dition; such an increase is abolished by phosphate. Consequently we can suppose that the inhibitory effect of histamine on ADP-induced aggregation, previously described by CONSTANTINE[4] could be due to the use of histamine-phosphate. We can put forward a similar supposition to explain the absence of any effect of histamine observed by O'BRIEN[2]. In actual fact, O'BRIEN[2] and CONSTANTINE[4] unfortunately do not specify the histamine salt they used. It seems interesting to quote also that we have obtained similar results with histamine and 48/80. As platelets are known to contain histamine, we can emphasize that, so far as we had observed, the effect of 48/80 above 9 µg/ml is independent of the concentration. Therefore we can suppose that histamine-liberator, even at low concentration, releases the maximum of histamine disposal. According to HUMPHREY[9], the content of histamine in the rat platelets is less than 1 µg/10^9 cells; on the contrary, by histamine addition every platelet could have at its disposal roughly 27×20^{-6} µg of histamine.

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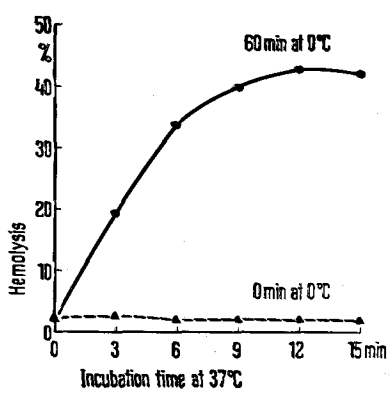
Hot-Cold Hemolysis: The Role of Positively Charged Membrane Phospholipids

The erythrocytes of several species exhibit a startling response to many bacterial exotoxins (e.g. *Cl. welchii* α-toxin or staphylococcal β-toxin): the classic 'hot-cold' hemolytic phenomenon; hemolysis caused by the lowest dilutions of some exotoxins might be incomplete or absent during incubation of susceptible erythrocytes for a fixed time at 37°C. However, additional incubation at 0°C, rather than inhibiting the hemolytic process, permits it to proceed to completion; hence the term 'hot-cold'.

In an attempt to explain this effect we advanced the working hypothesis that the 'hot' phase of the phenomenon may result from chemical alterations of the choline residues of membrane sphingomyelin. Our observation of lytic effects of the triiodide ion on the sheep, human, and rat erythrocytes fully supports this concept. The I_3^- ion was formed either non-enzymatically, by reaction of hydrogen peroxide with iodide, or enzymatically, using glucose oxidase and glucose as a source of H_2O_2 and horse radish peroxidase to oxidize iodide. Hemolysis was determined by measuring the amount of hemoglobin as hemin[1]. The lytic effects of I_3^- depend, *Caeteris paribus*, on the concentration of the triiodide ion identified by its absorption spectrum (max_1 at 3530 Å with molar extinction coeff. 26,400; max_2 at 2875 Å with *m/e* coeff. 40,000[2]) and determined using its max_1. The I_3^- induced hemolysis occurs between pH 6.0 and 7.9 with the optimum at pH 7.0. Hemolysis is usually complete within 60 min of incubation at 37°C. However, when the incubation time is shorter, or when less than optimal amounts of I_3^- are employed, the extent of the 'hot' hemolysis is reduced. Now, the erythrocytes left at 0°C, exhibit additional leakage of hemoglobin: the 'cold' effect which is proportional to the dose of I_3^- used in the 'hot' phase of the experiment or, with suboptimal time of incubation, to the duration of the 'hot' phase (Figure).



'Hot-cold' hemolysis of sheep RBC induced by I_3^-. Two series of duplicate tubes each containing 2.5 ml of 0.6% sheep RBC suspended in 0.1*M* potassium phosphate buffer, pH 6.9, and 0.8 ml of a solution of I_3^- (350 nanomoles; with molar ratio I_3^-/RBC phospholipid = 4.3) were incubated at 37°C ('hot' phase) and the percentage hemolysis determined in one series of tubes (▲---▲) at the indicated time intervals. At each time period duplicate tubes of the second series were removed to an ice bath and held for an additional 60 min ('cold' phase) and the percentage hemolysis again determined (●—●).

I_3^- hemolysis could be prevented by the addition of dipalmitoyl lecithin (Nutr. Biochem. Corp., Cleveland, Ohio; in form of sonicates) to the incubation medium before the initiation of the 'hot' phase. We have also demonstrated that this saturated lecithin binds I_3^- in a stable complex, apparently a triiodide, that can be identified by thin layer chromatography (Eastman Chromagram K301R2; continuous run in diethyl ether, methanol: 1:1). Similar interaction of I_3^- with $-N^+(CH_3)_3$ groups of membrane phospholipids, at pH 7.0 fully protonated (pK about 9.0) and accessible to influences of the medium[3], apparently leads to hot-cold hemolysis. Of the membrane phospholipids, the sphingomyelins which are net positive over wide pH[4], may well be the most active substrate for interaction with I_3^-. The erythrocytes of sheep, man, and rat show (in this order) both decreasing sensitivity to the lytic effects of I_3^- (Table), and decreasing contents of their sphingomyelins (14.66; 10.0; 4.91 nmoles/10^8 cells with the molar ratios total membrane phospholipid/membrane sphingomyelins of 1.58, 3.97, and 7.6, respectively[5–7]).

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Varying responses of the erythrocytes of sheep, man, and rat to agents which induce 'hot–cold' hemolysis

Agent	Sheep	Man	Rat
a) I_3^-	2.9	7.3	7.0–22.0
b) *Cl. welchii* Phospholipase C	0.50	0.92	31.0
c) *S. aureus* (strain R-1) β-toxin (G. M. WISEMAN[9])	2048	256	<4

a) For I_3^- the data are expressed as the molar ratio I_3^-/RBC phospholipid causing the maximum hemolytic response of 0.3% RBC at pH 6.9 in 0.1 *M* potassium phosphate buffer during 60 min of incubation with the standardized amounts of I_3^- at 37 °C. b) For *Cl. welchii* phospholipase C the data are expressed as micro-g of enzyme which causes 50% hemolysis in pH 6.5 isotonic saline containing 2 m*M* $CaCl_2$, during 30 min incubation of 0.3% RBC at 37 °C. c) The data[9] are expressed as dilutions of a β-toxin preparation giving the same hemolytic response at similar conditions (hemolytic units/ml).

Cl. welchii phospholipase C which releases the phophoryl-nitrogenous moiety from phosphatides of the erythrocyte membrane[8] shows a differential response of the erythrocytes of sheep, man, and rat similar to that induced by I_3^- (Table), and – under proper conditions – the 'hot-cold' phenomenon.

Our results support a view that the fixed positive charge of phospholipids (in particular, sphingomyelin) may regulate the leakage of hemoglobin through the erythrocyte membrane. Once facilitated this passive movement of hemoglobin proceeds and reaches an equilibrium in the 'hot' phase. When the cells are transferred into the ice bath before the equilibrium is attained the passive movement of hemoglobin proceeds until the equilibrium is reached ('cold' phase of the classical microbiology). The effect of 'hot-cold' hemolysis appears to be a common feature of the response of erythrocytes to any agent which alters or removes $-N^+(CH_3)_3$ groups of membrane phospholipids.

Zusammenfassung. Nachweis der Wirkung bestimmter Exotoxine von Bakterien auf die Erythrozytenmembran, wobei die Änderung der Permeabilität durch die Abspaltung von Cholin aus den Phospholipiden der Membran erfolgt.

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Antigen-Induced Mitosis in Liver Macrophages of Immunized Mice

Activation of macrophages appears to be essential to the expression of acquired resistance to many experimental infections[1,2]. Activated macrophages differ from 'normal' (non-activated) macrophages in having a greatly increased capacity to destroy intracellular micro-organisms, increased rates of phagocytosis and spreading on glass and an increased content of phase-dense and phase-lucent granules[1,3]. The process of activation is cell-mediated and immunologically specific[1,2,4]. Activation and the acquisition of resistance are generally accompanied by the development of delayed-type hypersensitivity[5] and the occurrence of mitosis in the macrophages of the peritoneal cavity[6], spleen and liver[7,8]. It has also been reported that mitosis can be induced in peritoneal macrophages by the re-injection of a soluble pure protein antigen into mice previously immunized with that antigen in Freund's adjuvant[9]. We have confirmed this observation; shown that the induction of mitosis is immunologically specific and is accompanied by morphological signs of activation; observed that mice which respond in this way also have delayed-type hypersensitivity to the antigen; and found that mitotic reactivity could not be conferred on normal mice by injection of high-titred antisera from immunized mice or rabbits[10]. In the course of these experiments we wished to determine whether the hepatic macrophages (Kupffer cells) which turn over very slowly – about once every 60 days[11] – and are thus a relatively fixed population, would also respond mitotically to antigenic challenge in immunized mice.

Non-inbred female SW mice were immunized by s.c. injections, 1 week apart of 100 µg of 4×recrystallized human serum albumin (HSA, Nutritional Biochemicals Corp.) as an emulsion of equal volumes of a saline solution with Freund's complete adjuvant (Difco). 4 weeks after the 2nd injection they received a s.c. injection of 2 mg HSA in 0.2 ml saline. Controls comprised immunized animals injected with saline only and non-immunized animals injected with HSA or saline. 20 h later they were injected i.v. with 100 µg vinblastine sulphate (Velbe, Lilly) in 0.5 ml saline. After a further 3 h they were killed by cervical dislocation and portions of the livers were removed and fixed in formol-saline. Sections 6 µm thick were cut from paraffin embedded blocks and stained with haematoxylin and eosin. The number of macrophages in mitosis was counted in 100 microscopic fields at a magnification of 100×. The abnormal mitotic figures were easily recognizable and those in Kupffer cells were distinguishable from the rare mitoses in hepatic parenchymal cells. In order to ensure that the results were not affected by distortion or compression of the tissue during processing,

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