Pathological Section.

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Dr. R. T. HEWLETT, President of the Section, in the Chair.

An Improved Method for Opsonic Index Estimations, involving the Separation of Red and White Human Blood Corpuscles.[1]

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THE opsonic index was introduced in 1905-06 by Sir A. E. Wright. It was for a few years extensively practised, but has fallen largely into disuse owing to its unreliability, which was realized by the profession, who were doubtless much influenced by the criticisms published by Mr. Greenwood and Dr. White, Dr. Hort, and others. In view of the value that an accurate method would possess in clinical work it seemed well worth considerable study, if the large liability to error could be removed, or at least reduced.

Opsonin is the name given by Sir A. E. Wright to the substance in human blood which is understood to cause the ingestion of bacteria by the leucocytes, and since this varies quantitatively in health or disease it is important that we should be able to measure its fluctuations in any patient's blood, its increase favouring recovery from the infection, and its deficiency assisting the disease.

In the opsonic test or estimation, leucocytes are set to ingest bacteria when mixed and incubated with normal serum, and the same operation is conducted with a diseased serum under similar conditions. In stained

[1] The main features of this work were conveyed to the Royal Society in a paper read March 14, 1912.

films the bacteria visible in, say, fifty of such leucocytes are counted in each case and the figures so obtained are contrasted. This ratio is used as a measure of the amount of opsonin in the two specimens of blood serum. The healthy serum is taken as the normal or control, and the diseased may of course be found to contain more or less, or the same—i.e., a normal amount of opsonin to the bacteria used, which may be any pathogenic organism.

From variations in the opsonic index of the serum of patients suspected of tuberculosis numerous diagnoses of tuberculosis have been made, and infection by certain other bacteria has also been presumed by opsonic comparisons on similar lines. Since opsonin has not been separated from the blood and we have no colour or simple test for its presence, the only practical method of its measurement at present available is this opsonic test or index.

Dr. Hort took a large sample of a patient's blood, divided it into several smaller volumes, disguised the identity of the specimens and obtained the opsonic index to the tubercle bacillus from several different operators simultaneously, at different pathological laboratories. These operators also measured the opsonic index of two sera which they did not know were identical. The results were discordant, and may be reviewed by reference to his paper.

Mr. Greenwood and Dr. White studied the mathematical liability to error arising from the method of counting the bacteria in a random sample of the leucocytes, instead of counting those in all of the leucocytes employed in each mixture (the accurate, but impractical course, on account of the labour involved). Since my work has followed the line of weakness indicated by him, it is necessary to give the important points of his work. If, for example, in any count 200 bacteria have been seen in the fifty leucocytes, it must be remembered that each leucocyte does not contain four bacteria, but their content is any number from 0 to about 25 or more, a series of numbers being added. Now, when a film is made in which the leucocytes contain all these varying numbers of bacteria, there is a great liability that the blanks (0 contents) and high contents (gluttons) are distributed unevenly in the film. Therefore, according as the operator begins counting in part of the field rich in one type of leucocytes or the other, the figure he will obtain will be a low or high one; the most significant point.

In their first communication Mr. Greenwood and Dr. White (R^1) concluded from a study of films prepared in Sir A. E. Wright's laboratory that the distribution of leucocytes (arranged with respect

to the number of ingested tubercle bacilli) is highly asymmetrical and surmised that the variations from the mean value, exhibited by samples of fifty or twenty-five, of the whole "population" of cells would be considerable. In the second paper (R) they analysed a count of no fewer than 20,000 leucocytes upon films prepared and counted by Dr. White. After elaborate mathematical analysis they concluded that a serum having precisely the same opsonic content as the standard might be expected once in seven trials to yield an index deviating from unity by as much as 33 per cent. in either direction. From this and further calculations they concluded that even in the case of samples of 100 it is doubtful whether single determinations which give values even beyond the limits of 1·3 to 0·7 can be regarded as satisfactory evidence of differentiation.

In the experiments I am about to detail, I had the valuable assistance of Dr. Cavendish Fletcher far into the exploratory stage, but unfortunately he had to abandon the work. The first idea was not directly associated with the error emphasized by Mr. Greenwood. It may be expressed as follows:—

Since the leucocytes act in the opsonic mixture as bacterial magnets, then if in the two mixtures there were very different numbers of them at work, this difference might cause a variation in the degree of phagocytosis in the two cases, apart from variations in opsonin. To test this point, the opsonic estimation was made of a sample of normal serum to the *Staphylococcus aureus*, and at the moment of withdrawal of the mixture from incubation part was withdrawn in a corpuscle-counting pipette, the remainder formed the films from which the ordinary opsonic figure was obtained by one worker and the other meanwhile counted the leucocytes which had been at work. This proceeding was repeated several times at a sitting with identical materials, and from four such sittings the results shown in Table A were obtained.

From this table the following points are apparent:—

(1) There was a considerable variation in the number of leucocytes obtained by successive dips into the vessel containing the "washed" corpuscles.

(2) This variation showed no definite association with a high or low figure obtained by the corresponding opsonic count.

(3) The opsonic figure obtained was liable to marked fluctuation, though it should have been constant, since all the materials were identical and the operator the same.

(4) There appeared to be the all-important constancy of the opsonic

figure when the numbers of blanks met during a fifty count was the same or nearly so.

TABLE A.—OPSONIC INDEX (OLD METHOD).

Repeated estimation of the opsonin to *Staphylococcus aureus* in normal serum and simultaneous enumeration of the leucocytes per cubic millimetre in each opsonic mixture.

Series	Leucocytes per cubic millimetre	Opsonic count per 50 leucocytes		Error *above* average	Error *below* average	Mean value	Blanks per 50 leucocytes	Worst error *above* average	Worst error *below* average
				Per cent.	Per cent.			Per cent.	Per cent.
1	1 15 × 200	279	$\frac{26}{1}$	—	4	292	9	—	—
	2 9 ,,	337		15	—		9	15	—
	3 14 ,,	285		—	2		7	—	—
	4 11 ,,	268		—	8		6	—	8
2	1 18 × 200	190	$\frac{16}{1}$	11	—	170	10	—	—
	2 16 ,,	258		52	—		3	52	—
	3 19 ,,	118		—	30		20	—	—
	4 10 ,,	114		—	33		20	—	33
3	1 14 × 200	185	$\frac{23}{1}$	8	—	170	16	—	—
	2 9 ,,	155		—	8		14	—	—
	3 6 ,,	213		25	—		13	25	—
	4 16 ,,	137		—	19		21	—	19
	5 36 ,,	180		5	—		13	—	—
	6 12 ,,	155		—	8		14	—	—
4	1 12 × 200	151	$\frac{15}{1}$	—	20	190	11	—	20
	2 7 ,,	191		—	—		7	—	—
	3 25 ,,	194		—	—		8	—	—
	4 10 ,,	224		17	—		4	17	—
		Corrected total		133	132				

Average error above or below the mean value ... $\frac{265}{18} = 14{\cdot}7$ per cent.

Worst error above ... ... 52 per cent.

Worst error below ... ... 33 ,,

All the materials were identical in each series, and the opsonic estimations by the same operator, and the observations made at about half-hour intervals.

The numerator = the highest bacterial content of any leucocyte in the series.
The denominator = the number of times it occurred in the series.

This last feature suggested that this large content variation might be due to uneven access between leucocytes and bacteria. However, the outstanding fault of the process in this large variation in the leucocyte content and its occurrence could only be due to difference of (1) *Appetite* or (2) *Opportunity*, or a combination of these factors.

(1) *Appetite.*—If the leucocytes have equally as good chances to pick up bacteria in the opsonic mixture, and yet show this variation, it must be presumed physiological, and there is no remedy.

(2) *Opportunity.*—It may be that all the leucocytes have similar appetites, but get very different opportunities to pick up bacteria owing to uneven distribution of the mixture.

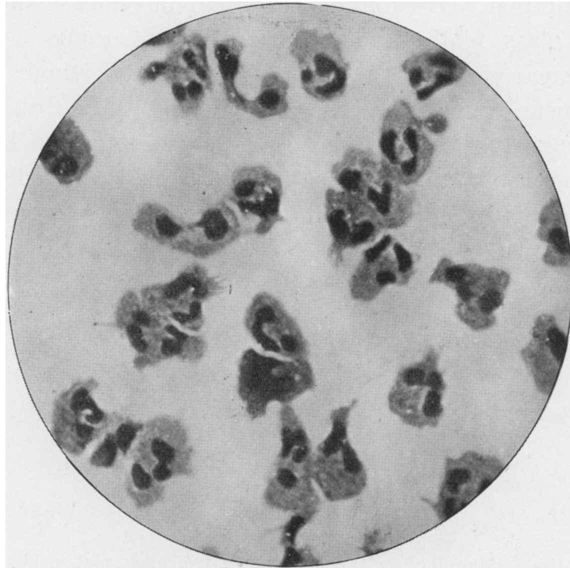

FIG. 1.

The polynuclear human leucocytes as seen on the plates of the "Ponder" cell, after washing the plate free of red corpuscles by saline. ($\frac{1}{6}$ obj.)

A scrutiny of the materials used in the old method showed two important defects:—

The Presence of Red Corpuscles.—Although white corpuscles only are concerned in the process, both red and white blood corpuscles are used; but since a bacterial suspension not exceeding 500 mm. per cubic centimetre is used, and washed blood corpuscles contain 5,000 mm. red, and 10 mm. white corpuscles per cubic centimetre, it is evident that for every leucocyte there are fifty bacteria provided; but surrounding this all-important leucocyte are 500 obstructing and useless red corpuscles. I therefore decided to abolish the red corpuscles, and this involved the *separation of red and white human blood corpuscles.*

The methods which were tried unsuccessfully included:—

Hæmolysis of the red corpuscles.

Agglutination by ferric chloride and filtration of the red groups.

Filtration of decalcified blood.

Sedimentation of decalcified blood after artificially raising the specific gravity.

However, Dr. Ponder's work on leucocytes furnished the nucleus of a successful method. He found that when blood is enclosed in a cell between two glass plates and incubated, there occurs a swarming of polynuclear leucocytes to the glass surfaces, to which they adhere firmly and appear remarkably distorted (fig. 1). My first problem was to get these leucocytes off the glass. This was found to be effected by citrate saline solution 1·5 per cent., 0·8 per cent. by di-sodium hydrogen phosphate, and by hypertonic salt solution and also by serum. After

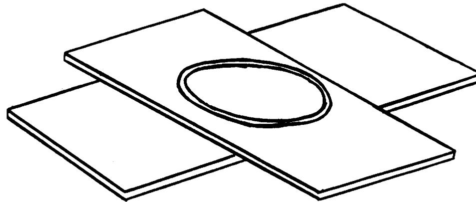

FIG. 2.

The modified "Ponder" cell, consisting of two glass plates, 1½ in. by 3 in., enclosing a cell, bounded by an india-rubber ring—the latter being filled with freshly shed human blood, and of nearly 1 in. inside diameter.

obtaining a large number in a test-tube in the citrate solution (to make films), I found they were highly unstable osmotically, and when transferred to normal saline the majority burst.

After experimenting with over 200 Ponder plates I realized that the leucocytes could only be obtained in bulk if favourable chemical conditions were ascertained (since incubation aggravated the bursting). The following method succeeded in supplying a majority of polynuclear leucocytes (the lymphocytes do not appear on the plates) in good condition, which could be incubated for fifteen minutes as in the opsonic index process. The detailed method is as follows:—

Blood is shed into a rubber ring (cell) sandwiched between two glass plates (fig. 2). This cell is incubated for twenty minutes at 37·4° C., removed from incubation, the cell is opened, the clot and ring removed,

and the plates washed with 1·25 per cent. saline to free them from red corpuscles and serum. After wiping the ring margin clear of more red corpuscles and dried serum, a few drops of cold NaCl 1·25 per cent. are poured on the leucocyte-laden area of each plate. These are replaced on the metal shelf of the incubator for fifteen minutes; when the plates are inspected, the previously distorted and stretched out polynuclears will be seen under a low objective to have become almost spherical and loose from the plate. By means of a long glass rod the fluid and floating leucocytes are swept into a small tube and concentrated by the centrifuge at moderate speed. After syphoning off the supernatant

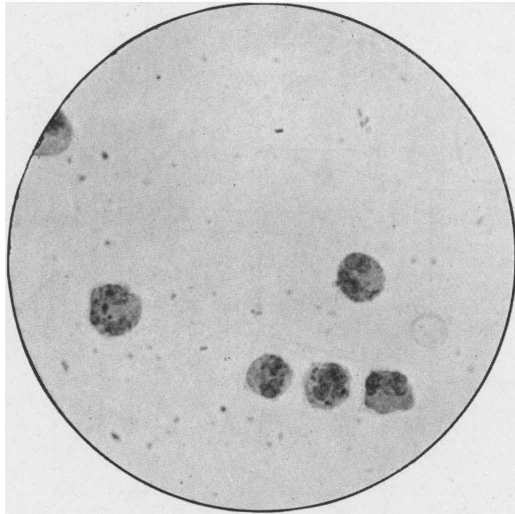

FIG. 3.

Human leucocytes (via "Ponder" plates) freed from red corpuscles, showing ingested staphylococci in opsonic process. ($\frac{1}{12}$ obj.)

fluid a very large number of human polynuclear leucocytes were obtained, 50 per cent. of which stand incubation with equal volumes of serum and the bacterial emulsion, the latter being made with 1·25 per cent. NaCl instead of normal saline (fig. 3).[1]

[1] Since half of the corpuscles survive, those destroyed are potential obstacles to mixing and even phagocytosis. But the ratio is now one obstacle to one leucocyte, instead of five hundred to one when using ordinary blood. It is possible to get a somewhat higher percentage of good leucocytes by other means (detailed elsewhere), but since the technique is more laborious I use the simpler method.

But before proceeding to test the opsonic process for the anticipated increased accuracy, the second defect of opportunity in the *old* method had to be attended to. In the *old* method the opsonic mixture (serum, bacteria, and washed blood corpuscles) was incubated for fifteen minutes, but even at the end of ten minutes the bulk of the corpuscles had settled to the bottom of the glass pipette, the supernatant fluid being clear. Since equality of opportunity cannot exist when this is permitted, this defect was remedied by keeping the mixture in slow rotary motion during incubation by means of the opsonic mill (fig. 4).

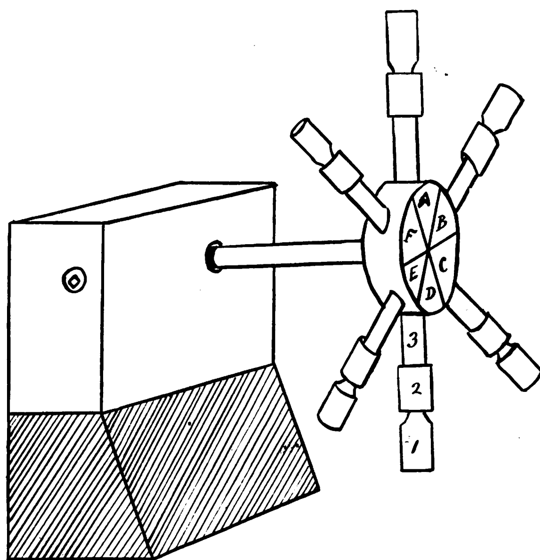

FIG. 4.

The Opsonic Mill. This clockwork-driven instrument, standing in the incubator, rotates the opsonic mixture and prevents sedimentation of the corpuscles. (Speed of rotation = one minute forty-five seconds per revolution.) 1, the shortened glass pipette; 2, india-rubber collar; 3, copper tube packed with fine copper wires.

A mechanism to prevent sedimentation of the opsonic mixture was devised by Rosenow (1906), and by Glynn and Cox (1912). The latter used a mechanism which rotated the pipette on its long axis in a horizontal plane, and their experiments showed no reduction of the error by its use when tested. Only a small benefit is to be expected from such an improvement, but it is probably inappreciable when the entire experimental error may be large, as in the old method. More-

over, their method of rotation is not ideal; for though sedimentation is prevented, there is no active mixing (from end to end of the pipette) induced by such a roller movement. In the device illustrated in fig. 4, not only does no settling occur, but experiments showed that the corpuscles pass up and down in the opsonic fluid during the changing positions of the pipette in the slowly moving wheel.

After a few trial experiments with the new materials I proceeded to test the opsonic index of the same serum repeatedly as in the experiments recorded (Table A), to ascertain whether the more even access and mixture of bacteria and leucocytes now improved the experimental error. The results are shown in Table B.

TABLE B.—OPSONIC INDEX (NEW METHOD).

Repeated estimation of the opsonin to *Staphylococcus aureus* in normal serum, using leucocytes only and the opsonic "mill." The series number indicates a set of four or six observations made at a sitting with the same set of materials.

Series	Opsonic count per 50 leucocytes		Error *above* average	Error *below* average	Mean value	Blanks	Worst error *above* average	Worst error *below* average
			Per cent.	Per cent.			Per cent.	Per cent.
1 Very thin bacterial emulsion	61		16	—		27	—	16
	72		—	—		17	—	—
	76	$\frac{7}{1}$	—	5	72	21	—	—
	79		—	9		19	—	—
	67		6	—		20	—	—
	84		—	15		14	15	—
2 Thin bacterial emulsion	117		—	13		13	13	—
	103		—	—		13	—	—
	114	$\frac{12}{2}$	—	11	103	12	—	—
	87		15	—		11	—	15
	91		11	—		10	—	—
	108		—	4		18	—	—
3 Medium bacterial emulsion	130		6	—		6	—	6
	147	$\frac{10}{1}$	—	5	139	9	5	—
	135		2	—		11	—	—
	144		—	3		8	—	—
4 Thick bacterial emulsion	160		10	—		4	—	10
	195	$\frac{14}{1}$	—	9	178	3	—	—
	197		—	10		6	—	—
	163		8	—		7	10	—
			74	84				

Average error above or below the mean ... $\frac{158}{20} = 7{\cdot}9$ per cent.

Worst error *above* the mean value ... ... 15 per cent.

Worst error *below* the mean value ... ... 16 ,,

The results recorded in this Table (B) show a marked reduction of the average and maximum error from the mean value, and this had occurred in spite of a fairly wide variation in the strength of the bacterial emulsion used. Believing that still higher accuracy might be obtained, I undertook a further series of tests, matching the emulsion used in Experiment 3, Table B (which had been fixed by heat) as a standard. I also decided to count 100 leucocytes, fifty from each of the two films made from the mixture. The results are shown in Table C and show a still higher level of accuracy than those of Table B, though visual matching of the emulsions (to produce an average phagocytosis of three bacteria per leucocyte) was not very successful.

TABLE C.—OPSONIC INDEX (NEW METHOD).

Repeated estimation of opsonin in normal serum to the *Staphylococcus aureus*, using leucocytes only, and the opsonic mill, and counting 100 leucocyte contents.

Series	50 leucocytes. Film I	50 leucocytes. Film II	Opsonic count	Mean value	Error from mean value +	Error from mean value —	Blanks per 100 leucocytes	Maximum deviation +	Maximum deviation —
					Per cent.	Per cent.		Per cent.	Per cent.
1	144	105	249	256	—	2	18	—	2
	127	144	271 $\frac{10}{2}$		6	—	18	6	—
	107	146	253		—	1	16	—	—
	131	119	250		—	2	20	—	—
2	142	178	320	324	—	1	10	—	—
	170	159	329 $\frac{14}{1}$		1	—	15	—	—
	150	160	310		—	3	16	—	3
	176	162	338		4	—	8	4	—
3	127	120	247	252	—	2	49	—	—
	128	103	231 $\frac{14}{1}$		—	8	40	—	8
	144	113	257		2	—	32	—	—
	144	132	276		9	—	40	9	—
4	99	112	211	230	—	8	25	—	8
	114	144	258 $\frac{9}{4}$		12	—	19	12	—
	121	109	230		—	—	23	—	—
	96	125	221		—	3	25	—	—
					34	30			

Average error above or below the mean value ... $\frac{64}{16}$ = 4 per cent.

Worst error *above* ... ... 12 per cent.

Worst error *below* ... ... 8 ,,

There was defective emulsification of the staphylococci in Series 3, evident in the "clumpy" films, but fortunately the entire results of the table can afford the handicap.

All the films of Tables B and C have been preserved.

SUMMARY.

The improvements described have produced :—

(1) A striking reduction in the liability to error of opsonic estimations when repeatedly tested.

(2) The results recorded by the *new* method (Table C) showed a liability to error of about one-quarter the magnitude of those recorded in Table A (*old* method), the conditions of experiment being almost comparable.

(3) The enhanced accuracy is associated with a much reduced range of microbic content of the leucocytes (0—14).

(4) The improved results are attributable to the more even distribution of bacteria amongst the leucocytes (by the removal of the red corpuscles) and by its maintenance during incubation in the opsonic mill.

(5) No observations were made of any variations in opsonin in health or pathological states.

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