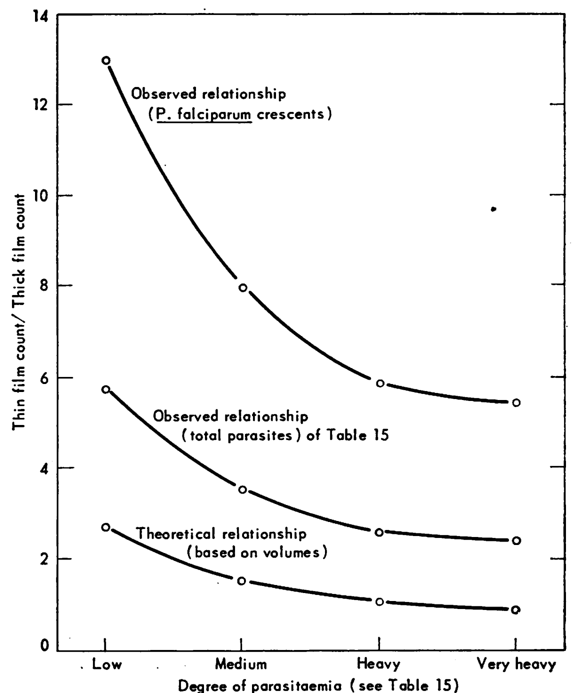


TABLE 18
RESULTS OF DETAILED SEARCH FOR CRESCENTS IN THICK FILMS ORIGINALLY NEGATIVE IN ROUTINE 100-FIELD EXAMINATIONS

Series	No. of subject	Results of 1 000 - field examinations			
		Thin films (0.064 mm³)		Thick films (1.6 mm³) [a]	
		No. of crescents	Time found (min)	No. of crescents	Time found (min)
Ibeshe 14/10	4	1	16	1	23
	15	1	26	1	29
	26	1	11	0	
Baiyeku 23/10	2	1	27	2	10, 13
	9	1	10	3	5, 16, 27
	15	1	30	1	20
Total crescents		6 (in 0.38 mm³)		8 (in 9.6 mm³)	
Equiv. crescent count (per mm³)		15.7		0.8	
Apparent loss in thick films [b]		—		94.7 %	

[a] *Average* thickness of film has been taken, as examination for 30 min is distributed all over film and not limited to the " best part ".

[b] Compared with value for thin films. If results are based on time of examination taken to find crescents, six were found in 120 min (0.26 mm³) in the thin films and eight in 142 min (7.6 mm³) in the thick films, again showing a heavy loss (95.5 %).

were carried out in the thick film with considerable care. In a specific search for crescents, in which other species or stages are ignored, it is not easy to see how so prominent a parasite could have been missed by an experienced observer.

The theoretical relationship, based on volume of blood examined, between thick- and thin-film parasite counts, is shown in Fig. 4. The observed relationships for total parasites and for *P. falciparum* gametocytes are also shown. The difference in efficiency of a thick film from its theoretical value as a result of the staining process is thus clearly demonstrated. It should again be stressed that all films were stained 24 hours after collection. The effect of the time factor between collection and staining on the loss of parasites in thick films is currently under study.

DISCUSSION

In previous work, mentioned in the introductory paragraph, we concluded that routine thick-film examination is relatively inefficient in the diagnosis of scanty parasitaemia. The results of the present investigation suggest that a high proportion of parasites is lost during staining. In a heavy infection this loss is of little importance, but in cases of low parasitaemia, common in African conditions and liable to become even more common in the later stages of malaria-eradication programmes, the loss could be of greater significance. In such cases the discovery of a parasite in the three-minute thick-film examination is made purely by chance. Although parasites are distributed fairly evenly throughout the blood (see Table 13), in very scanty positives the chances of finding a parasite are further diminished by the phenomenon of " grouping ". For subject No. 1 in Table 16, for example, four *P. falciparum* trophozoites were found in the last 120-fields' examination of the thin film. In the first 880 fields, careful search revealed no single parasite. Similarly, for No. 19 five rings were found in the first 135 fields, whereas the remaining 865 fields examined were negative. In a thin film this grouping covers a rather wider area, owing to the very thin film of blood. In a thick film, however, it accounts for the frequent occurrence of several parasites within one or two consecutive fields, surrounded by large numbers of negative fields.

The importance of this loss of parasites in the staining of thick films is considerable in epidemiological practice. Cases with scanty parasitaemia could be missed even if the routine examination of the thick smear were extended from three to 10 minutes. The marked loss of *P. falciparum* gametocytes may account for the reported infectivity of " negative " carriers in whom the routine examination failed to demonstrate the presence of a crescent. In fact, it is likely that a crescent count of as high as 20-30 per mm^3 could be missed in the normal thick-film examination. In view of the implications, it is clearly desirable that the conclusions drawn as a result of the present study be verified by other workers.

Clearly, all other things being equal, the larger the quantity of blood examined the greater is the likelihood of finding parasites. Fig. 4 shows the relationship between the routine examination of the theoretically perfect thick film and the long-term examination of the thin film. If no parasites were lost in staining, the microscopist would have two chances in three of finding a parasite count as low as one per mm^3 in a 10-minute examination of a thick film. To obtain the same results, a thin film would need to be examined for five-and-a-half hours, so this method is out of the question for very scanty parasitaemia. With the present staining technique, however, an examination time of 30 minutes with the thick smear would be required to give the same result (except in the case of crescents, which would demand a far longer examination, about one-and-a-half hours).

The thick film, with a better staining technique that retained the parasites, would therefore still be the most valuable tool in the diagnosis of scanty parasitaemia. The use of a concentration method, such as that described by Worth (1964), could be expected perhaps to demonstrate even lower parasitaemia than one per mm^3, but this method also would lose some of its advantage if there were a similar proportional loss of parasites in staining.

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RÉSUMÉ

Dans une étude antérieure, les auteurs ont attiré l'attention sur les inconvénients de l'examen courant des étalements épais pour déceler les parasitémies légères du paludisme. Ils ont également fait observer que la qualité des résultats obtenus par l'examen des étalements minces prélevés sur les mêmes sujets au même moment dépassait de beaucoup les prévisions faites compte tenu de la plus faible quantité de sang utilisée. Le présent travail visait à déterminer les raisons de l'infériorité apparente de l'étalement épais et de l'efficacité relativement supérieure de l'étalement mince.

Des paires de gouttes épaisses et d'étalements minces ont été prélevées sur un total de 233 sujets appartenant à différents groupes d'âge, et colorées au Giemsa suivant la technique habituelle. Les résultats de l'examen courant (100 champs) des étalements épais ont été ensuite comparés à ceux de l'examen différé (1000 champs) des étalements minces en tenant compte spécialement de la numération parasitaire, de la positivité et du diagnostic d'espèce. Pour rendre la comparaison valable, il était nécessaire de procéder à une évaluation quantitative de la surface moyenne et du volume de chaque type d'étalement. Cette étude a permis aux auteurs de calculer deux facteurs de conversion des numérations parasitaires par 1000 champs (étalements minces) et par 100 champs (étalements épais) en numérations par millimètre cube. L'application de ces facteurs aux résultats de la série d'examens a mis en évidence la nette supériorité de l'étalement mince sur l'étalement épais, à volumes de sang égaux. Par rapport à l'étalement mince, qui est fixé immédiatement, on constate qu'une proportion relativement forte de parasites (variant, selon l'espèce et le stade de développement, de 60 à 90%) disparaît pendant le processus de deshémoglobinisation et la coloration de l'étalement épais.

L'importance épidémiologique de cette constatation est discutée. Elle aiderait à expliquer certains cas d'infection d'anophélinés par des sujets chez lesquels les étalements sanguins sont en apparence négatifs; elle indiquerait d'autre part la nécessité urgente de mettre au point une méthode plus sensible de détection des parasitémies légères. L'utilisation de l'étalement mince n'est

pas une solution satisfaisante, en raison de la très petite quantité de sang examinée par unité de surface et du temps considérable que requiert la recherche des parasites. Les auteurs estiment qu'une amélioration de la technique de coloration ou l'adaptation aux conditions de travail sur le terrain de la méthode de concentration du sang permettraient un diagnostic plus efficace des parasitémies légères qui, déjà fréquentes en Afrique, se rencontreront sans doute plus souvent encore au cours des dernières phases des programmes d'éradication du paludisme.

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