tact, but suffered considerable distortion. The nuclei seemed to undergo partial homogenization, losing their normal chromatin pattern, while the nuclear membrane appeared to become thicker.

Further work will be necessary to determine whether these changes are caused by thermal or radiation damage from the incident triton beam. The cell damage, if caused by heating, may be ameliorated in future experiments by cooling the block with liquid nitrogen and eliminating the thermal insulation formed by the 100-μm Mylar supporting the cellulose nitrate film. Alternatively, cooling might be accomplished by conduction to a cold gas contained above the sample.

Although all erythrocytes fixed in deuterated formaldehyde produced radial track patterns, some phytohemagglutinin-treated lymphocytes exposed to deuterated thymidine did not (Fig. 2, c and d). Sparse tracks were noted over all nondeuterated control lymphocytes. It is inferred that the labeled cells were transformed by the phytohemagglutinin, while the cells associated with few tracks in excess of background were not transformed. The number of alpha particle tracks in individual clusters corresponding to lymphocytes were counted. The greatest deuterium content of a lymphocyte was estimated to be $(6 \pm 3) \times 10^9$ atoms. A lymphocyte fully labeled with methyl-deuterated thymidine is expected to incorporate 5×10^9 deuterium atoms during one cell division (*12*). Unexpectedly, a randomly oriented background track pattern was observed. We suspect that this was due to contamination of the Teflon film by detergent during the dispersion and fusion of the 0.2-μm tetrafluoroethylene particles which were used during the casting of the film. Any contaminant of tetrafluoroethylene that contains hydrogen should introduce deuterium in the absorber and result in randomly oriented background tracks. The radial track pattern associated with cells was readily distinguishable from the random background pattern, especially at low magnification. This pattern was not observed in the erythrocyte samples because the triton fluence—and hence the sensitivity—was lower by a factor of 50.

The system of deuterium micromapping described in this report is less sensitive than conventional tritium ARG, but it may prove useful for certain clinical studies of patients to whom it may be considered inappropriate to administer tritiated metabolic precursors or tritiated cells. As a particular example, the feasibility of labeling human lymphocytes with deuterated thymidine is demonstrated.

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18. We thank W. A. Scott, E. G. Reilly, and M. H. Bull for technical assistance, and R. A. Conard, L. E. Feinendegen, S. Perry, M. Friedkin, J. E. Cleaver, and S. R. Person for advice. This work was performed under the auspices of the U.S. Atomic Energy Commission.

27 June 1974; revised 12 August 1974

Singlet Excited Oxygen as a Mediator of the Antibacterial Action of Leukocytes

Abstract. *Human polymorphonuclear leukocytes kill a colorless mutant strain of* Sarcina lutea *much more readily than a carotenoid-containing strain. A similar protective effect has been reported in the organism during photodynamic inactivation, where it is attributable to the quenching of singlet excited oxygen by carotenoids. The findings with leukocytes support the suggestion that singlet excited oxygen acts as one of the mediators of their bactericidal action.*

Although oxidation has been considered one of the primary means whereby polymorphonuclear (PMN) leukocytes destroy ingested bacteria, the actual bactericidal cellular reactions have not been well characterized. The respiratory burst which accompanies phagocytosis in leukocytes was assumed by Iyer *et al.* (*1*) to be due to the formation of H_2O_2, and Paul and Sbarra (*2*) subsequently made direct measurements of an increase in H_2O_2 production by leukocytes during phagocytosis. Klebanoff and co-workers (*3*) extended these observations and suggested that a three-component system including myeloperoxidase, H_2O_2, and halide (Cl^- or I^-) is responsible for the bactericidal activity of PMN leukocytes. The role of these compounds as antibacterial agents in PMN leukocytes has been studied in patients suffering from chronic granulomatous disease, whose PMN leukocytes are unable to produce H_2O_2 when stimulated (*4*), and in patients with myeloperoxidase deficiency (*5*). In both of these cases, bactericidal activity is impaired.

Another hypothesis has been advanced to explain bacterial killing within leukocytes. Allen *et al.* (*6*) reported the appearance of chemiluminescence from human PMN leukocytes after stimulation with either bacteria or latex particles. They proposed that the chemiluminescence reflects the generation of singlet excited oxygen ($^1O_2$*), which acts as the bactericidal agent. The $^1O_2$* could be formed, according to Allen *et al.*, from the decomposition of an oxygen intermediate, such as the superoxide radical ($\cdot O_2^-$), formed during pyridine nucleotide oxidation. Several workers (*7*) have suggested that the spontaneous dismutation of $\cdot O_2^-$ produces $^1O_2$*, as follows

$$\cdot O_2^- + \cdot O_2^- \xrightarrow{+2H^+} H_2O_2 + {}^1O_2{}^*$$

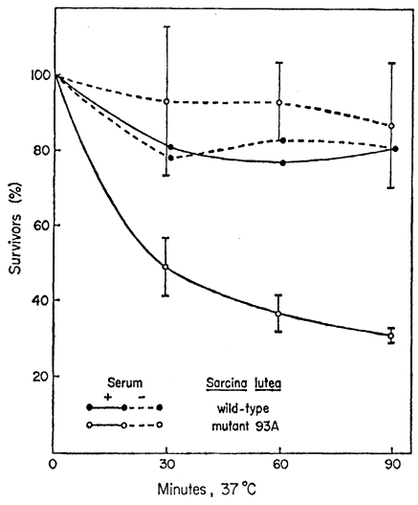

Fig. 1. Survival of carotenoid-containing wild-type *Sarcina lutea* and a pigmentless mutant strain, 93A, exposed to human PMN leukocytes. Flasks containing both leukocytes and pooled human serum were prepared as described in the text. Control flasks contained no serum. At time zero (2.5 to 7.5) × 10^6 live *S. lutea* (either wild type or mutant strain 93A) were added to both control and experimental tubes; the number of viable cells in the zero time sample was taken as 100 percent in calculating the percentage of survivors. Each data point represents three experiments; vertical lines denote 1 standard error and are plotted only for mutant strain 93A. No significant difference was found between the control (dashed line) and experimental (solid line) tubes for the wild-type pigmented strain. The colorless mutant strain 93A was readily killed by the human PMN leukocytes in the presence of homologous serum. The comparison between mutant strain with and without serum was significant ($P \leq 0.05$) after incubation for 90 minutes.

The enhanced production of $\cdot O_2^-$ by phagocytosing human PMN leukocytes has now been reported by Babior and co-workers (*8*). These authors have also observed an absence of $\cdot O_2^-$ production in leukocytes from patients with chronic granulomatous disease.

Another mechanism for producing $^1O_2$* from PMN leukocytes has been discussed (*9*). In the myeloperoxidase-H_2O_2-halide system, a halide could be oxidized to a hypohalite anion, which could then react with another molecule of H_2O_2 to produce $^1O_2$*. The latter system represents one of the chemical reactions for the production of $^1O_2$*, as described by Seliger (*10*).

$$H_2O_2 + Cl^- \xrightarrow{MPO} H_2O + OCl^-$$
$$OCl^- + H_2O_2 \rightarrow H_2O + Cl^- + {}^1O_2*$$

where MPO stands for myeloperoxidase.

Carotenoids have now been well documented as efficient $^1O_2$* quenchers in vitro (*11*) and have been assumed to function similarly in vivo (*12*), and we have taken advantage of this fact to test the hypothesis that $^1O_2$* is involved in the bactericidal action of human PMN leukocytes. We used a yellow coccus, *Sarcina lutea*, and a mutant strain, 93A, which appears to be a single-step mutation lacking colored carotenoid pigments (*13*). These two strains have been studied extensively (*13*) with respect to the ability of the carotenoid pigments in the wild-type strain to protect against the harmful effects of visible light excitation, presumably mediated through the generation of $^1O_2$*.

Human PMN leukocytes were isolated by the dextran sedimentation technique of Holmes *et al.* (*14*) and exposed briefly to a medium of low ionic strength to lyse erythrocytes, as described by Woeber *et al.* (*15*). Under these circumstances, preparations containing 90 to 95 percent PMN neutrophils could be routinely obtained. The leukocytes were suspended in Krebs-Ringer phosphate solution containing 0.1*M* glucose at *p*H 7.4 and an appropriate amount of pooled human serum was added to yield a final concentration of 10 percent serum. Overnight cultures of *S. lutea*, both wild type and mutant strain 93A, were washed and suspended in phosphate-buffered saline, *p*H 7.4, to a concentration of 10^8 cells per milliliter. The bacteria were added to the leukocyte preparation at a ratio of 1 to 3 bacteria per leukocyte, incubated at 37°C with shaking, and 0.1-ml portions were removed for enumeration of viable bacteria. The portions were diluted with 100 volumes of distilled H_2O and shaken vigorously to lyse the leukocytes and release any ingested bacteria. Suitable dilutions of these samples were plated in duplicate onto nutrient agar plates and incubated at 30°C for 48 hours before counting. The protocol is described in Table 1.

Our results, shown in Fig. 1, indicate that during a 90-minute incubation of PMN with wild-type *S. lutea* there was no significant killing either in the presence or absence of human serum. A different result was obtained when the colorless mutant strain, 93A, was incubated under similar circumstances. In the absence of human serum, there was no killing during the 90-minute incubation period. However, in the presence of both human PMN leukocytes and serum, the colorless mutant strain was rapidly killed.

These results, demonstrating the enhanced bactericidal effectiveness of human PMN leukocytes against nonpigmented as opposed to pigmented *S. lutea*, are very similar to the results obtained when the lethal photodynamic effect of endogenous or exogenous photosensitizers was studied (*12, 13*). In the photodynamic system, the photosensitizer generates $^1O_2$*, which is presumed to act as the lethal agent (*16*). Carotenoid pigments, which quench $^1O_2$*, act as protective agents against photodynamic inactivation (*11, 12*). The effect of the carotenoid pigments, both in quenching $^1O_2$* and acting as protective agents in vivo, is very much dependent on the number of conjugated double bonds in the molecule (*11*), which supports the hypothesis that these pigments function in vivo by quenching $^1O_2$*. Inasmuch as the absence of colored carotenoid pigments in mutant strain 93A of *S. lutea* renders those organisms susceptible to the bactericidal action of human PMN leukocytes, we conclude that $^1O_2$* may serve as one of the bactericidal agents of human leukocytes.

Table 1. Protocol for experiments and controls. Symbols: +, present; −, absent. Concentrations: leukocytes, 10^6 per milliliter; serum, 10 percent; *S. lutea*, (1 to 3) × 10^8 per milliliter.

Component	Tube number			
	1	2	3	4
Leukocytes	+	+	+	+
Serum	−	−	+	+
Sarcina lutea				
Wild type	+	−	+	−
93A	−	+	−	+

Another possible explanation for the effectiveness of carotenoid pigments in vivo in protecting against both photodynamic inactivation and the bactericidal action of PMN leukocytes is that the carotenoids may react directly with $\cdot O_2^-$, rendering it harmless. Yost and Fridovich (*17*) have demonstrated quite convincingly the importance of superoxide dismutase in imparting enhanced resistance to phagocytosis by *Escherichia coli* B, thus supporting the involvement of $\cdot O_2^-$ at some stage of the process of PMN action. Johnston *et al.* (*18*) have reported that both $\cdot O_2^-$ and H_2O_2 are necessary for PMN bactericidal activity, suggesting that hydroxyl radicals (·OH) may be involved in the process through the following reaction

$$\cdot O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$$

Although we do not know whether PMN leukocytes actually generate $^1O_2$*, their ability to make $\cdot O_2^-$ (*8*), coupled with their unique characteristic of lacking the enzyme superoxide dismutase (*19*), would enable any $\cdot O_2^-$ generated by the metabolic changes induced by phagocytosis to spontaneously undergo dismutation to $^1O_2$*, as described earlier (*7*). These observations raise the question of whether some forms of virulence may be associated with bacterial strains that are particularly effective in neutralizng the lethal effects of $^1O_2$*.

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17 May 1974; revised 2 July 1974

Mammalian Hepatic Lectin

Abstract. *A rabbit hepatic protein that specifically binds asialoglycoproteins is also a lectin that agglutinates untreated human and rabbit erythrocytes and neuraminidase-treated erythrocytes from rat, mouse, and guinea pig. Both binding of asialoglycoproteins and agglutination of erythrocytes appear to involve reaction on the same active sites of the hepatic protein.*

When the terminal sialic acid residues of their carbohydrate moieties are removed from a number of plasma proteins, the desialylated molecules are rapidly transferred from blood to hepatic parenchymal cells where they are catabolized by lysosomes (*1*). The transfer is initiated by binding of the desialylated protein to a hepatocytic plasma membrane glycoprotein that has been isolated, solubilized, and partly characterized (*2*). We report now on the erythrocyte-agglutinating ability of this tissue glycoprotein. Proteins exhibiting such agglutinating activity, collectively known as lectins, have been isolated from a variety of plants, invertebrates, and lower vertebrates (*3*). To our knowledge the rabbit hepatic binding protein (HBP) described herein is the first lectin of mammalian origin.

The protein was isolated from rabbit liver and purified by affinity chromatography on a Sepharose-asialoorosomucoid column (*4*). It was eluted by a solution of 1.25*M* NaCl, 20 m*M* ammonium acetate, and 0.5 percent Triton X-100, at *p*H 6.4, and extensively dialyzed against 20 m*M* ammonium acetate and 0.25 percent Triton X-100, at *p*H 6.4. To the dialyzed solution cadmium acetate was added to a final concentration of 20 m*M*. The solution was then kept for 30 minutes at 0°C; the precipitated protein was separated by centrifugation for 15 minutes at 10,000*g*, suspended in 2 m*M* cadmium acetate, recentrifuged, and resuspended in a solution of 1.25*M* NaCl containing 20 m*M* ammonium acetate, at *p*H 6.4. Solubilization, effected by the addition of one part of 0.2*M* disodium EDTA to 100 parts of suspension, was followed by a final ammonium sulfate precipitation of the protein and dialysis (*4*). The HBP, prepared in this way, is polydisperse, as characterized by electrophoresis, ultracentrifugation, and chromatography (*4*). The criteria by which purity of this polydisperse protein is estimated are complex (*4*).

Agglutination of red cells by HBP requires the presence of intact sialic acid residues on HBP, a *p*H above 6.5, and at least 0.003*M* calcium ions—conditions also essential to the ability of HBP to bind desialylated glycoproteins in vitro (*4*). Both the agglutination and

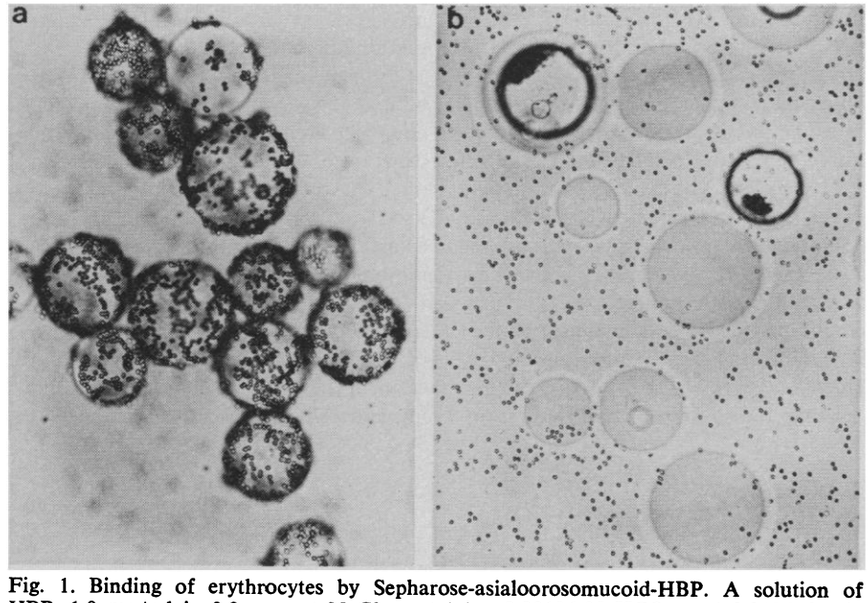

Fig. 1. Binding of erythrocytes by Sepharose-asialoorosomucoid-HBP. A solution of HBP, 1.0 mg/ml in 0.9 percent NaCl, containing 0.001*M* $NaHCO_3$, and 0.01*M* $CaCl_2$, *p*H 7.5, was passed through a small column containing 1.0 ml of CNBr-activated Sepharose 4B to which 3.0 mg of human asialoorosomucoid had been attached (*4*). After the column was washed with the same solution, the suspension of Sepharose beads was incubated for 10 minutes with either (a) human group A or (b) mouse erythrocytes (× 150).