Table 3. List of blood samples and plasma which initiated activity in inseminated spermatozoa

Source		pH	μg ammonium-N ml^{-1}
Barnacles			
Balanus balanoides	Blood	–	–
B. crenatus	Blood	–	–
Chthamalus montagui	Blood	–	–
Elminius modestus	Blood	–	–
B. hameri	Plasma	7.4	3.1–5.8*
Crabs			
Carcinus maenas	Plasma	7.6	6.4–7.8*
Cancer pagurus	Plasma	7.7	9.0–14.2*
Bivalves			
Mytilus edulis	Blood	–	–
Pecten maximus	Blood	–	–
Fish			
Dab (*Limanda limanda*)	Plasma	6.9	3.2–4.1*
Bird			
Quail (*Coturnix coturnix japonica*)	Plasma	8.2	3.2*
Human	Plasma	7.4	2.8–8.0 (normal range for human plasma)

* Determined by Conway diffusion method[8].

for signs of activity. Inactive inseminated spermatozoa only were used in the tests (table 2).

The results show that inseminated spermatozoa are more susceptible to ammonium ions than vesicula spermatozoa, since activity is initiated at lower levels of ammonium-N. Inseminated spermatozoa kept in sea water for 3–4 h remained inactive, so passage through the penis does not initiate activity[2]; at the end of this time the inseminated spermatozoa could no longer be induced to become active and were presumed to be dead.

Fluid collected by micropipette from the oviducal glands did not activate vesicula spermatozoa but readily activated inseminated spermatozoa. An instant 'explosion' of activity was observed when a portion of the inseminated spermatozoan mass was placed in a drop of oviducal gland fluid. Such activated spermatozoa had a mean speed of 159 μm/sec (range 105–200 μm/sec) at 12 °C. The epithelium lining each of the oviducal glands produces an elastic sac into which the eggs pass at oviposition[6]. When an 'acting female' is in a receptive state the oviducal glands are invariably swollen with fluid. How this fluid arises is not known at present, but a clue to its origin lies in the fact that the blood of *B. balanoides* initiates high activity in inseminated spermatozoa. The epithelium lining the oviducal glands at the time of insemination is reduced in size and the underlying basement membrane is very thin[6], so filtration of the blood across the lining could give rise to the oviducal gland fluid.

The discovery that the blood of *B. balanoides* activates inseminated spermatozoa stimulated the testing of the blood of other animals (table 3). Although all the samples tested initiated activity in inseminated spermatozoa, the blood of *Cancer pagurus* was the most effective. None of the blood samples activated vesicula spermatozoa. The high levels of activity initiated by blood could not be mimicked by the ammonium ion solutions alone, even when adjusted to blood pH values. It is concluded that a further factor must be present in the blood (and oviducal gland fluid).

As only a small amount of blood can be collected from *B. balanoides*, the blood from either *B. hameri* or *Cancer* was used in the further experiments. An ultrafiltrate of *B. hameri* plasma (Amicon UM 20 membrane, which according to the makers has a mol. wt cut-off at 20,000) was still active and initiated high activity in inseminated spermatozoa. Boiling the filtrate for several min so that its ammonium-N level becomes negligible completely destroys the activity. In a more detailed experiment plasma from *Cancer* was boiled for different lengths of time and subsampled for bioassay. The activity progressively decreased as the ammonium-N level fell from 9.0 μg ml^{-1} to 2.8 μg ml^{-1}.

Although the evidence is partially circumstantial, there is reason to believe that the activation of inseminated *B. balanoides* spermatozoa is initiated, in part, by ammonium ions; a further factor normally present in the blood (and oviducal gland fluid) is necessary for maximum activity. Oviducal gland fluid is undoubtedly the activating fluid and is released just prior to oviposition[9]. The initiation of activity of the spermatozoa is synchronized for once activated the gametes remain so for only 5–6 min. Oviducal gland fluid also appears to cause the progressive breakdown of the gelled mass of inseminated spermatozoa; breakdown may be the result of enzymic and/or pH effects, or it may simply be that the spermatozoa once activated are able to swim clear of the mass. This is important because it means that there is continuous release of activated spermatozoa throughout the oviposition period, so ensuring successful fertilization.

9 E. J. Conway, in: Microdiffusion analysis and volumetric error. Crosby Lockwood and Son Ltd, London 1950.

Resolution of small G_1 and G_2 populations of L1210 leukemia by flow microfluorometric analysis aided by velocity sedimentation[1]

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Summary. L1210 leukemic cells grown in vitro were subjected to kinetic analysis using a flow microfluorometer. A single broad peak was found for the DNA content distribution if unfractionated cells were used; prior fractionation using lg velocity sedimentation allowed the separation of small peaks with smaller (G_1) and larger (G_2) DNA contents from the dominant S phase peak with intermediate DNA content.

Initial kinetic analysis of our in vitro L1210 leukemia line using flow microfluorometric techniques failed to demonstrate the typical double peaked DNA content distribution seen for other L1210 leukemias, usually grown in vivo[4]. One explanation for our result was that a preponderance of cells in the S-phase of cell cycle could mask the identification of smaller numbers of cells in the G_1 and G_2 phases of cycle. The validity of this explanation is reported here.

Materials and methods. Our L1210 leukemia was obtained in 1969, and adapted to grow in liquid medium in 1973. Alternate in vivo/in vitro passage has been maintained since then. The in vitro culture medium consists of Fischer's medium (Flow Laboratories) supplemented with horse serum (10% v/v) obtained within the institute, and containing β-mercaptoethanol at a final concentration of 60 µmoles/l. Cultures are grown in a total volume of 5 ml of medium in tissue culture flasks (Falcon Plastics) initially containing 3×10^5 leukemic cells. These cells grow to a density of approximately 1.5×10^6 cells/ml in 3 days, when they are subcultured. Separation on the basis of cell size was performed using a 'staput' velocity sedimentation gradient with a volume of 500 ml. 10 ml of 0.10%, 0.35% and 0.70% bovine serum albumin (BSA) in Hanks' buffer were added sequentially to 10 ml of Hanks' buffer containing the cells from a single culture, immediately after which a 1–3% BSA gradient was constructed[5,6]. Cells were allowed to sediment for 2.5 h, the cone volume discarded, and 30 ml fractions collected. Fractions were spun, resuspended in Tyrodes buffer, counted, and then fixed in formaldehyde for 24 h at 4 °C. After fixation, 1 ml samples of each fraction were stained with 100 µl of a 1 mg/ml acridine orange solution, and DNA content distributions obtained using a Cytofluorograph® (Biophysics Corp.).

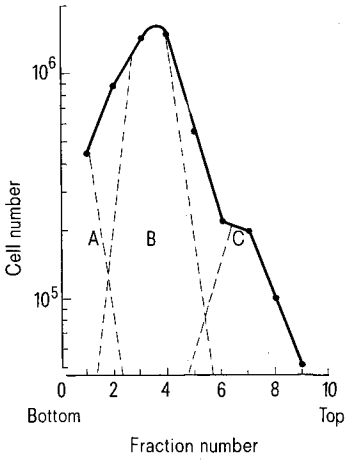

Fig. 1. Number of L1210 cells versus velocity gradient fraction number. 9 fractions were collected and counted electronically. Open points connected with solid line represent actual data, dashed lines are fit on the basis of DNA content distributions discussed in the text.

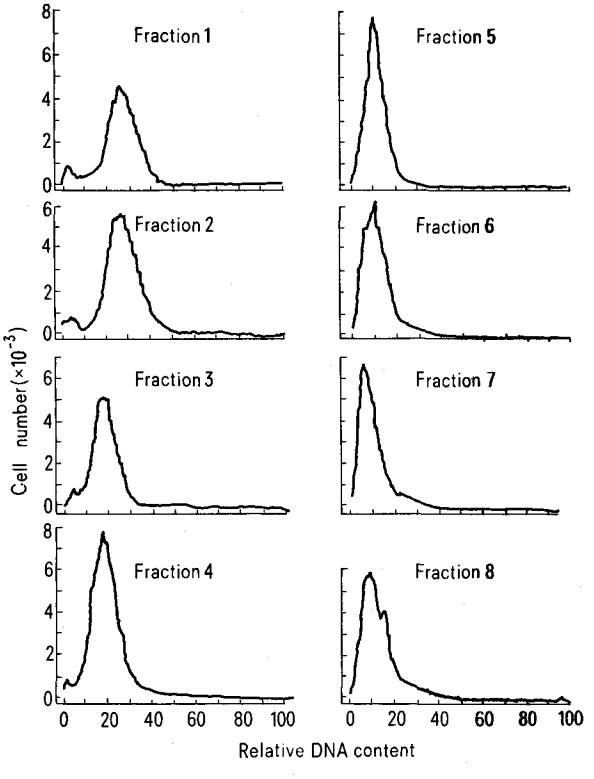

Fig. 2. Analysis of the relative DNA contents of L1210 cells from each of 8 velocity gradient fractions. The cell fractions in figure 1 were used, except for fraction 9 which contained too few cells for analysis.

Results and discussion. After velocity sedimentation, 9 fractions were obtained after discarding the cone volume. A plot of cell number versus fraction number is seen in figure 1. The distributions indicated by the dashed lines are assigned on the basis of the DNA content information discussed below. Peaks correspond to fraction 2 (curve A), fractions 3 and 4 (curve B) and fraction 7 (curve C). The DNA content distributions of each of the fractions is seen in figure 2. By placing all the distributions on one axis, 3 patterns can be distinguished with fractions 5,6,7 and 8 (curve C), grouped together with a low (G_1) DNA content, fractions 3 and 4 (curve B) with an intermediate (S) DNA content, and fractions 1 and 2 (curve A) with a high (G_2) content. By reference to the numbers of cells in distributions A, B and C the distribution of cells in the various cell cycle phases can be calculated to be approximately 20% in G_1, 60% in S and 20% in G_2. We have preliminarily evidence which suggests that the cell cycle distribution obtained is dependent on the length of time between subculturing, with increasing numbers of cells in G_1 as the culture ages. This is in accord with other culture systems in which cells reach plateau phase. In exponentially growing cultures in vitro the vast majority of cells appear to be in S-phase, making it impossible to discern small G_1 and G_2 populations without prior separation and thus confirming our prediction.

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