which, when the automatic apparatus is used, is valid.

A more serious difficulty is that of standardization and is not peculiar to Pearson's method; it is solved by the use of a commercial standard serum of the Versatol type. Only comparison with a standard serum of good quality is valid (Rivin *et al.*, 1958), for artificial titrated solutions never have exactly the same degree of humidity as the serum and the slightest difference in the quantity of water causes enormous differences in the intensity of Liebermann's or Salkowski's reactions.

Since the time of the circuit is rigorously constant, it has been possible to study the influence of continual variations in temperature (Fig. 2). A temperature of 65°C. appears best because between 56°C. and 72°C. variations have very little effect on the development of the colour; thus even a small variation in the thermostat does not produce appreciable errors (Fig. 3). Furthermore, at this temperature the levels of the colour for free cholesterol and for esterified cholesterol are identical (Girard and Assous, 1962).

SUMMARY

The use of an automatic apparatus with a strictly constant time for the reaction and the mixing processes eliminates the errors observed in the manual methods.

An installation is proposed in which the temperature is fixed in such a way that reproducibility is good such as was never achieved manually with series including many samples.

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Separation of human lymphocytes and monocytes using an 'oil bottle'[1]

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Several methods of separating lymphocytes and monocytes based on specific gravity differences between lymphocytes and other types of cells have been described (Kline, 1955; Ottesen, 1954; Seal, 1959; Tullis, 1952; Ventzke, Perry, and Crepaldi, 1959). The viability of the isolated cells has usually been tested by indirect methods (Jago, 1956; Lalezari, 1962; Lapin, Horonick, and Lapin, 1958). The technique to be described is simple, requiring only one centrifugation; it is reliable, and uses equipment which can be readily purchased. No haemolytic agents are used to eliminate the red blood cells. The viability of the cells obtained using this technique has been directly assayed by the ability of these cells to proliferate in subcutaneously placed micro-diffusion chambers in human subjects.

METHOD

All glass surfaces were siliconized and all equipment and reagents were sterilized. Sixty to 80 ml. of blood was collected with a plastic tube[2] and led directly into a 100 ml. graduated flask containing 20 ml. of 6% dextran in isotonic saline (M.W. 188,000)[3] and 1 ml. of 10% E.D.T.A. The flask was stoppered and inverted gently eight times. The erythrocytes were allowed to settle by gravity for 20 minutes at room temperature. The supernatant plasma was placed in a 100 ml. 'oil bottle' with a 0·5 ml. stem 4 cm. long, with a diameter of 3·8 mm. (Corning Glass Co., Corning, N.Y.). The 'oil bottle' was then spun at 2,500 r.p.m. for 30 minutes in a no. 2 international centrifuge. To hold the 'oil bottle' special cups (international no. 395) were used.

All the cellular elements of the plasma were now layered in the narrow dependent stem of the 'oil bottle' with the platelets uppermost, followed by the lymphocyte-monocyte layer, then the polymorphonuclear cell layer, and finally a button of red cells at the bottom. The line of demarcation between these layers was easily seen (Fig. 1). The supernatant plasma was poured off. The thick platelet layer was removed using a Pasteur disposable pipette[4] and a rubber bulb.

To remove the lymphocyte-monocyte layer a narrow glass pipette 10 cm. long was fashioned from the long stem of a disposable pipette. The tip of this pipette should

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[2]Blood Collection Set no. 36, Abbott Labs., Chicago, Ill.

[3]Pharmachem Corp., Bethlehem, Pa.

[4]Transpets, Clay-Adams, New York City

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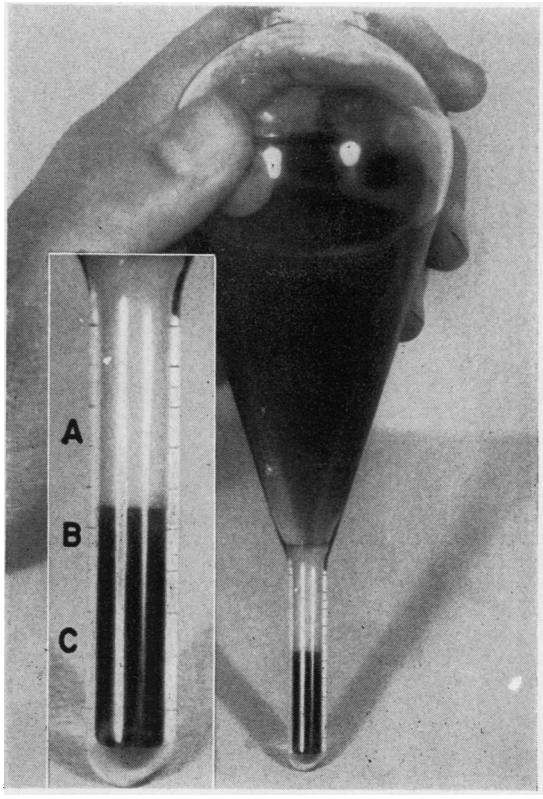

FIG. 1

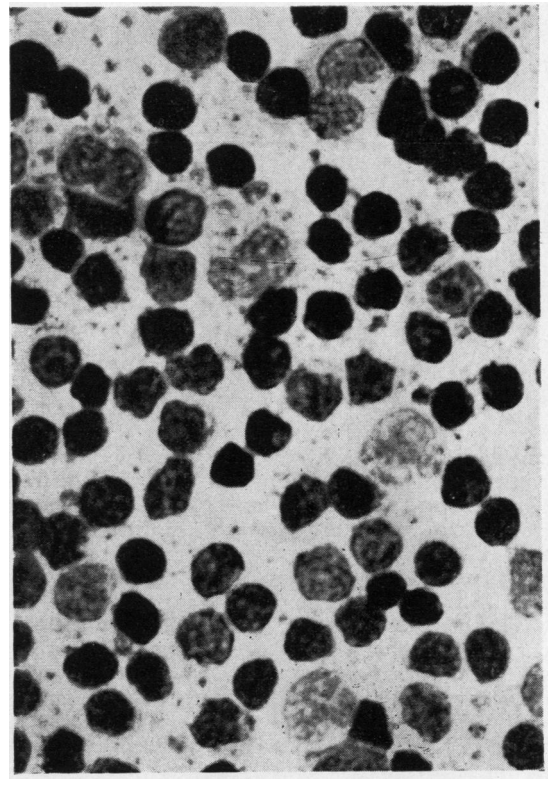

FIG. 2

FIG. 1. *Appearance of 'oil bottle' after centrifugation. Cellular elements are layered in narrow dependent portion of tube. A = platelet layer, B = lymphocyte-monocyte layer, C = polymorphonuclear leucocyte and red cell layer.*
FIG. 2. *Giemsa-stained smear of lymphocyte-monocyte layer from patient no. 4.*

be about 0·5 mm. in diameter. It was then connected to a mouthpiece by a short glass adapter and a piece of polyethylene tubing. The fine glass pipette was guided into the narrow stem of the 'oil bottle' and then the narrow tip of the pipette was placed just within the lymphocyte-monocyte layer. Gentle mouth suction was applied to draw the lymphocytes and monocytes into the pipette.

Aliquots of the lymphocyte suspension were spread on glass slides and stained with Giemsa stain. Differential counts were performed on these preparations. Staining of the cells with trypan blue was performed according to the method of Pappenheimer (1917).

To test viability directly, lymphocytes, both of autologous or homologous derivation, were sealed in an Algire type micro-diffusion chamber constructed of Millipore filter type HA (0·45μ pore size) and lucite. Chambers, each containing about 10^6 lymphocytes, were placed subcutaneously into 32 patients. These chambers were removed two to four weeks later and stained with Giemsa and Feulgen stains.

RESULTS

Table 1 shows the result of 10 consecutive lymphocyte separations in people with normal total white blood counts and differentials.

The degree of purity of the lymphocyte-monocyte fraction ranged from 99·5% to 91·0%. The number of red cells present per 100 white blood cells ranged from zero to 8. The morphology of the cells obtained with this technique was normal as judged by Giemsa staining (Fig. 2). Trypan blue staining showed 0% to 3% of the separated lymphocytes to be stained.

These lymphocyte-monocyte fractions placed in diffusion chambers were transformed into fibroblasts and giant cells, indicating that these cells were viable. The growth of homologous lymphocytes was no different than that of autologous lymphocytes. The persistence of the cellular homograft was proven by the presence of the female chromatin marker in the nuclei of female cells growing in diffusion chambers implanted into male patients.

TABLE I

RESULTS OF 10 LYMPHOCYTE SEPARATIONS

Case No.	Total W.B.C. (per c.mm.)	% Lymphocytes and Monocytes in Differential Count	Differential after Separation per 200 Cells		No. R.B.C. per 200 W.B.C.
			Lymphocytes + Monocytes	Polymorphs	
1	6,100	23	199	1	None
2	10,950	27	193	7	None
3	7,250	32	182	18	2
4	5,600	46	194	6	3
5	7,050	43	199	1	6
6	5,450	40	198	2	16
7	7,000	34	195	5	4
8	7,000	27	198	2	6
9	8,000	32	199	1	2
10	6,000	19	199	1	6

DISCUSSION

The oil bottle is used commercially to measure the amount of sediment in oil. The advantage of the oil bottle for collecting human lymphocyte-monocyte fractions is that in one step it concentrates the cellular elements from a large volume of plasma into a narrow tube, and thus circumvents the need for multiple centrifugations and pooling of lymphocytes from several ordinary test tubes. Also the calculation necessary for various types of 'middle constricted' bottles is not needed. Because only one centrifugation is required, the time between the collection of blood and the final collection of lymphocytes is under one hour. The viability of cells collected in this way is demonstrated by their ability to proliferate in subcutaneously placed micro-diffusion chambers. Since the minimal numbers of cells necessary to 'seed' a diffusion chamber is not known, the percentage of the viable cells present cannot be determined using this method. However, trypan blue staining indicated a high percentage of viable cells. This technique should also be suitable for finding tumour cells in peripheral blood, since tumour cells and lymphocytes are usually found in the same fraction; large numbers of pure platelets or polymorphonuclear leucocyte fractions may also be harvested using this technique.

SUMMARY

Attention is called to a commercially available 'oil bottle', well suited for the purpose of separating lymphocytes and monocytes and other cell fractions from human blood. A technique utilizing this bottle has been found to be rapid, simple, and reliable; viable lymphocyte-monocyte fractions are obtained.

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