hydrocortisone and RU-38486 (50 or 150 mg/kg) were not significantly different (0.11 ± 0.04 and 0.11 ± 0.02 IU/mg of protein, respectively) from control rats.

Conversely, as shown in figure 3, α-glucosidase-specific activities were not significantly different in 22-day-old normal rats and 22-day-old rats previously injected with RU-38486 (25 mg/kg) for 7 days.

These results suggest that: 1) before 14 days, hydrocortisone induces α-glucosidases by a direct interaction with its own receptor; 2) during the third postnatal week, the spontaneous rise in α-glycosidase activities is not prevented by RU-38486. This absence of an effect of RU-38486 between 15 and 22 days is in agreement with the progressive loss of glucocorticoid responsiveness by α-glycosidases during this period [13]; at the end of the fourth week, adrenalectomized and sham operated rats have identical α-glycosidase specific activities [2, 14]. Several studies support the hypothesis that normal development of α-glycosidases during the third week is not dependant upon glucocorticoids, but is regulated by an intrinsic timing mechanism. Sucrase and maltase develop normally in fetal rat or mouse intestine that is transplanted into the stable hormonal environment of an adult animal [15 – 17]. The most conclusive evidence was given by Kwo-Yih Yeh and Holt [3] who showed that day 5 isografts expressed sucrase activity in 13-day-old hosts; at a time at which the host intestine did not. Our experiments give additional evidence that during the third week of postnatal life the normal development of trehalase, sucrase-isomaltase and maltase-glucoamylase is most probably independant of glucocorticoids.

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Kinetics of chemo-attraction of polymorphonuclear leukocytes towards N-formyl peptide studied with a novel polycarbonate (Nuclepore) membrane in the Boyden chamber

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Summary. The motile responses of human polymorphonuclear leukocytes (PMN) to N-formyl-methionyl-leucyl-phenylalanine (FMLP) in the Boyden chamber using a new sparse-pore polycarbonate membrane (pores 3 μm in diameter and occupying 0.1 % of surface area) were compared with those demonstrated by using a standard polycarbonate (Nuclepore) filtration membrane (pores 3 μm in diameter and occupying 5 % of surface area). Motility of PMN in gradients of FMLP using the new membrane was not influenced by chemokinetic effects of the factor, and the 'background' migration of the cells was minimal. However, motility of PMN in gradients of FMLP using the standard membrane was found to be influenced by chemokinetic effects of the chemotactic factor, and the 'background' or 'control' migration (in the absence of chemotactic factor) of the cells was substantial. Greater directional migration of PMN according to steepness of the gradient of chemotactic factor was demonstrated with the use of the new membrane. The new membrane may be of considerable value in the further study of the chemotactic responses of PMN.
Key words. Polymorphonuclear leukocytes; chemotaxis; N-formyl peptide.

N-formyl peptides [1], especially N-formyl-methionyl-leucyl-phenylalanine (FMLP) [2] are chemotactic for polymorphonuclear leukocytes (PMN) and the latter substance has become a widely-used factor for assessing the motility and chemotactic activity of these cells. The technique used by most authors for testing chemotaxis of leukocytes towards FMLP has been the Boyden chamber [3], according to which, the chemotactic factor is placed in solution in the lower compartment of a two-compartment chamber and a suspension of PMN is placed in the upper compartment. The cells are allowed to sediment onto, and then migrate into or through, a cellulose-ester filtration membrane separating the two compartments and motility is determined according to the number of cells migrating to the lower surface of the membrane [1, 3] or the distance which the 'leading front' of cells migrate into the membrane [4 – 6] in given incubation period.

Chemotaxis is usually measured as the difference between such assessments and the corresponding cell motility in chambers containing no chemotactic factor ('background' or 'control' migration).

Nevertheless, when assessed by Boyden chamber techniques, the 'control' migration of PMN in the absence of chemotactic factor can be increased by non-chemotactic factors such as albumin in the medium [7 – 9]. This phenomenon of increased random migration of PMN under the influence of a chemical factor has been referred to as 'chemokinesis' [10, 11] and FMLP, when present on both sides of the membrane in equal concentrations (i.e. no gradient) has been found to have such chemokinetic effects [12].

In an attempt to overcome the problem of distinguishing chemotaxis from chemokinesis, a technique of 'chequerboard' analysis has been described [4, 6, 9] in which the migra-

tion of PMN into membranes is assessed over ranges of absolute concentrations and with various gradients. Nevertheless, high control migration rates are not reduced, and separation of chemotaxis from chemokinesis by this method remains difficult[13].

As an alternative to cellulose-ester membranes in the Boyden chamber, the use of a polycarbonate membrane (Nuclepore Corporation, California) which is 10 µm thick and has pores 3 µm in diameter, occupying 5% of surface area, with an average inter-pore distance of 3.9 µm has been described[14, 15]. Migration is assessed as the proportion of cells migrating to the lower surface from the upper side of the membrane in a given period. This membrane allows shorter incubation periods, and is favoured by some authors[16, 17] but does not solve the problems of high control migration rates and the separation of chemokinetic effects of chemotactic agents in the Boyden camber.

The major reason for the failure of these membranes in the Boyden chamber has been suggested to be that the cells on the upper surfaces are provided with no alternative direction to move other than that measured as chemotactic (i.e. into or through the membrane)[18]. Accordingly, the present author[15] designed a sparse-pore, polycarbonate (Nuclepore) membrane, which was shown to separate chemotactic and chemokinetic movement of PMN in response to *Escherichia coli* culture filtrate when used in Boyden-type chambers in the manner described for standard polycarbonate membrane[14]. This new membrane is 10 µm thick, and has 3 µm pores occupying 0.1% of surface area (average inter-pore distance approximately 48 µm). The principle of the design of the new membrane is that the increased distances between the pores provide ample area for cells to move (even when chemokinetically stimulated), on the upper surface without meeting a pore and moving through to the lower surface of the membrane. However, cells chemotactically attracted to a substance emanating from the lower compartment of the chamber are able to move directly to the pores, and pass through, appearing on the lower surface. Thus both control migration (in the absence of factor) and chemokinetic migration (presence of factor, without gradient) are markedly reduced. Chemotactic movement is established as the percentage of total cells (top and bottom) on the lower surface at the end of the incubation period.

The present paper reports migration of PMN through the new membrane in comparison to standard polycarbonate membrane, towards various concentrations of FMLP, together with a chequer-board analysis of movement of PMN through both membranes.

Materials and methods. N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma) in 10^{-2} M solution in dimethyl sulfoxide was a kind gift from Dr A. Ferrante, Adelaide Childrens Hospital. It was diluted in Hanks solution buffered with 20 mM hepes at pH 7.2 (Hanks-hepes) with 10% autologous plasma to 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M. Autologous, buffered, heparinised plasma was obtained as the supernatant of the PMN separation technique (see below) and used in 10% final dilution, to approximate the conditions encountered by PMN in inflammatory exudates.

Polymorphonuclear leukocytes were separated from heparinised whole blood by the one-step Hypaque-Ficoll method of Ferrante and Thong[19-21]. PMN were washed twice in Hanks-hepes and resuspended in Hanks-hepes with 10% autologous plasma in a final concentration of 1×10^6/ml. All steps were carried out at 37 °C to avoid delays of onset of migration at the beginning of incubation, and because this method preserves maximal morphological reactivity of PMN[22, 23].

The polycarbonate membranes, of sparse-pore (special lot 41A6, fig. 1) and standard types (lot 62B5D12, fig. 2) were generously provided by the Nuclepore Corporation (California). The minimum inter-pore distances of these membranes had been previously calculated as 48 ± 18.5 µm and 3.9 ± 2.8 µm[15]. Before use, the membranes were washed twice in absolute ethanol to remove the coating of polyvinylpyrrolidone used as a wetting agent (see manufacturer's information).

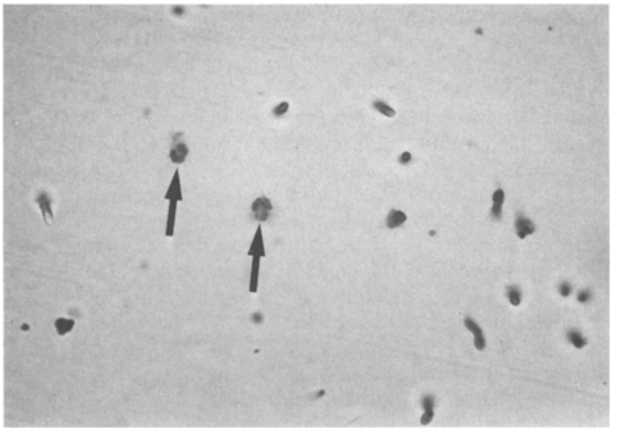

Figure 1. Photomicrograph of new, sparse 3-µm pore diameter polycarbonate (Nuclepore) membrane, showing PMN (arrowed) on one surface. Haematoxylin stain, bright-field illumination, × 350.

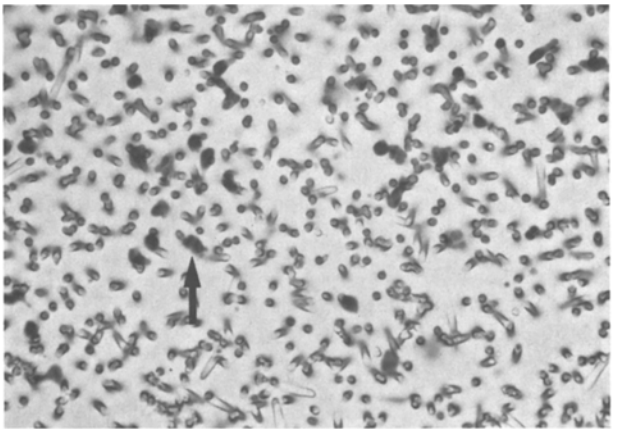

Figure 2. Photomicrograph of standard 3-µm pore diameter polycarbonate (Nuclepore) membrane. PMN are arrowed. Haematoxylin stain, bright-field illumination, × 350.

The Boyden chamber used in these experiments was modified from the adapted Boyden chamber described by Wilkinson[6]. The chamber consisted of a 13-mm diameter disk of polycarbonate membrane (glossy surface to the inside) which was glued with a silicone (Dow Corning) sealer to the end of a cylinder cut from the barrel of a plastic centrifuge tube. This closed cylinder was then placed membrane end down in a well of a tissue-culture plate (24 wells per plate, Linbro, Flow Labs, Virginia) in the bottom of which was a 12-mm diameter ring of silicone rubber. The space inside this ring (10-mm diameter) of rubber and between the floor of the well and the membrane constituted the lower chamber, while the lumen of the barrel constituted the upper chamber.

For the assay of chemotaxis of PMN toward various concentrations of FMLP in new and standard membranes, the lower compartment of each chamber was filled with 0.4 ml FMLP or control solution and the upper compartment was lowered into place. Then 0.3-ml cell suspension was placed in

the upper compartment to begin the incubation. After 20 min at 37 °C, the membranes were fixed with 4% formaldehyde in saline for 10 min, washed and stained with hematoxylin. After drying in a 60 °C oven, the membranes were mounted between glass slides and coverslips using 'Entallan' (Merck, Darmstadt) medium and examined with a light microscope using bright field illumination. Cells were counted on the bottom surface of the membrane as a percentage of total cells (top and bottom) in 10 random fields of each membrane. These percentages were averaged in groups of three membranes, with means and standard deviations of these averages being calculated for each group.

To carry out the chequer-board analysis, 0.4 ml of various concentrations (10^{-7} M, 10^{-8} M, 10^{-9} M) of FMLP or control solution were placed in the lower compartments of triplicate chambers, and 0.3 ml of PMN suspended in the same concentrations of FMLP or control solution in the upper compartments. If the concentration of FMLP was higher in the lower compartment, a positive gradient was therefore present, and if the concentration of FMLP was lower in the lower compartment, a negative gradient was established. Incubations and processing of membranes were carried out as above, and chemotaxis was assessed in the same way.

Results. In preliminary experiments, the presence of 0.1% dimethyl sulfoxide in media was found to have no effect on the migratory response of PMN towards dilute *E. coli* filtrate across either standard or new polycarbonate membranes.

Migration of PMN through the new membrane was slight when either 10^{-5} M or 10^{-9} M FMLP or 'control' solution was present in the lower compartment (fig. 3). Migration was 26 ± 6.0% and 24 ± 7.1% of total cells with 10^{-6} M and 10^{-8} M FMLP respectively and 50 ± 2.8% with 10^{-7} M FMLP in the lower compartment.

Migration through the standard membrane was 25 ± 5.5% when 10^{-5} M FMLP was present in the lower chamber and 54 ± 10.0%, 57 ± 5.7% and 56 ± 12.1% when 10^{-6} M, 10^{-9} M FMLP and 'control' solution respectively was present in the lower chamber. Migration was maximal (>90%) with both 10^{-7} M and 10^{-8} M FMLP. The proportions of PMN on the lower surfaces were therefore higher for all concentrations of FMLP when the standard membrane was used rather than the new membrane, and the measured effects of 10^{-7} M and 10^{-8} M were similar.

In the chequer-board studies of PMN movement, migration of PMN through the new membrane was always less than 5% of total cells when the concentrations of FMLP were

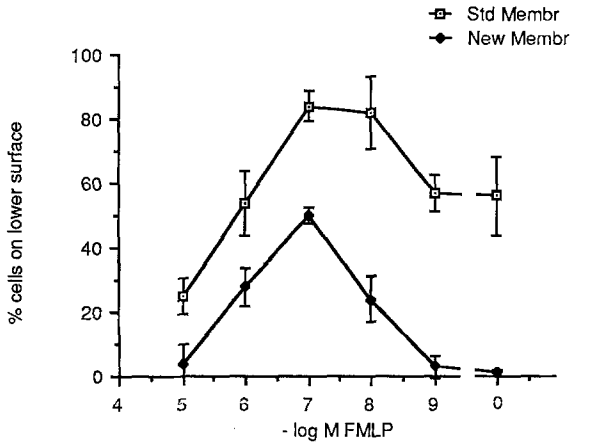

Figure 3. Graph indicating motile responses (assessed as % cells on the lower surface of membrane after incubation for 20 min at 37 °C) of PMN to various concentrations of N-formyl-methionyl-leucyl-phenylalanine (FMLP) using new and standard polycarbonate membranes.

equal, or a negative gradient was present (table 1). In the presence of a positive gradient, the proportion of cells found on the lower surface of the membrane correlated with the gradient present. Thus when 10^{-7} M FMLP was below the membrane, migration was higher with the cells in control solution than when the cells were in 10^{-9} M or 10^{-8} M FMLP (58 ± 9.5%, 37 ± 11.2% and 19 ± 5.6% respectively. Similarly, if 10^{-8} M FMLP was below the membrane, migration was higher if the PMN were in control solution than if the cells were in 10^{-9} M FMLP (34 ± 12.5% and 10 ± 8.3% respectively).

In chequer-board analysis of migration of PMN across the standard membrane (table 2), movement of the cells was higher in the presence of a positive concentration gradient of FMLP than in no gradient or a negative gradient for 10^{-7} M, 10^{-8} M, and 10^{-9} M of the chemotactic factor. Furthermore, the migration was greater with the higher gradients at each absolute concentration of FMLP. Thus when 10^{-7} M FMLP was present below the membrane, migration was 69 ± 12%, 74 ± 5.5% and >95% when the cells were in 10^{-8} M, 10^{-9} M and control solutions respectively. When 10^{-8} M FMLP was present below the membrane, migration was 80 ± 2.8% and more than 95% when the cells

Table 1. Chequer-board analysis of migration of PMN towards FMLP using the new (sparse 3-μm diameter pore) polycarbonate (Nuclepore) membrane

		% cells (mean ± SD) on lower surface of membrane Molar concentration of FMLP below membrane			
		0	10^{-9}	10^{-8}	10^{-7}
Molar concentration of FMLP above membrane	0	<5	22 ± 4.0	34 ± 12.5	58 ± 9.5
	10^{-9}	<5	<5	10 ± 8.3	37 ± 11.2
	10^{-8}	<5	<5	<5	19 ± 5.6
	10^{-7}	<5	<5	<5	<5

Table 2. Chequer-board analysis of migration of PMN towards FMLP using standard 3-μm diameter pore polycarbonate (Nuclepore) membrane

		% cells (mean ± SD) on lower surface of membrane Molar concentration of FMLP below membrane			
		0	10^{-9}	10^{-8}	10^{-7}
Molar concentration of FMLP above membrane	0	41 ± 12.7	64 ± 12.1	>95	>95
	10^{-9}	51 ± 5.5	58 ± 9.3	80 ± 19.8	74 ± 5.5
	10^{-8}	45 ± 18.0	40 ± 12.0	59 ± 4.2	69 ± 12.2
	10^{-7}	49 ± 8.4	41 ± 8.8	48 ± 6.6	66 ± 8.1

were in 10^{-9} M and control solution respectively. However, the background or control migration was 41 ± 12.7% of cells in the absence of FMLP. Furthermore, the migration of PMN in the non-gradient presence of the highest concentration (10^{-7} M) of FMLP was 66 ± 8.0%. The difference between the migrations of cells in the non-gradient presence of FMLP and the complete absence of the factor is an indication of the chemokinetic effect of the factor and was therefore substantial in these experiments using the standard membrane.

Discussion. The present studies have shown that chemotactic migration of PMN towards FMLP can be demonstrated in a Boyden chamber without significant influence of either control (background) migration or chemokinetic effect of the agent by the use of a sparse-pore (0.1% of surface area) polycarbonate (Nuclepore) membrane, confirming earlier observations using *E. coli* as chemoattractant[15]. While the percentage of cells migrating to the lower surface is greater when a membrane having pores (5% of surface area; 'standard' Nuclepore membrane) is used, both the control migration and chemokinetic migration contribute to the total percentage of cells on the lower surface at the end of incubation. The present results show in addition, that migration of PMN is maximal in the highest gradient of concentration between the two compartments of the Boyden chamber, supporting earlier observations made using visual assay techniques[24]. However, the precise concentration of FMLP to which the cells respond is better defined using the new membrane in these experiments, since the migration of cells towards 10^{-7} and 10^{-8} M could not be distinguished with standard polycarbonate membrane, while with the use of the new membrane, maximal responses of PMN were demonstrated with 10^{-7} M FMLP.

This concentration (10^{-7} M) of FMLP providing a maximal chemotactic response across the new membrane is slightly higher than that reported by other authors[6, 25–27], but the discrepancy could be accounted for by the fact that many of the cells are initially separated at a distance from the widely separated pores, and the chemotactic factor forms a gradient on the upper surface of the membrane by diffusion or convection from the upper mouths of the pores. Thus many of the cells would be responding to a lesser concentration of FMLP. Such an explanation of the mechanism by which polycarbonate membranes are suitable for measuring chemotactic responses of PMN is different to the suggestion by Horwitz and Garrett[14] that the cells respond to a gradient formed only by diffusion along the intramembranous length of the pores.

Nevertheless, the new polycarbonate membrane is demonstrated to be superior to standard membrane for distinguishing chemotaxis from chemokinesis using 10^{-6} M and 10^{-8} M as well as 10^{-7} M FMLP, and thus in many circumstances, the former may be the membrane of choice for use in the Boyden chamber. The studies show further that comparisons of motility of PMN using the two membranes can be used to study the relative chemokinetic and chemotactic effects of factors affecting the locomotion of these cells.

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A new chromosome number for *Bombina* (Anura, Discoglossidae)

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Summary. The karyotype of a primitive discoglossid anuran, *Bombina maxima*, native to southwestern China, has $2n = 28$ chromosomes with 6 large and 8 small bi-armed homologous pairs. This is a higher chromosome number than described for other *Bombina* species, all of which have $2n = 24$.
Key words. Bombina maxima; Amphibia; Discoglossidae; karyotype.