rectly classified 88 percent of the birds that were subsequently sexed by dissection.

13. The courtship sequence in the moorhen has been described by N. A. Wood [*Br. Birds* **67**, 104 (1974)] and by Petrie (*10*). Courtship attempts are initiated by a bird approaching another and performing a characteristic neck-arching display. The relative frequency of observations of neck-arching by marked males and females is an indication of how often each sex initiates courtship.
14. These results could be a consequence of an excess of marked females. However, of 223 marked adults, 125 were male and 98 were female. This difference was not statistically significant (binomial test, $P = .08$).
15. An agonistic encounter, as defined here, is one in which an animal, by approaching or threatening, causes another to withdraw, or one in which two animals physically fight. Chasing was the most commonly observed encounter, amounting to 75 percent of the encounters observed (*10*).
16. Almost all agonistic encounters had a clear outcome (those that did not were not included in subsequent analyses). One bird was considered dominant if it clearly elicited avoidance or withdrawal or defeated its opponent in a fight. Each individual's performance in aggressive encounters was expressed as the percentage of opponents dominated. This measure takes account of interactions between pairs of birds where only one bird was marked.
17. Relative body weight has been shown to be an important factor determining the outcome of aggressive interactions in other bird species (for example, in the dunnock *Prunella modularis* [M. E. Birkhead, *Ibis* **123**, 75 (1981)].
18. If only those females that were weighed at the same time of year are considered (that is, excluding those whose weights were estimated), the correlation between female weight and the proportion of agonistic encounters won is significant (1979: $r_s = .58$, $P < .05$, $N = 10$; 1980: $r_s = .54$, $P < .05$, $N = 11$).
19. Theoretically, competition among females could occur as a result of a shortage of available male partners in flocks. However, flocks contained approximately equal numbers of males and females. For example, the mean proportion of males and females in one flock during the 1978–79 winter was 49.7 percent and 46.5 percent, respectively (3.8 percent could not be reliably sexed as they were subadult). Moreover, agonistic encounters between females that determined access to males always occurred in the presence of other males. Most of the marked nonterritorial unpaired birds were males: 6 of 10 were males in 1979 and 11 of 15 were males in 1980. See also (*14*).
20. G. A. Clark, *Condor* **81**, 193 (1979).
21. J. A. Bailey, *J. Wildl. Manage.* **32**, 835 (1968).
22. The index involves the assumption that shape remains approximately constant over the size range considered.
23. The fat weight used was the sum of four discrete fat pads, those in the inguinal region and those covering the patella on both sides of the body. The sample was collected in November 1980; birds were dissected within 3 days of death.
24. Unpaired birds were easily identified as they formed small flocks on the area during the breeding season (*10*). The number of unpaired males cannot be expressed as a percentage of paired males in order to estimate the proportion of nonbreeding individuals in the population because the groups of nonbreeding birds may have contained birds from outside the study area.
25. Nests were found by searching the area at weekly intervals and by observation of breeding pairs. After nests were found they were visited at intervals of 1 to 3 days. It was thus possible to record the days on which clutches were started and finished, and the length of the incubation period, with an accuracy of ±3 days. The sample includes pairs for which the start of incubation and the outcome of nesting attempts (successful and unsuccessful) were known.
26. Nest predation rates can be as high as 69 percent (*10*). The following potential predators were observed on the study area: stoats (*Mustela nivalis*); rats (*Rattus norvegicus*); carrion crows (*Corvus corone*); jackdaws (*Corvus monedula*); jays (*Garrulus glandarius*); magpies (*Pica pica*); and foxes (*Vulpes vulpes*).
27. It was not possible to obtain an equivalent result for pairs that were successful in hatching eggs since the sample was too small.
28. The number of eggs that a female can lay does not appear to be a major factor limiting reproductive performance in polyandrous species [D. Lack, *Ecological Adaptations for Breeding in Birds* (Methuen, London, 1968)]. This may also be the case in moorhens; one polyandrous female laid eggs for a second male after laying three clutches for her first male (*10*).
29. Material was not available at this time of year to check this result by dissection. The sample combined 22 males measured in January 1979 with 25 males measured in February 1980. The correlation between male condition and bill length (an alternative measure of size) also gave a negative correlation ($N = 47$, $r = -.35$, $P < .02$).
30. Small males may be in better condition because they have lower absolute food requirements than larger birds. The equation derived by J. Aschoff and H. Pohl [*J. Ornithol.* **111**, 38 (1970)] provides an estimate of heat production (M) of a resting bird: $M = 0.0317\ W \exp 0.726$ where W is weight in grams and M is expressed in kilocalories per hour per bird. Substituting the weight of the smallest male moorhen measured in January ($W = 313$ g) yields $M = 2.055$, and substituting the weight of the largest male ($W = 458$ g) yields $M = 2.709$. Thus, the larger bird had a 32 percent higher heat production and a correspondingly greater energy expenditure. The higher food requirement of larger birds might become critical when the food supply, or time available for feeding, is limited. This is the case for moorhens in winter when there is a significant 12 percent decline in mean adult weight between November and January (mean November weight, 436 g; mean January weight, 386 g; $t = 4.44$, $P < .001$) (*10*).

31. I thank P. Walker for allowing me to work on his land; the Wildfowl Trust, Slimbridge, for providing dissection material; L. Huson, M. Gosling, and H. W. Norton for statistical advice and comment; A. F. G. Dixon for providing the initial opportunity to do this work and for helpful comment; T. R. Halliday, P. H. Harvey, and anonymous referees for their comments on earlier drafts; and L. M. Gosling for his continual support and for helpful discussion. Financial support was provided by the Science Research Council.

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Neutrophil Pseudoplatelets: Their Discrimination by Myeloperoxidase Demonstration

Abstract. *Neutrophils, especially in acute infection or the myeloid leukemias, may shed platelet-sized particles that can readily be distinguished from true platelets because they contain neutrophil myeloperoxidase. This enzyme, unlike platelet peroxidase, is not inhibited by glutaraldehyde. The myeloperoxidase and acid hydrolase levels and continuous plasma membranes of these cell-like particles suggest that they are functional cellular entities. They further differ from platelets in that they contain nuclear remnants, occur in bacteria-laden pus and inflammatory exudates, are ingested by macrophages, and do not adhere to each other or aggregate. They could be involved in the immune response to pathogens or contribute to trauma and healing by facilitating deployment of neutrophil acid hydrolase, neutral protease, and myeloperoxidase.*

While using improved cytochemical procedures for demonstrating peroxidases in granulocytic leukocytes by light and electron microscopy (*1*), we observed peroxidase-positive, platelet-sized particles in the blood of many patients. These particles differ from platelets in that they contain a glutaraldehyde-resistant myeloperoxidase (MPO) rather than platelet peroxidase, which is completely inhibited by glutaraldehyde (Fig. 1, A and B) (*2*). MPO is considered a virtual marker for the neutrophil (*3*), the most common leukocyte in peripheral blood. Unlike platelets, the newly elucidated particles may contain nuclear fragments and appear to be formed from neutrophils. We have named them neutrophil pseudoplatelets (*4*).

In most samples of blood showing such fragments, neutrophils with cytoplasmic extensions or with membranes separating their cell bodies from one of these budding moieties could readily be seen (Figs. 1 and 2). The high levels of MPO and acid hydrolases present in these particles and their continuous plasma membranes suggest that they are functional cellular entities, not merely fragments of degenerating neutrophils.

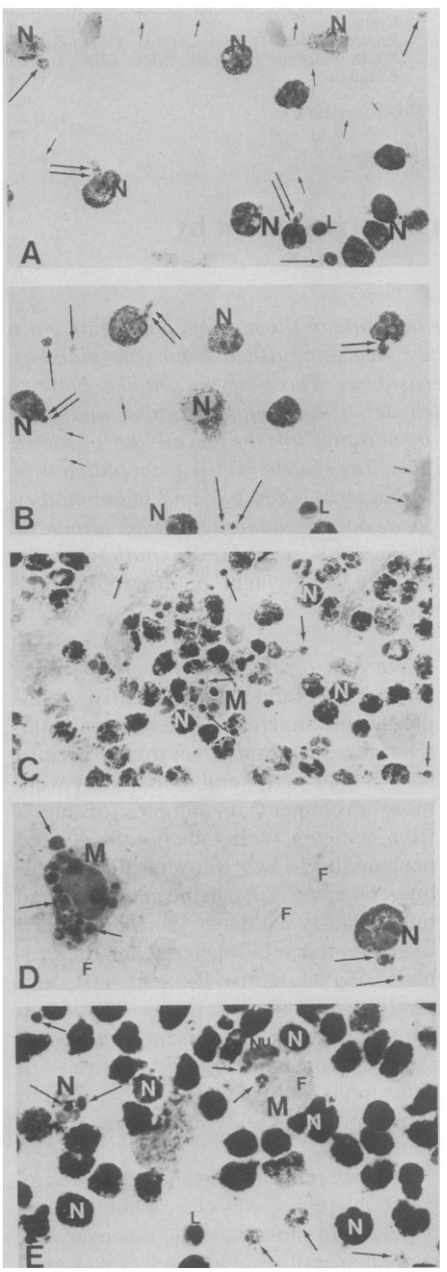
We first observed neutrophil pseudoplatelets in blood and bone marrow samples from myeloid leukemia patients, who may have many neutrophil precursors in their peripheral blood. They were more prominent in samples of blood from patients with infection-associated neutrophilia. They were most conspicuous, however, in samples of pus and inflammatory exudates, which contained large numbers of bacteria and neutrophils (Fig. 1, C to E); platelets were rarely seen in these samples. That pseudoplatelets were not present in some pus samples consisting almost entirely of neutrophils indicates that they are not merely products of dying or degenerating neutrophils.

The size range of particles classified as pseudoplatelets was the same as for platelets. In most patients, however, the median size of pseudoplatelets was considerably larger than that of platelets. Pseudoplatelets showed less of a tendency to assume dendritic or contracted forms than platelets and, unlike platelets, did not appear to adhere to each other or aggregate.

The discrimination of pseudoplatelets from platelets was rarely possible with Romanowsky-type stains such as the

Wright-Giemsa stain, widely used to study cellular morphology and the relative number of different types of leukocytes present in a blood film. Cytochemical methods for demonstrating leukocyte glycogen or acid hydrolases were not helpful because of the presence of these substances in platelets and in leukocytes other than neutrophils.

Pseudoplatelets were not limited to blood, pus, and inflammatory exudates. They also seemed to be present in sections of frozen tissue and in semithin and ultrathin sections of inflamed tissues rich in neutrophils. In these preparations, however, a section through a pseudoplatelet could not be positively discriminated from a section through a neutrophil or through a cytoplasmic extension of a neutrophil.



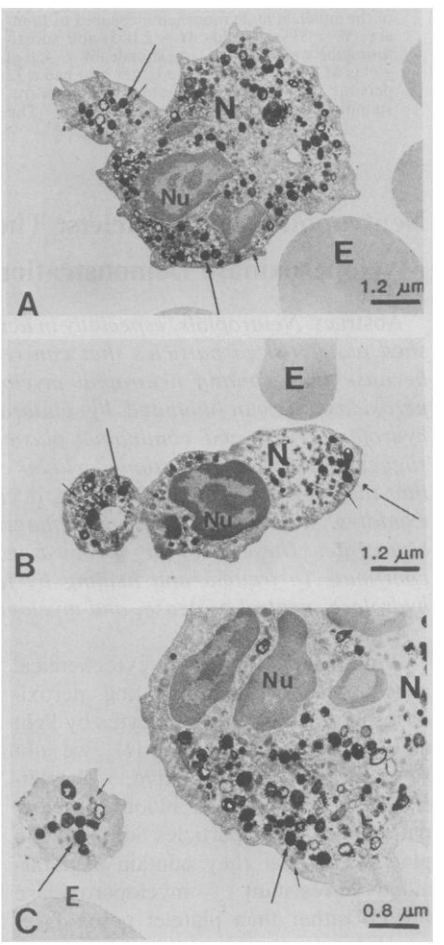


Fig. 1 (left). (A and B) Photomicrographs of human leukocyte preparations treated for the histochemical demonstration of their peroxidases (*1*). Neutrophil pseudoplatelets (large arrows), by virtue of their granular staining for myeloperoxidase activity, are readily discriminated from the barely visible platelets (small arrows), whose peroxidase activity is completely inhibited by the glutaraldehyde fixation. Some neutrophils (*N*) are seen budding (double arrows) to form pseudoplatelets. A dying neutrophil with enlarged, weakly stained nuclear lobes is visible in the center of (B). Nuclei in the neutrophils and lymphocytes (*L*) are visible because of the counterstain. (×950) (C to E) Smears of exudates (pus) from acutely inflamed oral lesions treated for peroxidase demonstration (*1*). Pseudoplatelets (arrows) can be observed inside macrophages (*M*) and neutrophils as well as adhering to these cells. In (E) a cluster of fusiform bacteria (*F*) is visible inside a macrophage that also contains pseudoplatelets (arrows). The nucleus (*Nu*) of this cell is polarized. Magnifications: (C) ×600, (D) ×1200, and (E) ×900. Fig. 2 (right). (A to C) Electron micrographs showing progressive stages of pseudoplatelet formation by budding from a parent neutrophil. Careful inspection reveals unstained secondary granules (small arrows) and primary granules (larger arrows), stained for myeloperoxidase activity, in both the neutrophil cell body and the forming or completely formed pseudoplatelet. *E*, erythrocyte; see legend to Fig. 1 for abbreviations.

The identification of separating or detached pseudoplatelets was unequivocal in films of peripheral blood, bone marrow, or exudates stained by our rapid, precise MPO procedure (*1*) and examined by light microscopy. In these preparations the whole cell or particle was visible, not merely a section of it. That the neutrophil cytoplasmic processes and pseudoplatelets seen in the blood films were not artifacts of the smearing process was shown by their identification with light and electron microscopes in blood and enriched-leukocyte suspensions cast in epoxy slides (Cast-A-Slide, Ted Pella, Inc.) (Fig. 2) (*5*). These cells were harvested, fixed, incubated for demonstration of MPO activity, and embedded entirely while in suspension; they were never smeared or allowed to settle on a surface.

There appear to be alternative mechanisms of pseudoplatelet formation that are analogous to those of platelet production. The almost invariable presence of neutrophils with cytoplasmic extensions in samples containing pseudoplatelets suggests budding from the parent neutrophil. However, the frequent appearance of the nuclear remnants in pseudoplatelets indicates that some type of fragmentation of the parent neutrophil, more extensive than the demarcation process in the megakaryocyte periphery (*6*), may also occur.

The relation of pseudoplatelets to neutrophils is supported by the comparable activities and inhibitor sensitivities of their MPO and by the ultrastructural similarity of their granules. The electron microscope occasionally revealed the ellipsoidal primary neutrophil granules in both neutrophils and pseudoplatelets. This type of granule is not observed in platelets.

Fragmentation of leukemic leukocytes to form platelet-sized particles, which causes spuriously high platelet counts, is well documented (*7*). Since cytoplasmic extensions were infrequently observed in neutrophil precursors and since far larger numbers of pseudoplatelets were observed in the blood of patients with infection-associated neutrophilia and in inflammatory exudates and pus, a derivation of most pseudoplatelets from mature neutrophils is likely.

Some samples of pus and inflammatory exudates consisting almost entirely of neutrophils appeared to be devoid of pseudoplatelets and bacteria. This suggests an association between these fragments and some types of pyogenic bacteria. We frequently observed (i) macrophages ingesting pseudoplatelets in samples rich in bacteria (Fig. 1, C to E) and

(ii) pseudoplatelets and vacuoles containing bacteria in the same macrophage. Pseudoplatelet formation increases the surface area of neutrophils and the contact of their MPO with bacteria. Phagocytosis of pseudoplatelets by macrophages could be involved in their processing of bacterial antigens and in their complex interactions with lymphocytes and plasma cells.

The role of pseudoplatelets in inflammation, however, may not be strictly constructive or reconstructive. Their presence in synovial fluid in arthritic joints (*8*) could contribute to trauma as well as healing by facilitating the distribution of lysosomal hydrolase, neutral protease, and MPO. Indeed, differences in size and possibly chemotactic responses between pseudoplatelets and neutrophils could result in important differences in their distribution during inflammation. Neutrophils perform their most important functions at extravascular sites. The mature neutrophil, with its lobulate nucleus, is generally considered to be an end-stage cell. This nuclear segmentation may facilitate the cell's deformability and passage through the walls of blood vessels. Nuclear hypersegmentation was prominent in neutrophils in our pus samples, which contained many bacteria and pseudoplatelets with nuclear fragments. This suggests that pseudoplatelet formation is a natural consequence of the nuclear lobulation in the neutrophil that augments its role in acute inflammation. Moreover, the small size of the pseudoplatelet apparently permits its deployment to areas relatively inaccessible to the larger neutrophil.

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References and Notes

1. The procedures used were modifications of published methods [R. C. Graham, Jr., and M. J. Karnovsky, *J. Histochem. Cytochem.* **14**, 291 (1966); J. S. Hanker, J. Laszlo, J. O. Moore, *Histochemistry* **58**, 241 (1978); J. S. Hanker *et al.*, *Cancer Res.* **39**, 1935 (1979)]. The light microscopic cytochemical procedure (detailed directions are available from Sigma Chemical Co., St. Louis) has an advantage over earlier 3,3'-diaminobenzidine procedures since it permits the MPO of neutrophil-series cells to be easily distinguished from other granulocyte peroxidases, such as the principal peroxidases of eosinophils and basophils in glutaraldehyde-fixed specimens.
2. J. Breton-Gorius, F. Reyes, G. Duhamel, A. Najman, A. C. Gorin, *Blood* **51**, 45 (1978); D. Catovsky, A. D. Crockard, E. Matutes, M. O'Brien, in *Histochemistry: The Widening Horizons*, P. J. Stoward and J. M. Polak, Eds. (Wiley, Chichester, England, 1981).
3. MPO is present in primary or azurophilic granules of neutrophil-series leukocytes and, to a far lesser extent, in monocytes [P. Murphy, *The Neutrophil* (Plenum, New York, 1976)].
4. For a detailed description of this research, see the paper by J. S. Hanker, W. W. Ambrose, E. T. Arnn, P. E. Yates, and B. L. Giammara (*Histochem. J.*, in press). See also J. S. Hanker and B. L. Giammara, *J. Cell Biol.* **95**, 28 (Abstr.) (1982).
5. B. L. Giammara and J. S. Hanker, *Stain Technol.*, in press; *Proc. Electron Microsc. Soc. Am.* **40**, 358 (1982).
6. J. H. Wright, *J. Morphol.* **21**, 263 (1910); R. P. Becker and P. P. DeBruyn, *Am. J. Anat.* **145**, 183 (1975); M. Shaklai and M. Tavassoli, *J. Ultrastruct. Res.* **62**, 270 (1978).
7. S. A. Stass, M. L. Holloway, R. B. Slease, H. R. Schumacher, *Am. J. Clin. Pathol.* **68**, 350 (1977); J. O. Armitage, J. A. Goeken, J. R. Feagler, *J. Am. Med. Assoc.* **239**, 433 (1978).
8. J. M. Riddle, personal communication.
9. Supported by Navy Medical Research and Development Command contract N00014-82-K-0305 and NIH awards DE 02668 and DE 05333. We are indebted to W. W. Ambrose, P. E. Yates, and S. Carbone for excellent technical assistance; J. O. Moore, T. M. Hassell, J. Nash, and H. J. Towle for patient samples; E. T. Arnn, J. Laszlo, and R. P. White for enlightening discussions; and J. Weaver for typing and collating the manuscript.
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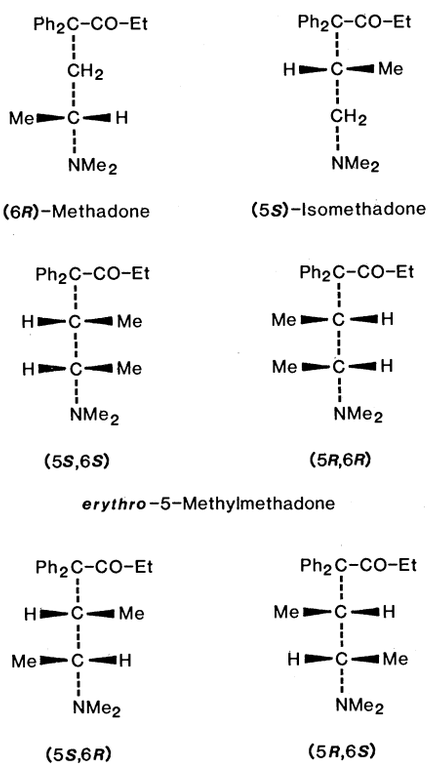
Methadone Conformation and Opioid Activity

Abstract. *The inactive methadone analog* threo-*5-methylmethadone has a solid-state conformation in which the nitrogen is antiperiplanar to the tertiary carbon C(4). Since* threo-*5-methylmethadone exhibits no opioid agonism either in vivo or in vitro, methadone analogs probably do not have this conformation when bound to an opioid receptor. The potent agonist (−)-*erythro-*5-methylmethadone has a solid-state conformation in which the nitrogen atom is rotated back toward the phenyl rings on the quarternary carbon, suggesting that this unusual conformation is the active one.*

The analgesic activity of methadone and some of its derivatives is attributed to its direct binding to opioid receptors (*1*, *2*). There is ample evidence that stereochemistry plays a dominant role in determining opioid receptor affinity (*3*, *4*). However, the inherent flexibility of the methadone molecule has made it difficult to identify unambiguously the molecular conformation that is responsible for receptor binding. The principal source of conformational flexibility in the methadone molecule is the presumed rotation about bonds linking the nitrogen atom and the quarternary carbon atom (Fig. 1A). Crystallographic and spectral analysis of methadone and isomethadone indicated that an antiperiplanar (*5*) arrangement of the charged nitrogen atom and the phenyl-substituted carbon atom may be one of the pharmacophoric conformations (*6*).



Addition of a 5-methyl substituent to methadone altered its activity. In three standard tests the (−) enantiomer of *erythro*-5-methylmethadone was at least five times as potent as (−)-methadone and (−)-isomethadone, whereas the *threo* racemate was totally inactive (*7*–*9*). Inspection of space-filling models showed that the flexibility of methadone is greatly restricted by 5-methyl substitution. The conformation of (−)-*erythro*-5-methylmethadone (Fig. 1C) in the solid state (*7*) is one in which the nitrogen atom is +clinal ($\tau = 97°$) to the phenyl-substituted carbon atom. Molecular mechanics calculations indicate that this is the minimum energy conformation (*10*). Hence the +clinal conformation may be more important than the antiperiplanar arrangement for receptor interaction of methadone and its derivatives. Analysis of the crystal structure of inactive *threo*-5-methylmethadone was undertaken to provide further information about conformational requirements for activity.

threo-5-Methylmethadone has the extended conformation (Fig. 1D). Molecular mechanics calculations indicate that this conformation of the *threo* isomer is