mean luminosity of the ground), as well as the standard error were calculated for each variant.

The results of these determinations are shown in the Figure. Between the variants to which filtrates were added simultaneously with slide insemination and the controls, no significant statistical differences appear. The addition of any product after 2 days of incubation markedly activated the multiplication during the following 3 days. The stimulatory effect of the autolysed culture filtrate was however particularly strong, suggesting a notable action of the catabolic products of mycobacteria on their proper multiplication. Although the present results must be considered as only preliminary, they show that, by introducing certain agents in the Youmans' medium after 2 days of incubation of mycobacteria, one may expect some promoting of mycobacterial growth.

The method we have used offers certain obvious advantages: (1) it is theoretically very accurate, (2) it is highly sensitive and (3) it allows the quantitative study of the initial multiplication of a very small inoculum. In order to improve the adherence of the mycobacteria on the slide, which is not satisfactory after 6–8 days of incubation, it would be necessary to increase the paraffin ratio in hexane, or to use silicone-coated slides[7].

Résumé. On a utilisé une technique microspectrofluorométrique quantitative afin de pouvoir déterminer la multiplication initiale d'un très faible inoculum de la souche $H_{37}Rv$ de *Mycobacterium tuberculosis*. Cette technique a permis de mettre en évidence l'effet fortement stimulant d'un filtrat de culture de la même souche sur la multiplication bacillaire précoce, à condition que ce filtrat soit ajouté au milieu de culture après deux jours d'incubation des mycobactéries.

R. Racotta[8] and Al. Ciures

Clinical Institute of Phthisiology and Department of Biophysics, Medico-Pharmaceutical Institute Bucharest, (Rumania), 9th January 1967.

[7] K. Higashi, S. Tsukuma and M. Naito, Am. Rev. resp. Dis. *85*, 392 (1962).

[8] Present address: Institute for Biology, Rumanian Academy, Bucharest 17 (Rumania).

Analysis of a Turbidimetric Method for Quantitatively Estimating Cell Aggregation

Attention has been centred recently on improving methods to illuminate cell contact and aggregation. Roux[1] and Herbst[2] at the turn of the century, and Holtfreter[3,4] employed cell dissociation methods and cell reaggregation techniques to test the effects of chemical agents on adhesion and selective association of cells.

It is only recently, however, that methods for quantitatively estimating cell aggregation have been devised. A standardized procedure for estimating cell aggregation was described by Moscona[5]. This method is an excellent one for studying 'histotypic' aggregation. Curtis and Greaves[6] used a 'flocculation' technique for measuring cell aggregation based on the principles which obtain for passive colloidal particles[7].

The main purpose of the present investigation has been to analyse the turbidimetric method for quantitatively estimating cell aggregation and to demonstrate that it is as suitable for studying aggregation of embryonic chick cells as it was shown to be by Born[8] for studies on platelet clumping in vitro.

The method has been used for studies on aggregation of embryonic chick fibroblasts[9,10] and a standardized procedure is described.

Suspensions of embryonic chick fibroblasts are prepared as follows: nine-day-old chick embryos provided muscle tissue which is cut up into small fragments in warm Hanks' balanced salt solution (Hanks' B.S.S.) to remove red blood cells. The tissue is placed in 0.25% trypsin (Difco 1:250) in Hanks' B.S.S. for 10 min at 37 °C and dispersed into single cells by flushing through a pipette. The cells are centrifuged at 200 *g* for 5 min, washed in warm Hanks' B.S.S. and finally resuspended in Hanks' B.S.S. without phenol red which interferes with the optical density readings.

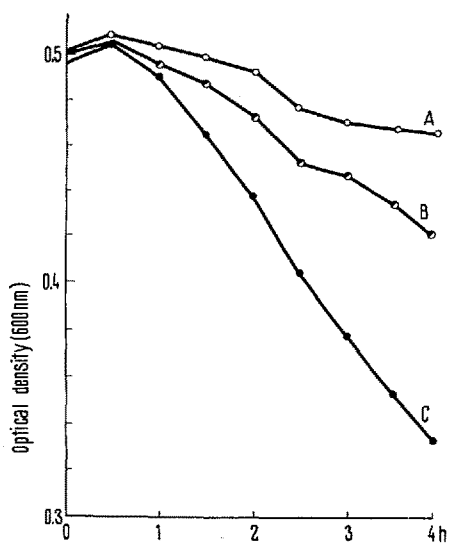


Fig. 1. The effect of the speed of rotation on the optical density of aggregating embryo-chick fibroblasts. A, 750 rpm; B, 600 rpm; C, 450 rpm.

[1] W. Roux, Arch. EntwMech. Org. *1*, 43 (1894).
[2] C. Herbst, Arch. EntwMech. Org. *9*, 424 (1900).
[3] J. Holtfreter, J. exp. Zool. *93*, 251 (1943).
[4] J. Holtfreter, Revue can. Biol. *3*, 220 (1944).
[5] A. Moscona, Expl Cell Res. *22*, 455 (1961).
[6] A. S, G. Curtis and M. F. Greaves, J. Embryol. exp. Morph. *13*, 309 (1965).
[7] J. T. G. Overbeek, in *Colloid Science* (Ed. H. R. Kruyt; Elsevier, Amsterdam 1952).
[8] G. V. R. Born, Nature *194*, 927 (1962).
[9] B. M. Jones, Nature, *205*, 1280 (1965).
[10] V. A. Knight, B. M. Jones and P. C. T. Jones, Nature *210*, 1008 (1966).

A 2 ml sample of cell suspension is placed in a siliconized test-tube (50 · 10 mm) which is inserted in a perspex arrangement fixed to the test platform of an absorptiometer (E.E.L. Quantitrator with Unigalvo 20 attached). The perspex holder is heated by circulating water to keep the enclosed test-tube containing the cell suspension at the temperature required.

The cell suspension is rotated by a stirrer which is a rod covered with siliconized glass (3.5 · 1.0 mm). The rod is rotated in the tube by the revolving magnet underneath the test platform of the absorptiometer.

The beam of white light which passes through the preparation has its intensity decreased by 50% with a Neutral Density filter (Kodak N.D. 0.3) to bring the light intensity into a convenient value for the particular cell system used. A band-pass filter (E.E.L. No. 607) removed all the components of the resultant white light other than that at a wave-length of 600 nm ± 15, which impinges on the photocell. At this wave-length absorption of light by the preparation is at a maximum.

The galvanometer moving spot is set at infinity on the scale by adjusting the dark current and readjusting to zero with reference to distilled water. The galvo scale, along which the spot moves, ranges from 0–100, corresponding to the absolute range 0–2 for optical density. As aggregation of the cells occurs there is a decrease in optical density (Figure 1; curve C) and an increase when aggregates disperse[10].

The speed of rotation of the stirrers is the same as that of the substage rotating magnet, the speed of which can be determined by using a Dawe Transistor Stroboflash. A reed (magnetically operated) switch is inserted within the body of the titrator in such a position that it is actuated by the stirrer magnet. The switch triggers the stroboflash which acts as an electronic rev. counter. The speed of rotation can be checked during the course of the experiment without disturbing the preparation.

Figure 1 shows how differences in the speed of rotation effect aggregation. Aggregates obtained optimal size after 4 h when the speed of rotation of the stirrer rod was 450 rpm (Figure 2). At higher speeds aggregates were smaller. At speeds lower than 450 rpm, the larger aggregates tended to gravitate and remain in the lower part of the tube

Cell aggregation and the correlated changes in optical density in a rotated cell suspension was also affected by the concentration of cells used (Figure 3). Exploratory results reveal that the most suitable cell density is about $7 \cdot 10^6$ cells/ml.

Viability of chick fibroblasts under the conditions of the test was not impaired. Stains such as lissamine green[11] and trypan blue were used. Cells which do not

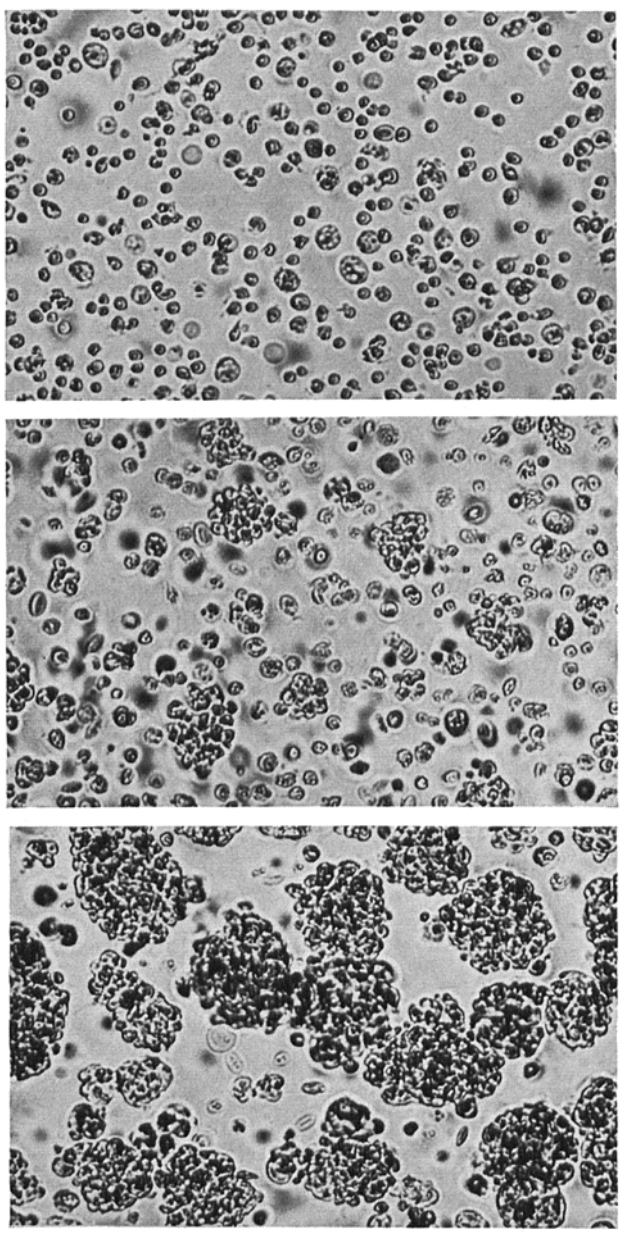


Fig. 2. Aggregation of cells when rotated at 450 rpm in the turbidimetric method. Samples of a cell suspension taken at different times during the 4 h experimental period. Top: single cells at 0 h; centre: initial aggregates formed at the end of 1 h; bottom: aggregates produced at the end of the 4 h period when the process of aggregation had come to an end.

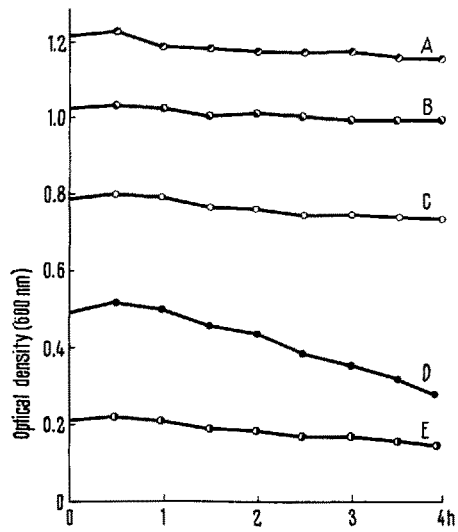


Fig. 3. The effect of different concentrations of cells on optical density of aggregating embryo-chick fibroblasts: A, $16 \cdot 10^6$ cells/ml; B, $14 \cdot 10^6$ cells/ml; C, $11 \cdot 10^6$ cells/ml; D, $7 \cdot 10^6$ cells/ml; E, $5 \cdot 10^6$ cells/ml.

[11] R. J. GOLDACRE and G. SYLVEN, Br. J. Cancer *16*, 306 (1962).

absorb these stains are considered viable. Damaged and dead cells take up the stain readily.

The pH of the Hanks' B.S.S. in which the cells were rotated was 7.4. At the end of the experiment the pH of the Hanks' had dropped to 7.2.

It is possible to measure changes in optical density by connecting an automatic pen recorder to the galvanometer. This is useful for recording changes over a short period as when platelet clumping occurs in a matter of minutes in the presence of A.D.P.[12]. When cell aggregation is a gradual process, there is no problem in accurately recording the correlated decrease in optical density by taking readings at appropriate times[13].

Résumé. Une technique a été établie pour estimer quantitativement l'aggrégation des cellules des tissus dissociés par une méthode turbidimétrique. Des fibroblastes de l'embryon de poulet ont été mis en rotation sur un absorbtiomètre. Pendant l'aggrégation des cellules vivantes, on observe une diminution de la densité optique de la suspension mésurée par un galvanomètre.

ISABEL CUNNINGHAM
and J. H. R. HIRST

Zoology Department, University College of Wales, Aberystwyth (Wales, U.K.), 6th February 1967.

[12] W. F. J. CUTHBERTSON and D. C. B. MILLS, J. Physiol. *168*, 29P (1963).

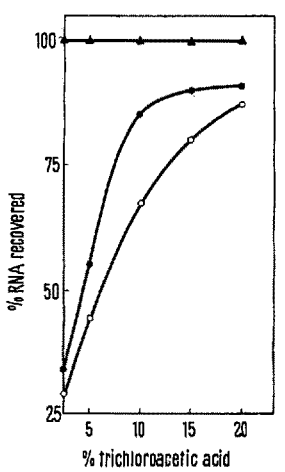
[13] I thank Professor B. JONES for his advice and encouragement, Mrs. ALISE HOWSE for her skilled technical assistance. I am also grateful to the British Empire Cancer Campaign for financial support.

On the Prevention of Loss of RNA into Lipid Solvents

In the current methods for delipidation of acid-precipitated liver homogenates, in which ethanol or acetone is used as the first of the lipid solvents, considerable amounts of RNA are lost into the solvent phase[1-3]. Such losses have been shown to be minimal if 96% ethanol containing 10% potassium acetate is used instead of aqueous ethanol[4]. It will be shown in the present note that 100% recoveries of RNA could be obtained from the acid precipitates if dioxane is used as the first of the lipid solvents.

Rat liver cytoplasmic fraction, prepared according to the method of PALADE and SIEKEVITZ[5], was used in these studies, since maximum losses of RNA into the lipid solvents have been shown to occur from this fraction[2]. Duplicate samples were treated with increasing concentrations of cold trichloroacetic acid and after 10 min centrifuged in the cold at 2500 rpm, for 5–10 min. The precipitates were extracted thrice by centrifuging with cold trichloroacetic acid of the same concentrations, and were then treated individually either with 96% ethanol or acetone or dioxane. In other experiments, mixed solvents (ethanol-ether, 2:3; ether-dioxane, 2:3; acetone-dioxane, 2:3; or acetone-ether, 2:3) were used for the first extraction. Precipitates obtained with low concentrations of trichloroacetic acid dissolve in small amounts of dioxane. However, complete reprecipitation occurs when the dioxane added is about 8-fold excess of the amount of acid in the precipitates. All operations were carried out at 2–4 °C except treatment with dioxane, which was done at 5–8 °C in order to avoid freezing. The sediments were then successively extracted twice with ethanol-ether (3:1) and twice with ether. RNA in the final precipitates was extracted with hot 5% trichloroacetic acid and determined by the orcinol reaction as described by SLATER[6].

RNA recovered from samples precipitated with different concentrations of acid, and subsequently extracted with cold ethanol or acetone or dioxane, is shown in the Figure. Treatment with cold ethanol or acetone results in an appreciable loss of RNA into the solvent phase. The losses are more marked in samples precipitated with low concentrations of trichloroacetic acid. Thus after precipitation with 2.5 or 10% acid, the RNA recoveries after ethanol treatment are 30% and 87% and after acetone treatment, 35% and 90% respectively. However, RNA equivalent to that present in the samples not subjected to any lipid solvent treatment, could be



Recovery of RNA from trichloroacetic acid precipitated cytoplasmic fraction after extraction with 96% ethanol (o—o) or acetone (●—●) or dioxane (▲—▲). Amounts are expressed as % of RNA present in samples precipitated with 2.5 or 5% trichloroacetic acid, which were not extracted with lipid solvents.

[1] P. R. VENKATARAMAN and C. U. LOWE, Biochem. J. *72*, 430 (1959).
[2] P. R. VENKATARAMAN, Biochim. biophys. Acta. *39*, 352 (1960).
[3] T. HALLINAN, A. FLECK and H. N. MUNRO, Biochim. biophys. Acta. *68*, 131 (1963).
[4] W. J. STEELE, N. OKAMURA and H. BUSCH, Biochim. biophys. Acta. *87*, 490 (1964).
[5] G. E. PALADE and P. SIEKEVITZ, J. biophys. biochem. Cytol. *2*, 171 (1956).
[6] T. F. SLATER, Biochim. biophys. Acta. *27*, 201 (1958).