The exposures required for greater degrees of erythema (and, presumably, darker tanning) cannot be readily determined. At wavelengths below about 280 nm, the erythema produced by three times the MED does not reach the same degree of redness nor last as long as that produced by three times the MED at wavelengths around 300 nm (*9*). Van der Leun (*13*) argued that the difference between the erythema produced by short wavelengths (250 to 280 nm) and that produced by wavelengths between 280 and 320 nm reflects two independent types of erythema. It is not known whether delayed tanning is associated with both types, or, if so, whether both types are equally effective. Thus, a person using a tanning booth, where a large fraction of the erythemally effective irradiance is at shorter wavelengths relative to solar irradiance at 30°N (Fig. 2C), might be exposing himself to burning rays that may not induce tanning as effectively as the somewhat longer rays associated with sun-induced erythema. Indeed, the salon operator reported that the tan obtained following sunlamp-induced erythema differs qualitatively ("orangish") from that following sun-induced erythema.

While achieving the erythema required for tanning a person is also exposing himself to skin cancer–inducing radiation. Although for humans the action spectrum for skin cancer induction is not known, for hairless mice it is very similar to that at which damage to DNA occurs (*14*). We shall assume that the DNA-damage action spectrum (*15*) is applicable to an assessment of the induction of nonmelanoma skin cancer in Caucasians and compare DNA-damaging UV radiation from the sunlamp with that from the sun. Table 1 shows that the irradiance from sunlamps at 33 cm contains about 26 times more DNA-damaging UV radiation than sunlight at 30°N. The ratio of DNA-damaging irradiance to erythemally effective irradiance is 1.00 for sunlight at 30°N. For a sunlamp it is 6.65 (26.2/3.94). Therefore, a sunlamp produces nearly seven times more DNA damage per unit of erythema than the sun.

Although the action spectrum for DNA damage is applicable to thin-skinned hairless mice, it may not be applicable to humans because some UV radiation is absorbed by the outer layers of the skin, preventing it from reaching the basal cell layer. To provide an idea of how skin transmission might affect this, Table 1 shows comparable values calculated with a weighting function comprised of the action spectrum for DNA damage and a transmission curve for untanned Caucasian epidermis (*16*). Even with this assumption, there is still 7.55 times more DNA-damaging UV radiation from sunlamps per unit time than there is from the sun at 30°N, or 1.92 (7.55/3.94) times more DNA damage per unit of erythema.

One means of ameliorating this problem would be to use cellulose acetate filters. (Because the transmission of UV radiation declines gradually during irradiation as a result of photodegradation of the filters, they would need to be changed every few days to maintain sufficient irradiance.) Cellulose acetate blocks the shorter wavelengths (Fig. 2A). For fluorescent sunlamps, a 5-mil filter reduces the erythemally effective irradiance by a factor of 2.6, so the exposure duration for an MED would be increased by a factor of 2.6. However, the filter would reduce the DNA-damaging UV radiation dose by a factor of 5.5 and the skin-transmitted, DNA-damaging dose by 3.3, so there would be a net improvement. However, even when equipped with such filters, tanning booths still cause more DNA damage per MED than the sun.

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7. The representation is as follows:

$$E(\lambda) = 4.278 \times 10^{-4} e^{\lambda/37.65} \times (1 + e^{(\lambda-299.1)/2.354})^{-0.6724} + 1.119 e^{-(\lambda-250)^2/206.5}$$

where $E(\lambda)$ = effectiveness as a function of wavelength (λ) normalized to 1.0 at 300 nm.
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Compartmentalization of Cyclic AMP During Phagocytosis by Human Neutrophilic Granulocytes

Abstract. *Immunocytochemistry shows that early during phagocytosis of zymosan, adenosine 3',5'-monophosphate (cyclic AMP) appears on the cell surface before the phagosome is internalized. The appearance of cyclic AMP on the cell surface is coincident with that of granule products and regulatory subunit of type I cyclic AMP-dependent protein kinase. Guanosine 3',5'-monophosphate is not associated with the initiation site of phagocytosis, but is observed throughout the granular cytoplasmic region. This sharply localized accumulation of cyclic AMP may serve as a signal for the initiation of phagocytosis.*

The signal for activating the effector systems that initiate phagocytosis by neutrophils is unknown. Since the first phase of phagocytosis involves physical and chemical contact of the neutrophil plasma membrane with the object to form the phagosome, it seems possible that, as with other bulk transport processes, adenosine 3',5'-monophosphate (cyclic AMP) is synthesized at the site of contact and provides the signal for phagocytosis. Although biochemical studies have addressed this problem, the results are apparently conflicting. For example, cyclic AMP has been reported to enhance (*1*, *2*), inhibit (*3*), and have no effect on (*4*) phagocytosis. The polarity of structure of the neutrophil is crucial in terms of movement and phagocytosis. Phagocytic stimuli are confined to minute areas of the cell surface and the resulting events proceed within a limited cell volume. Hence, we used immunocytochemical

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methods to study the location of cyclic AMP and guanosine 3′,5′-monophosphate (cyclic GMP) and the translocation of granules during formation of the phagosome. We report here that cyclic AMP is specifically localized at the site of the newly forming phagosome, and its appearance there is coincident with that of granule products and regulatory subunit of type I cyclic AMP-dependent protein kinase (R^1). The coincident localization of cyclic AMP and R^1 during the initial encounter of the particle with the plasma membrane suggests either specific compartmentalization of cyclic AMP (*5*) or that cyclic AMP serves as a signal to regulate endocytosis.

Monolayers of human neutrophils were prepared (*6*) and challenged for phagocytosis for 30 seconds with zymosan opsonized with normal type AB human serum. Phagocytosis was stopped by washing the cells with 1 percent paraformaldehyde. Cells were stained with immunofluorescent conjugates for surface binding or were fixed further with formaldehyde, methanol, and acetone (FMA) and then stained for surface and intracellular binding of antigens (*7*). During phagocytosis, granule products leak out into the extracellular fluid as a result of degranulation before the object is completely enclosed in the phagosome (*8*). Since unstimulated cells contain no granule products on their surface, the site of the forming phagosome can be ascertained with paraformaldehyde-fixed cells by observing granule products that appear on the cell surface in close proximity to the object (*6*). Paraformaldehyde fixation therefore offers the opportunity to distinguish early phagocytic events (formation of the phagosome) from later events in which the phagosome has been completely internalized. Antigens are observed both on the surface and within cells with FMA fixation.

Cells were stained simultaneously with two antiserums labeled with contrasting fluorochromes (*9*) in order to compare the distribution of two antigens in a single cell. The azurophil and specific granules were identified with fluorescent labeled antiserums against myeloperoxidase and lactoferrin, respectively (*6*). In some instances 0.01 percent methyl green, a nuclear counterstain, was used to permit orientation of the phagocytized particle with respect to the nucleus. Specificity and preparation of antiserums to myeloperoxidase and lactoferrin (*9*), cyclic AMP and cyclic GMP (*10*), and R^I (*11, 12*) have been published. Briefly, staining was blocked by absorption of its respective immunogen by the labeled antiserum, either in solution or on a solid phase. Serums obtained before immunization did not stain cells. Cells were examined with a Leitz fluorescent microscope with epi-illumination, using filter pack H for fluorescein excitation and filter pack N for rhodamine, both with edge filter K480 to minimize quenching. Photographs were taken with Kodak Ektachrome 400 film push-processed to 1600.

Figures 1 and 2 show that unstimulated neutrophils fixed with FMA were stained throughout their cytoplasm for cyclic AMP, cyclic GMP, and granule markers. Granular staining was observed for cyclic GMP, myeloperoxidase, and lactoferrin, whereas the cyclic AMP distribution appeared diffuse. This is in agreement with immunocytochemical observations of cyclic GMP in the zymogen granule area of pancreatic acinar cells (*13*). Cells fixed similarly with paraformaldehyde were not stained by the conjugates, indicating that cyclic AMP, cyclic GMP, myeloperoxidase, and lactoferrin were absent from the surfaces of unstimulated cells.

Upon phagocytic challenge, myeloperoxidase and lactoferrin (Fig. 3) appeared simultaneously on the cell surface and outlined the forming phagosome. Some of the particles observed by phase microscopy were not stained, presumably because phagocytosis had not been initiated or the particles were completely internalized and thus inaccessible to the antibodies. Figure 4 shows that intense staining for cyclic AMP was also observed in association with the forming phagosome. In contrast, cyclic GMP was not detected on the cell surface. To show that the cyclic AMP was being observed during the early stages of phagocytosis, before closure of the phagocytic compartment, cells were fixed for detection of surface antigens and stained simultaneously for cyclic AMP and either myeloperoxidase or lactoferrin. Figures 5 and 6 show that cyclic AMP and granule markers were observed on the cell surface at the same time; we never observed cyclic AMP on the cell surface without granule markers. To observe the staining of the phagosome, the focal plane was adjusted above the nuclear plane. Similar immunoreactivity was observed without the nuclear counterstain.

While differences in intracellular distribution between cyclic AMP and cyclic GMP are not distinctive in unstimulated cells (Fig. 1), they are dramatic after phagocytosis (Fig. 7). Intense cyclic AMP staining was observed in association with only a few particles, presumably those in a precise phase of engulfment. Cytoplasmic staining for cyclic AMP was less intense than the staining associated with the particles (*14*). In contrast, cyclic GMP remained distributed throughout the cytoplasm after phagocytic stimulation. Changes in the intensity of staining for cyclic GMP after phagocytosis could not be evaluated with these techniques. Occasionally staining for cyclic GMP appeared brighter in the cytoplasmic area closest to the zymosan particles that were reactive for cyclic AMP, but cyclic GMP was not observed on the surface (Fig. 4).

These results show that cyclic AMP increases only at the site of particle-cell contact during formation of the phagosome. Similar results were obtained for the interaction of lymphocytes with latex particles (*15*). We suggest that adenylate cyclase is activated on selective regions of the neutrophil membrane, and the newly synthesized cyclic AMP is localized at adjacent sites of the phagosome. This compartmentalization of cyclic AMP at the site of particle-cell contact is probably important for initiation of phagocytosis. It is of interest that a rapid rise in the cyclic AMP concentration was observed biochemically in neutrophils incubated with chemoattractants (*16*) or phagocytotic stimuli (*17*). Thus an increase in cyclic AMP induced by surface stimulation by both chemotactic factors and phagocytic stimuli represents an early event in neutrophil activation. Accumulation of cyclic AMP may provide a source of the energy necessary for pseudopod fusion or granule-membrane fusion, as suggested by Park *et al.* (*1*), or cyclic AMP may be regulating glycogenolysis (*17*). Studies of phagolysosome fusion of *Acanthamoeba* showed that cyclic AMP accelerates the fusion process (*18*). Alternatively, cyclic AMP accumulation may represent an epiphenomenon.

Since cyclic AMP modulation is believed to involve cyclic AMP-dependent protein kinases and a neutrophil cyclic AMP-dependent protein kinase has been reported (*19*), we looked for the immunocytochemical evidence for localization of R^I during phagocytosis. Like cyclic AMP, R^I was observed around the phagosome in stimulated cells fixed with paraformaldehyde and not on the surfaces of unstimulated cells (data not shown). However, R^I was found not only with the forming phagosome, but also throughout the cytoplasm of stimulated cells (Fig. 8). The coincident appearance of cyclic AMP and R^I at the initiation site of phagocytosis suggests that the cyclic

AMP detected by immunocytochemistry may be bound to R^I. The observation that R^I is present throughout the cytoplasm with little detectable cytoplasmic cyclic AMP suggests that stimulated neutrophils have little cyclic AMP bound to R^I cytoplasmic receptors (*14*). It appears that phagocytic stimulation causes rearrangement of the protein kinase configuration so that R^I and cyclic AMP are exposed to the fluorescent conjugates at the initiation site on the cell surface. Further studies are needed to prove this. Sites for phosphorylation might be a membrane protein associated with the granules or plasmalemma, or microfilaments, which are the principal structures of the pseudopodia (*20*).

There is evidence that both cyclic GMP and Ca^{2+} are involved in the secretory process (*8, 21*). The striking difference in distribution between cyclic

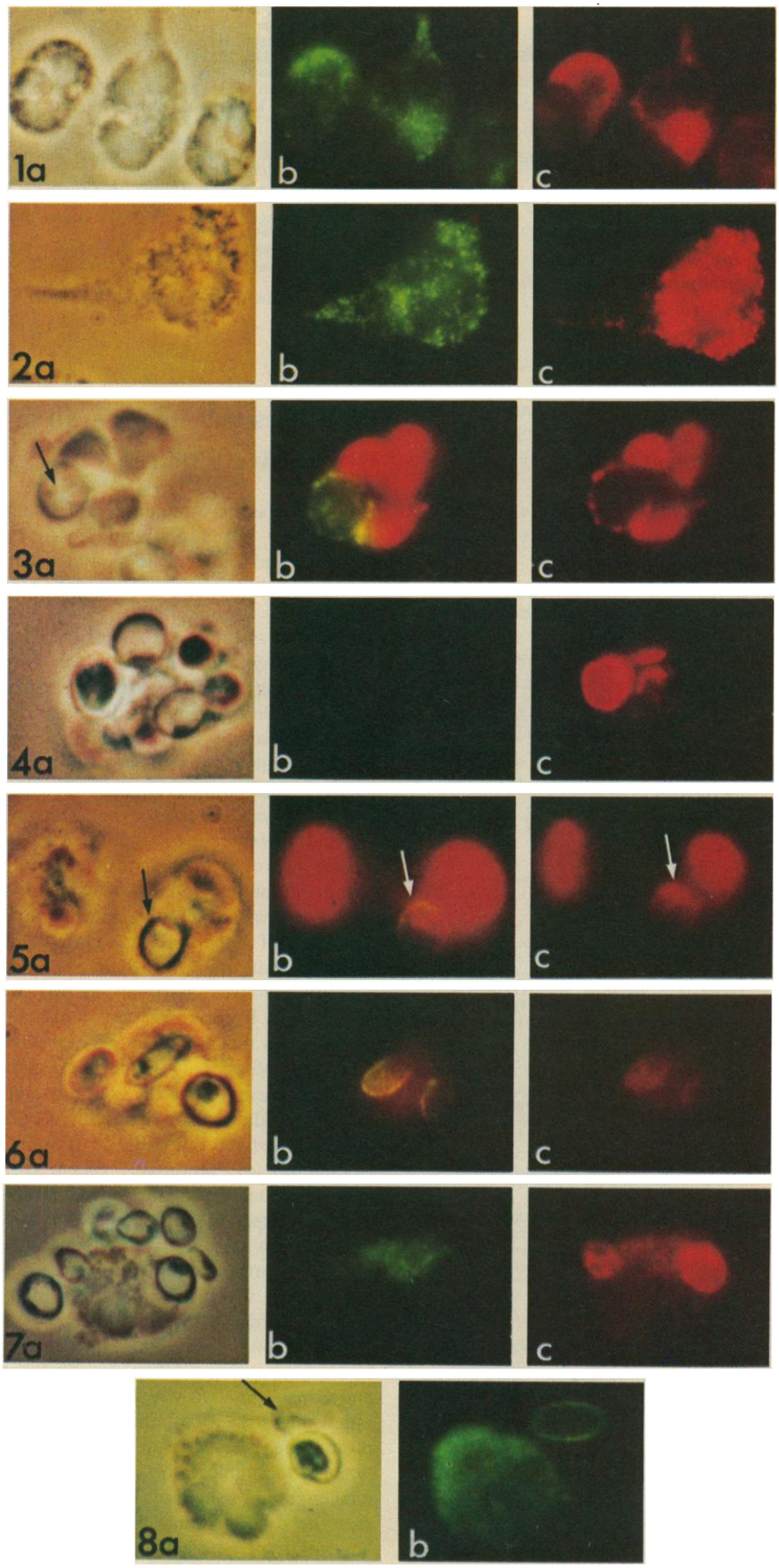

Fig. 1. Intracellular binding of cyclic GMP (b) and cyclic AMP (c) of unstimulated neutrophils after FMA fixation. Cyclic AMP is diffusely distributed throughout the cytoplasm; cyclic GMP staining is somewhat more granular. In this and the following figures, phase microscopy is shown in (a). Fig. 2. Intracellular binding of myeloperoxidase (b) and lactoferrin (c) of unstimulated neutrophils after FMA fixation. Both granule markers are distributed at the cell periphery, cytoplasm, and uropod. Subtle differences in the distribution of myeloperoxidase and lactoferrin are seen. Fig. 3. Surface binding of myeloperoxidase (b) and lactoferrin (c) of neutrophil challenged with zymosan. Cells were counterstained with methyl green to permit nuclear orientation. One zymosan particle is outlined by both markers (arrow), presumably as granule contents leak from incompletely fused phagolysosomes. Fig. 4. Surface binding of cyclic GMP (b) and cyclic AMP (c) of neutrophil challenged with zymosan. Note that the neutrophil is associated with more than five particles, but only three show binding for cyclic AMP. Cyclic GMP is not observed. Fig. 5. Surface binding of myeloperoxidase (b) and cyclic AMP (c) after challenge with zymosan and counterstaining with methyl green. Cyclic AMP was always observed coincident with the azurophil granule marker (arrow). Fig. 6. Surface binding for lactoferrin (b) and cyclic AMP (c) after challenge with zymosan and counterstaining with methyl green. Cyclic AMP was always observed coincident with the specific granule marker. Fig. 7. Intracellular binding of cyclic GMP (b) and cyclic AMP (c) after fixation with FMA in neutrophils challenged with zymosan. Intense cyclic AMP staining is associated with only a few particles, whereas cyclic GMP is observed throughout the cytoplasm. Fig. 8. Intracellular cytoplasmic binding of R^I (b) after fixation in neutrophils challenged with zymosan. The R^I is also associated with one particle in the process of ingestion (arrow); the other particle is negative, probably because phagocytosis has not been initiated.

AMP and cyclic GMP during phagocytosis implies a difference in mechanism of action for these nucleotides. Since cyclic GMP is consistently found within the granular cytoplasmic region of the cell, it is likely, as others have suggested (*8, 13, 21*), that cyclic GMP plays a role in the functional process of secretion.

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Food Dyes and Impairment of Performance in Hyperactive Children

We discuss here a number of issues which were not directly addressed by Swanson and Kinsbourne (*1*) but which are essential to proper interpretation of their results.

The first issue relates to criteria for diagnosing hyperactivity. Although there is continuing debate regarding the propriety or utility of using the diagnostic label "hyperactivity" (hyperkinetic syndrome, minimal brain dysfunction), the label remains widely applied. The descriptors of and criteria for the syndrome are undergoing change (*2*), but as far as we are aware, Swanson and Kinsbourne (*3*) are the only advocates of using drug response as a confirmatory criterion for a diagnosis of hyperactivity. Thus, it is possible that many of their so-called "nonhyperactives" would indeed be considered hyperactive by most researchers in this area. The general position of professionals is perhaps most clearly represented by the continuing efforts to predict *which* hyperactive children will respond favorably to stimulant medication (*4*). Moreover, recent data indicating that the cognitive and behavioral responses of hyperactive and normal children are qualitatively similar (*5*) appear to invalidate unequivocally any further use of drug response as a diagnostic criterion in hyperactivity.

The second point concerns the between-group focus taken by Swanson and Kinsbourne (*1*) which overlooks an important aspect of the data. Interpreting the three-way interaction by analyzing the simple interaction effects of challenge and test time, they concluded that the dye challenge affects performance of hyperactives but not of "nonhyperactives." However, even a cursory examination of their figure 1 (*1*) reveals that the performance of the two groups was similar under dye challenge but differed on placebo. Thus the interesting between-group difference, and the cause of the significant three-way interaction, is the contrasting placebo functions. While we do not dispute the correctness of their analysis, concurrent examination of the effects of test time within group X challenge conditions is essential to a complete understanding of the results. Comparison of group means derived from their figure 1 suggests significant and similar deteriorations in performance over time in all conditions except the hyperactives after placebo challenge. Thus the data do not permit the conclusion that "the performance of the nonhyperactive group was not affected by the challenge with the food dye blend." Under placebo conditions baseline data reflected the expected superiority of the "nonhyperactive" group, but by the final test session their performance had fallen to a level similar to the hyperactive group which showed no change. Why did the "nonhyperactives" manifest the observed deterioration in performance under the placebo condition? This question must be answered before the findings of the challenge study can be interpreted unambiguously.

Finally, the children in the study (*1*) were involved in a "controlled implementation of the Feingold diet" although no assessment of the diet treatment itself is presented. It should be emphasized that the clinical significance of any "challenge effect" will not be established until it is demonstrated that the same children (or some subset) giving a challenge response aiso show a good diet response in controlled study. Only then could we attribute the day-to-day behav-

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