has not previously been shown to have a significant effect on the neutrophil[12]. The chemoattractant material appears to be a lipid, released spontaneously into surroundings from fibrillar structures which lie on the outside of the bacterial cell[13]. The data obtained from this investigation suggests that the AP fractions and the PLS from *P. acnes* are important as chemotactic factors, and supports the concept of lipids as attractants. The potent chemoattractant effect of AP's and PLS, in addition to the depressive effects of PLS in direct contact with the granulocytes may be an essential factor in the development of the inflammatory lesions seen in acne vulgaris.

1 D.J. Gould, W.J. Cunliffe and K.T. Holland, J. invest. Derm. *68*, 251 (1977).
2 W.L. Lee and A.R. Shalita, J. invest. Derm. *70*, 219 (1978).
3 M.S. Puhvel and M. Sakamoto, J. invest. Derm. *71*, 324 (1978).
4 S. Abrahamsson, L. Hellgren and J. Vincent, Experientia *34*, 1446 (1978).
5 L. Hellgren, G. Selstam and J. Vincent, Experientia *35*, 196 (1979).
6 L. Hellgren, B. Lindblom, J. Vincent and L. Wilhelmsson, Experientia *35*, 1298 (1979).
7 S. Abrahamsson, N. Engström, L. Hellgren and J. Vincent, in preparation.
8 E. Berlin, L. Hellgren, O. Thulesius and J. Vincent, Experientia, in press (1980).
9 L. Hellgren, M. Romanus and J. Vincent, in preparation.
10 H. Gnarpe, J. Belsheim and S. Persson, Infection *6*, suppl 1, 98 (1978).
11 H. Gnarpe and J. Belsheim, in manuscript.
12 P.C. Wilkinson, G.J. O'Neill and K.G. Wapshaw, Immunology *24*, 997 (1973).
13 R.J. Russell, R.J. McInroy, P.C. Wilkinson and R.G. White, Immunology *30*, 935 (1976).

Net ATP synthesis by running the red cell calcium pump backwards

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Summary. Ca^{2+} loaded inside-out vesicles from human red blood cells, yielding Ca^{2+} into a Ca^{2+} free medium with 4 mM EGTA, 2 mM ADP and 10 mM phosphate, produced an excess of 14.9 pmoles · min^{-1} · (mg protein)$^{-1}$ of ATP compared to controls in which the transmembrane Ca^{2+} gradient was abolished by the ionophore A 23 187.

The human red blood cell membrane is equipped with an ATP-fuelled Ca pump[2-4] which under physiological conditions extrudes Ca^{2+} ions from the cytosol against a gradient of 10^3–10^4. Its normal performance can be symbolized as:

$$n\,Ca_i^{2+} + ATP \rightarrow n\,Ca_0^{2+} + ADP + PO_4^{3-}$$

(Ca_i^{2+}, Ca_0^{2+} = internal and external calcium). It is of considerable interest to know whether such an active transport system is reversible as a whole, i.e. whether an (electro) chemical gradient (of Ca^{2+}) can run down in such a way that the energy is not dissipated but appears as bond energy in a chemical compound (ATP). In order to test this point one must measure the change in ATP concentration after having made some (or all) concentrations on the right larger, and those on the left smaller than normal. The Na pump[5-8] and the Ca pump of the sarcoplasmic reticulum[9] have been run backwards successfully. Confirming an earlier report[10] Rossi et al.[11] recently demonstrated that in ATP depleted, PO_4^{3-} enriched human red cells 2 mM external Ca^{2+} concentration is sufficient to induce $^{32}PO_4^{3-}$ incorporation into ATP. However, in those experiments the net ATP concentration decreased significantly during the process, which leaves room for doubt whether the energy for ADP^{32}P synthesis was indeed derived from the Ca^{2+} gradient rather than from concomitant ATP hydrolysis.

In the present experiments inside-out vesicles were prepared from fresh human red cells essentially according to Steck and Kant[12] (see legend to table). After separation on a dextran cushion 60% of the material was present as closed inside-out vesicles as judged from latent acetylcholinesterase[12]. The vesicles were first loaded with Ca by pump action in a medium containing 20 or 40 μM Ca^{2+}, 2 mM ATP and excess Mg^{2+}. They were subsequently washed in buffered KCl solution. This procedure resulted on average in 21.5±2.4 mM (SEM, n=21) internal Ca^{2+} concentration (for an estimate of vesicle volume[17]. The pellet was resuspended by vigorous shaking in a Na-free medium containing 10 mM PO_4^{3-}, 2 mM ADP, 4 mM EGTA, 5 mM Mg^{2+} and 20 μM P^1, P^5-di(adenosine-5′-)pentaphosphate, a potent adenylate kinase inhibitor[13], and incubated for 15 min at 37 °C. (When necessary, contaminating ATP in ADP (Boehringer) was removed by eluting 200 μmoles at a time from a 15×1.2 cm column of Dowex SBR (type I) anion exchange resin with 0.02 N HCl in the cold, followed by neutralisation with KOH and freeze-drying). ATP concentration was measured by the luciferin-luciferase method, comparing samples with standards having the same composition except for ATP concentration. Crude firefly extract (Sigma) was chromatographed on a 100×2.6 cm G 100 Sephadex column[14,15] to free it from adenylate kinase. Luminescence was assayed by photon counting either with an EMI 6097 photomultiplier tube without wavelength discrimination or on a Packard scintillation spectrometer. Calibration curves were exponential in the first and linear in the second case.

The conditions used (described in detail in legend to the table) seemed appropriate to induce reversal of the pump direction since addition of ADP caused an extra leak of Ca^{2+} of 239±76 pmoles · min^{-1} · (mg protein)$^{-1}$ (6 experiments, P=0.024) from the vesicles during 15 min at 37 °C. The table summarizes experiments demonstrating that this ADP dependent Ca^{2+} movement was accompanied by net ATP synthesis. After loading the vesicles with Ca^{2+} the suspension was divided into 2 portions which were treated identically except that one (control) was treated with 10 μM A 23 187, a rather Ca-specific ionophore, in order to abolish the Ca^{2+} gradient across the membrane. It may be seen from the table that in the presence of a Ca-gradient across the membrane an extra ATP production of 14.9 pmoles · min^{-1} · (mg protein)$^{-1}$ took place. There was a large basal ATP formation, probably attributable to incomplete adenylate kinase inhibition. On the other hand there must have been some ATP consumption by the Mg^{2+} stimulated ATPase. Its activity in vesicle preparations was 0.85 nmoles · min^{-1} · (mg protein)$^{-1}$ at 100 μM ATP (8 experiments).

Mean rate of ATP formation by Ca^{2+} loaded inside out vesicles during 15 min at 37 °C. In controls the Ca^{2+} gradient was abolished prior to exit phase by ionophore A 23 187 (10 µM)

Experiment number	ATP formed (pmoles · min^{-1} · (mg protein)$^{-1}$) 1 Control (ionophore)	2 Vesicles with Ca-gradient	3 2-1
1	117	112	− 5
2	137	167	30
3	40	73	33
4	59	62	3
5	280	290	10
6	217	232	15
7	86	98	12
8	73	94	21
		Mean	14.88
		SEM±	4.56
		p for deviation from zero:	0.014

Vesicle preparation according to Steck and Kant[12] with the following modifications: White membranes prepared as described earlier[16]. To initiate vesiculation white ghosts (0.2 mg · ml^{-1} protein) were incubated for 30 min at 0 °C and 15 min at 37 °C in (mM) 0.5 tris-Cl, 0.05 dithiothreitol (pH 8.5 at 22 °C) and pelleted at 28,000 × g for 20 min in the cold. The pellets were resuspended in a small volume of the same solution and passed 4 times through a 26-gauge needle. The homogenate was layered on a dextran cushion (8.01 g/100 ml, density 1.03 g/ml, Sigma clinical grade, mol.wt 80,700) and centrifuged for 2 h at 44,000 × g in the cold. The band floating on top of the cushion was collected and washed 3 times with 10 mM tris-Cl (pH 7.4) in the cold. The vesicles were resuspended in 140 mM KCl, 20 mM tris-Cl (pH 7.4) to a final concentration of about 1.5 mg · ml^{-1} protein.

Ca loading: vesicles (4.5 mg protein) incubated for 20 min at 37 °C in 30 ml of (mM) 140 KCl, 20 tris-Cl, 2 $MgCl_2$, 0.5 Mg-ATP (Boehringer), 0.02 or 0.04 $CaCl_2$ (pH 7.4). After cooling to 2 °C they were washed 3 times with approximately 20 ml per mg protein of (mM) 140 KCl, 20 tris-Cl (pH 7.4) in the cold.

Ca exit phase (shown in table): the pellets were resuspended in 4 ml of (mM) 140-200 KCl, 20 tris-Cl, 5 $MgCl_2$, 4 H_4-EGTA, 10 KH_2PO_4, 2 ADP or K_2ADP (Boehringer), 0.02 P^1,P^5-di(adenosine-5′)pentaphosphate (Ap5A) as Li-salt (Boehringer) (freshly prepared and neutralized with tris to pH 7.4 at 37 °C), protein concentration ~ 1 mg · ml^{-1}. Subsequently the suspension was divided into 2 parts. To one of them (control) 10 µM A 23187 was added (stock solution 10 mM in dimethylsulfoxyde (DMSO)), the other received only DMSO. After 5 min incubation at o °C (long enough to abolish the Ca gradient in the presence of the ionophore) samples for zero-time measurements were taken, the rest was incubated at 37 °C for 15 min; reaction stopped by cooling in ice. After centrifugation for 10 min at 28,000 × g the clear supernatant was collected for ATP determination (stored frozen for 3 h).

ATP determination: With EMI photomultiplier tube: Samples diluted and Na_2 ATP standards ($0.4\text{–}2 \times 10^{-7}$ M) made up in (mM) tris-SO_4 20, $MgSO_4$ 10, tris-EGTA 2, pH 7.75 (solution a). Standards contained an amount of incubation medium corresponding to sample dilution. 0.5 ml of this was mixed with 0.5 ml of luciferin (Sigma) $8 \cdot 10^{-6}$ g · ml^{-1} in solution a and the reaction started by adding 50 µl of luciferase solution from column 1:5 in (mM) glycine 50, Na_2HAsO_4 10, Na-EDTA 1, pH 7.4. Photons were counted for 30 sec at 10, 70 and 130 sec and the result extrapolated to zero-time. All reactants were stored in ice, measurements took place during warming to room temperature.

On Packard instrument: 1 ml of solution a was added to above reaction mixture, standards were $6 \cdot 10^{-9}$–$2 \cdot 10^{-7}$ M. Measurement at 0 °C at fixed time after starting reaction. Response to ADP and Ap5A contained in samples was negligible. A 23187 in the highest concentration ever present in the assay mixture (0.5 µM) did not reduce the luminescence (response with A 23187 was 105 ± 10% (SEM) of control (p = 0.65) in 6 replicates).

Ca-efflux measurement: (6 experiments. Result see text). Loading media contained additionally 0.03 µCi ^{45}Ca per ml. To measure ^{45}Ca content vesicles were separated from medium by filtration on Millipore membranes (pore size 0.45 µm) and washed with cold 140 mM KCl, 20 tris-Cl, pH 7.4[17,18]. Radioactivity was assayed by liquid scintillation counting (filter dissolved in 1 ml 2-ethoxyethanol + 10 ml xylene-Triton X100 (1 + 1) with 4 mg · l^{-1} Omnifluor (NEN). (A 23187 was a generous gift of Eli Lilly Co.)

Studies on disrupted whole membranes gave a K_m of 0.4 mM (unpublished) (Na + K − ATPase was of no concern since the media were Na-free and Ca + Mg − ATPase activity in open membranes was quenched in the reversal experiments by the presence of EGTA, keeping the Ca^{2+} concentration $< 10^{-8}$ M). The fraction of ATP splitting by the Mg-ATPase due to the excess ATP formed by the Ca^{2+} gradient ought to be added to the numbers in column 3 of the table. However, assessing Mg-ATPase activity is impossible since the absolute values of the ATP concentration are unknown. This is so because ADP was also added to the standards which caused its ATP contamination (of µmolar concentrations in the sample) to cancel in the measurements. Therefore zero-time values for ATP concentrations are not absolute figures. Yet from a rough estimate it seems unlikely that the Mg-ATPase correction could bridge the gap between ADP dependent Ca^{2+} release and ATP formation. It seems that Mg-ATPase, measured separately, under-estimates ATP-consuming processes in the vesicle experiments.

If the total free energy change

$$\Delta G_t = -\Delta G^0 - RT\ln\left\{\frac{[ATP]}{[ADP]\cdot[PO_4]}\right\} + nRT\ln\left\{\frac{[Ca_0^{2+}]}{[Ca_i^{2+}]}\right\} + nV_mF$$

is negative, the system does work, if it is positive it absorbs work. With ATP = $2.5 \cdot 10^{-6}$ M (measured in experiment 8 of the table), ADP = 2 mM, PO_4^{3-} = 10 mM, Ca_i^{2+} (medium) $\leqslant 10^{-8}$ M, Ca_0^{2+} (inside vesicles) = 21 mM and assuming zero membrane potential (V_m) and 30.55 kJ · mole^{-1} for ΔG^0 of ($ATP + H_2O \rightleftharpoons ADP + PO_4^{3-}$), ΔG_t is +12.32 kJ · mole^{-1} for n = 1 and +49.84 kJ · mole^{-1} for n = 2. Thus there is no thermodynamic objection to the result found, regardless of whether the stoichiometry is 1:1 or 2:1 for Ca:ATP.

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2 H.J. Schatzmann, in: Topics in Membranes and Transport, vol.6, p.126. Ed. F. Bronner and A. Kleinzeller. Academic Press New York 1975.
3 B. Sarkadi and D.C. Tosteson, in: Transport Across Single Biological membranes, p.117. Ed. G. Giebisch et al. Springer Verlag 1979.
4 B. Sarkadi, Physiol. Rev., submitted.
5 P.J. Garrahan and I.M. Glynn, J. Physiol. *192*, 237 (1967).
6 A.F. Lant and R. Whittam, J. Physiol. *199*, 457 (1968).
7 I.M. Glynn and V.L. Lew, J. Physiol. *207*, 393 (1970).
8 R.L. Post, K. Taniguchi and G. Toda, Ann. N.Y. Acad. Sci. *242*, 80 (1974).
9 M. Makinose and W. Hasselbach, FEBS Letters *12*, 271 (1971).
10 H.G. Ferreira and V.L. Lew, J. Physiol. *252*, 86 (1975).
11 J.P.F.C. Rossi, P.J. Garrahan and A.F. Rega, J. Membrane Biol. *44*, 37 (1978).
12 T.L. Steck and J.A. Kant, in: Methods in Enzymology, vol. 31, p.172. Ed. S. Fleischer and L. Packer. Academic Press New York 1974.
13 P. Feldhaus, T. Fröhlich, R. Goody, M. Isakov and R.H. Schirmer, Eur. J. Biochem. *57*, 197 (1975).
14 R. Nielsen and H. Rasmussen, Acta chem. scand. *22*, 1757 (1968).
15 A. Lundin, A. Rickardsson and A. Thore, Analyt. Biochem. *75*, 611 (1976).
16 H.J. Schatzmann, J. Physiol. *235*, 551 (1973).
17 B. Sarkadi, J.D. MacIntyre and G. Gardos, FEBS Letters *89*, 78 (1978).
18 A. Wüthrich and H.J. Schatzmann, Cell Calcium *1* (1980), submitted.