the rates of sterol synthesis in a number of extrahepatic tissues is consistent with the view that sterol synthesis in these organs is controlled by cholesterol carried in one of the serum lipoprotein fractions. Presumably, this mechanism is similar or identical to that described in the isolated fibroblast where low density lipoproteins have been shown to control the level of HMG CoA reductase (*6*). Second, it is of interest that only seven of the twelve extrahepatic tissues tested in this study manifested enhanced sterol synthesis (*11*). It is not known whether the remaining tissues would also have shown increased rates of synthesis if the period of observation has been more prolonged. Alternatively, it is conceivable that these tissues are subject to a different control mechanism. Third, it is impressive that the rate of sterol synthesis in the derepressed small intestine actually exceeded the rate in the liver of fed animals, that is, approximately 300 nmole per gram of tissue per hour. In contrast, in the other responsive tissues such as lung and kidney the observed rates of synthesis were still low relative to those in the gastrointestinal tract and liver. This finding again emphasizes the quantitative importance of these two organs to net sterol balance in the body. Fourth, cholesterogenesis in the liver is clearly regulated either directly or indirectly by remnants of chylomicrons, bile acids, and various hormones. Whether the liver also is regulated by serum lipoproteins to any significant degree cannot be determined on the basis of these studies because of the profound fasting effect that is undoubtedly present in the animals treated with APP for 4 days.

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Calcium Influx Requirement for Human Neutrophil Chemotaxis: Inhibition by Lanthanum Chloride

Abstract. *Calcium fluxes of human neutrophils measured in the presence of chemotactically active serum showed a marked stimulation of calcium-45 uptake from the media. Chemotactically inactive serum did not cause an influx of calcium. The magnitude of the calcium influx due to activated serum is sufficient to trigger contractile systems previously described in muscle cells. Lanthanum chloride inhibited the chemotactic response of human neutrophils to activated serum. Lanthanum in concentrations that suppressed chemotaxis also inhibited the calcium influx caused by activated serum. The data support a direct role of calcium influx in chemotaxis of neutrophils.*

The ability of a cell to translate a chemical gradient into directional motility, the phenomenon of chemotaxis, has interested cell biologists for many years (*1*). Chemotaxis of neutrophils has been demonstrated in vitro and the nature of the attractant characterized (*2, 3*). However the intermediary steps coupling the chemotactic agent to oriented motion remain essentially unknown. Colchicine and other antitubulins inhibit chemotaxis (*4*) and divalent cations appear to be required for neutrophil motility (*5*). Isolation of actomyosin-like proteins from both guinea pig and horse neutrophils (*6*) has raised the question of whether calcium activation of a contractile mechanism is involved in the directional movement of neutrophils. To answer this we measured the relationship between the in vitro chemotactic response and calcium fluxes of neutrophils exposed to activated serum. Lanthanum ion (La^{3+}), which inhibits transmembrane calcium movement, was employed to determine the role of calcium movement in chemotaxis.

The depressant effect of lanthanum on isolated frog heart contractions was demonstrated as early as 1910 (*7*). Since that time the specific antagonism of calcium binding by lanthanum has been elucidated (*8*). Smooth muscle is a contractile system requiring calcium and is quite sensitive to inhibition by lanthanum. The calcium influx required to sustain contraction of smooth muscle cells can be selectively blocked by concentrations of lanthanum around 0.1 m*M*. Millimolar concentrations of lanthanum are further capable of inhibiting calcium efflux in smooth muscle (*9*). Lesseps (*10*) demonstrated by electron microscopy the inability of lanthanum to penetrate embryonic chick cells. This suggests that lanthanum is selectively effective at the plasma membrane.

Employing a modification of the Boyden technique (*2*), we investigated the chemotactic responsiveness of isolated neutrophils to activated serum in the presence of La^{3+}. Neutrophils were collected from normal volunteers and isolated by sedimentation of blood in 3 percent dextran, followed by hypotonic lysis of contaminating red cells. The leukocyte preparation contained at least 85 percent neutrophils and less than 15 percent mononuclear cells. The cells were resuspended in Gey's medium and washed in a balanced salt solution (BSS) containing 135 m*M* NaCl, 4.5 m*M* KCl, dextrose (1 g/liter), 10 m*M* Hepes buffer (*p*H 7.2), 1.3 m*M* $MgCl_2$, and 1.5 m*M* $CaCl_2$. The final concentration of cells was adjusted to 2.0×10^6 neutrophils per milliliter. Lanthanum was added in the form of $LaCl_3$. Serum was obtained from normal volunteers, activated by incubation with endotoxin, and employed in the concentration that resulted in maximum chemotactic response (3 percent by volume in BSS). Samples in chemotaxis chambers were incubated in triplicate for 90 minutes, at which time the filters were removed, fixed, and stained, and the neutrophils that migrated to the lower surface were counted in 20 high-power fields.

Figure 1 demonstrates the effect of La^{3+} on the chemotactic response of neutrophils to activated serum (AS). Cell

viability, as assessed by exclusion of trypan blue (*11*), was at least 95 percent and was unaltered during incubation by concentrations of $LaCl_3$ up to 4.0 m*M*. Lanthanum was not chemotactic and its effect was consistent whether it was placed on both sides of the filter at equimolar concentrations or solely in the compartment containing the cells. The concentration of La^{3+} that resulted in half-maximal inhibition (the 50 percent effective dose or ED_{50}) was $5 \times 10^{-4}M$; there was no consistent effect at 0.2 m*M* and almost complete inhibition at 1.0 m*M*. Increasing the external calcium concentration from 1.5 to 4.0 m*M* decreased the inhibition by 0.75 m*M* $LaCl_3$ from 75 to 40 percent ($P < .005$).

Random motility, as measured by the number of migrating cells in the absence of a chemotactic gradient, was also inhibited by concentrations of La^{3+} greater than 1.0 m*M*, but the effect was variable below 1.0 m*M*. The gross morphology and the adherence of the neutrophils to the substratum of the chemotaxis filters were not adversely effected by the presence of $LaCl_3$. Human monocytes showed a similar if not more sensitive response to lanthanum.

To follow calcium movement as a function of chemotaxis, neutrophils at a final density of 5×10^6 cell/ml were incubated in 5 ml of BSS with ^{45}Ca (10 to 30 μc/ml) for 15 minutes to reach equilibrium. The incubation was then continued in the presence of either AS or the chemotactically active purified split product from

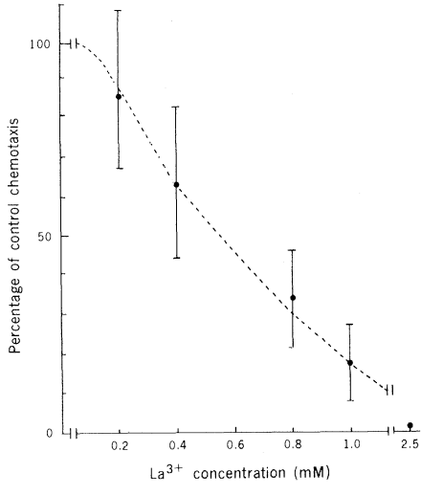

Fig. 1. Effect of varying concentrations of $LaCl_3$ on chemotaxis of human neutrophils to AS. Lanthanum was added in the indicated molar concentration to both sides of a filter, with AS in the lower compartment only. Data are expressed as percentage response of cells exposed only to AS. Values are means for at least four experiments performed in triplicate. Error bars indicate the standard error.

Table 1. Influence of several chemotactic factors on cellular calcium accumulation after 10-minute incubation in BSS. Calcium-45 (32.5 μc/ml) was added to the medium and equilibrated for 15 minutes before the addition of the stimulant. Cells were harvested on Millipore (0.44-μm) filters and washed with 4 m*M* La^{3+} in BSS. The amount of calcium that entered was calculated from a specific activity of 6.55×10^{11} counts per gram of calcium and assuming a cellular weight of 10^{16} daltons. Concentrations used were 3 percent by volume AS and ĀS, 30 percent by volume C5a, and $1 \times 10^{-6}M$ NFP. The significance of the results compared to those obtained with ĀS was calculated by Student's *t*-test for unpaired data.

Factor	Activity (counts per 2.5×10^6 cells)	Ca^{2+} influx (μmole per gram of cells)
ĀS	201 ± 40.5	
NFP	325 ± 58.6*	12.4
AS	1209 ± 17.5†	83.5
C5a	1415 ± 147†	100.0

*$P < .025$ ($N = 6$). †$P < .005$ ($N = 6$).

the fifth component of complement (C5a) (*12*). The results were compared to those obtained with cells exposed to heat-inactivated serum (ĀS) as a control. At various times thereafter cells were harvested by centrifugation at 250*g*, resuspended, and washed with BSS until the ^{45}Ca activity in the wash was equal to the background. The cells were then lysed and counted by liquid scintillation. An alternative technique used to collect the cells was passing the cell preparation through 0.44-μm Millipore filters and washing the retained cells. This method yielded similar results. Since the majority of calcium binding sites are extracellular (*9*), even in cells with no extracellular matrix (*13*), lanthanum can be used to compete with extracellular calcium binding (*9, 14*). In these experiments lanthanum was used during the wash in a concentration of 4 m*M* to strip extracellular bound calcium from the cells. An additional feature of lanthanum at millimolar concentrations is its ability to inhibit calcium efflux (*15*), thus partially sealing the internal milieu of the cell against calcium egress during the washing procedure.

Figure 2 represents the accumulation of ^{45}Ca by neurotrophils in the presence of AS or ĀS. The observed stimulation due to AS occurs within minutes and by 15 minutes ^{45}Ca uptake levels off at values up to 400 percent greater than control values.

The effect of lanthanum on calcium influx was determined by incubation with La^{3+} concentrations up to 1.0 m*M*. The La^{3+} could be added to the neutrophil suspension either when the ^{45}Ca was introduced or at the time of stimulation with chemotactic factors. In either case a dose-dependent decrease in cellular uptake of ^{45}Ca was observed, and the dose-response curve was nearly identical to that observed in the chemotaxis assay with an ED_{50} of $6 \times 10^{-4}M$. Likewise, 0.2 m*M* La^{3+} was without effect and 1.0 m*M* La^{3+} produced a result similar to that found with ĀS (Fig. 2). A similar influx of calcium was observed with C5a. *N*-Formylmethionylphenylalanine (NFP), a chemotactic factor not derived from serum (*16*), at $1 \times 10^{-6}M$, produced a smaller but significant calcium influx (Table 1).

Neutrophils incubated for 10 minutes with AS accumulated 83 μmole of Ca^{2+} per gram of tissue (Table 1). This may be compared with 91 μmole of Ca^{2+} per gram of tissue reported by Bianchi (*17*) for maximum activation of skeletal muscle actomyosin.

The calcium influx results presented here differ from those of Gallin and Rosenthal (*18*), who reported no demonstrable stimulation of calcium influx associated with the chemotactic fraction of activated serum. However, the influx experiments of Gallin and Rosenthal were performed in calcium-depleted media. Extracellular bound calcium on the leukocytes may be displaced by a chemotactic factor and this may explain the discrepancies. The use of lanthanum to wash extracellular bound calcium from the cells eliminates these difficulties and

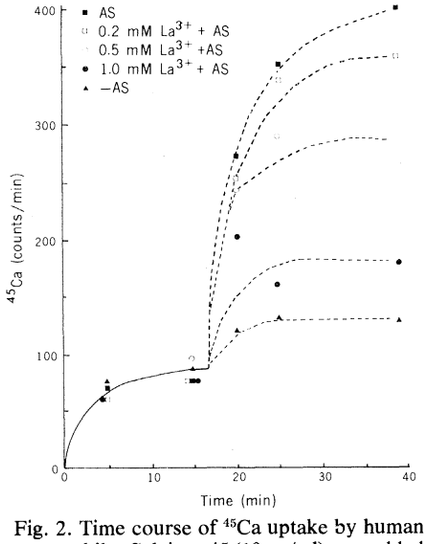

Fig. 2. Time course of ^{45}Ca uptake by human neutrophils. Calcium-45 (10 μc/ml) was added at time zero to the medium containing 2.5 cell/ml in BSS. After 15 minutes, to allow for equilibration, the cells were exposed to the appropriate concentrations of $LaCl_3$ and AS. Serum incubated at 56°C for 30 minutes without endotoxin (ĀS) was employed as a control. Samples (2 ml) were placed in BSS with 4 m*M* $LaCl_3$ and harvested by centrifugation.

enables one to make more meaningful assumptions concerning the intracellular fraction of the label. The stimulation of influx due to purified C5a and to NFP gives supportive evidence that the influx due to AS is related to its chemotactic potential.

Gallin and Rosenthal (*18*) demonstrated a stimulation of calcium efflux by activated serum. We also observed an increase in the washout of label from cells incubated for 1 hour in a medium containing ^{45}Ca and exposed to AS. Lanthanum chloride in concentrations up to 1.0 m*M* did not inhibit this effect. The stimulation of calcium efflux by AS may be due to release of a bound intracellular pool of calcium, which is subsequently pumped from the cell. Wilkinson (*19*) has postulated that intracellular release of calcium has a role in neutrophil chemotaxis. However, the data presented here suggest that release of an intracellular pool is insufficient to promote chemotaxis or that such a pool is dependent on calcium influx.

It appears that calcium influx is correlated with chemotaxis, and the amount of calcium that moves into the cell is sufficient to activate a contractile process. The observed calcium influx is unlikely to be an energy-requiring event since the gradient of Ca^{2+} across the plasma membrane would favor calcium movement into the cell as in other well-studied cellular systems. An interaction between La^{3+} and the chemotactic factor is not supported by the data, since concentrations of La^{3+} below 1.0 m*M* selectively blocked calcium influx due to AS. Furthermore, La^{3+} inhibited cellular motility in the absence of a chemoattractant. The role of Ca^{2+} efflux may be secondary since concentrations of La^{3+} that blocked chemotaxis inhibited only calcium influx. Although there was no gross chemical gradient of the chemotactic agents during our calcium flux experiments, these agents may exert their cellular effects in the absence of a gradient. To induce chemotaxis, the gradient would provide direction through a sensing mechanism. The directional polarization may be separate but related to the contractile action initiated by the absolute concentration of the attractant.

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Cytochrome P-450 Induction by Phenobarbital and 3-Methylcholanthrene in Primary Cultures of Hepatocytes

Abstract. *The characteristic hepatocellular changes resulting from phenobarbital administration in vivo, namely an increase in the levels of cytochrome P-450 and proliferation of membranes of the smooth endoplasmic reticulum, have been demonstrated in primary cultures of nonreplicating hepatocytes on floating collagen membranes. Addition of methylcholanthrene to the medium resulted in an increase in cytochrome P-448 within 48 hours, whereas the phenobarbital induction of P-450 required 5 days. These results demonstrate that responses induced in adult liver cells in vivo by phenobarbital can be reproduced in cultured hepatocytes, contrary to previous reports.*

Cytochrome P-450 plays a crucial catalytic role in the activities of several liver microsomal enzymes which, known collectively as mixed function oxidases, are involved in the activation of carcinogens and the detoxification of drugs by liver (*1*). The concentration of cytochrome P-450 in liver is increased by the administration in vivo of a number of chemical compounds, including phenobarbital and 3-methylcholanthrene (MC) (*2*). The molecular forms of cytochrome P-450 induced by these two compounds exhibit difference spectra with absorption maxima at different wavelengths (*3*); they also give different signals when tested by electron paramagnetic resonance (*4*). We use the terms cytochrome P-450 and cytochrome P-448 for the cytochromes induced by treatment with phenobarbital and MC, respectively (*5*).

In addition to its effect on cytochrome P-450, phenobarbital administered to animals causes a characteristic proliferation of membranes of the smooth endoplasmic reticulum (SER) in the cytoplasm of hepatocytes and a simultaneous increase in the levels of several microsomal mixed function oxidases (*2*, *6*).

In the systems of primary cultures of hepatocytes reported so far (*7*, *8*), the administration of such compounds as phenobarbital and MC has not resulted in quantitative changes in cytochrome P-450 levels, nor has it elicited the characteristic, phenobarbital-associated proliferation of SER membranes. The inability to induce cytochrome P-450 and to elicit the characteristic responses to phenobarbital administration in primary cultures of hepatocytes has restricted the use of these systems in studies of drug and carcinogen metabolism in vitro. It has also raised the question of whether it is possible to induce in any culture system the phenobarbital-characteristic form of cytochrome P-450 and the associated proliferation of SER (*8*).

Here we report the induction of cytochrome P-450 by phenobarbital, and cytochrome P-448 by MC, in primary cultures of adult rat hepatocytes on floating collagen membranes. We also report that the proliferation of vesicles of smooth endoplasmic reticulum occurs in the cytoplasm of the cultured hepatocytes after the administration of phenobarbital.

In a system (*9*) for the primary culture of parenchymal hepatocytes on floating collagen gels, the cultured hepatocytes