QUANTITATIVE CYTOCHEMICAL OBSERVATIONS ON THE CONTROL OF RESPIRATION IN POLYMORPHONUCLEAR NEUTROPHIL LEUCOCYTES OF AMPHIUMA TRIDACTYLM

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SUMMARY

Quantitative effects of altering oxidative phosphorylation and respiration on the activity of the enzyme menadione reductase (NAD[P]H₂:2-methyl-1,4-naphthoquinone oxidoreductase, E.C. 1.6.5.2), in stabilized polymorphonuclear neutrophil leucocytes of Amphiuma tridactylum, were studied by amplitude-contrast microscopy and microspectrophotometry.

In a general way, the rate of enzymic activity was proportional to ADP concentration and inversely proportional to the concentration of ATP. Terminal respiratory blocking by azide produced selective subtotal inhibition. Uncoupling of phosphorylation by dinitrophenol produced complex results.

Neutrophils of A. tridactylum, irrespective of their stage of maturation in the circulating blood, could be subdivided into 3 metabolic classes: Class I cells, of low enzymic activity (predominantly mitochondrial), greatly activated by ADP, somewhat activated by ATP, and only slightly inhibited by dinitrophenol; Class II cells, twice as active as Class I (in which the endoplasmic reticulum and idiozome were as active as the mitochondria), further activated by ADP used alone or with dinitrophenol, and unaffected by ATP or dinitrophenol; Class III, hyperactive cells (enzymic localization identical with that in Class II), inhibited by ATP and dinitrophenol, and not activated by ADP. Some of the mitochondria of Class III neutrophils retained nearly a third of their reductase activity when the reaction mixture contained 10⁻⁴ M azide.

There is reason to believe that Class I neutrophils may form a reserve population of vegetative cells; in vitro, they can be transformed into Class II cells when a high concentration of ADP is added. Class II and Class III cells are potentially capable of amoeboid movement and phagocytosis. The metabolic mobilization of neutrophils could be interpreted as being controlled by 2 different feedback mechanisms: activation by ADP in cells of Classes I and II, and inhibition by ATP in cells of Class III.

INTRODUCTION

The hypothesis that the relative concentrations of inorganic phosphate (Pᵢ), adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP) regulate the rate of cellular respiration by feedback control at the coupling sites between electron-transfer and oxidative phosphorylation has gained wide acceptance in biochemistry (reviews by Lehninger, 1965, 1967, 1970; Racker, 1965; Green & Maclennan, 1967; Loewy & Siekevitz, 1969). Electrons donated by the glycolytic pathway, tricarboxylic acid and oxidative pentose cycles, reduce NAD or NADP. They are then linked to
the respiratory pigments by a small group of flavoprotein enzymes (the E.C. 1.6 group, Anonymous, 1961), which accept electrons from NADH₂ and NADPH₂ and donate them to the cytochromes (usually, cytochrome b in mitochondria):

\[
\begin{align*}
&\text{ADP} + P_i \\
&NADH_2 \xrightarrow{f_p} Q \xrightarrow{\text{cyt } b} \text{cyt } c \xrightarrow{\text{cyt } a} \text{O}_2 \\
&\downarrow \text{ATP} \uparrow \text{ATP} \uparrow \text{ATP}
\end{align*}
\]

where solid arrows represent electron transport; broken arrows, oxidative phosphorylation; \(f_p\), a flavoprotein; \(Q\), a quinone intermediate (Crane, Hatefi, Lester & Widmer, 1957; Green & Maclennan, 1967). An increase in concentration of ADP and \(P_i\) leads to an increase in electron transfer by feedback reinforcement; an increase in ATP slows down electron transfer by feedback inhibition. A self-balancing homoeostatic system of energy conservation is postulated, and overwhelming evidence in support of this hypothesis has accumulated (reviews by Green & Maclennan, 1967; Lehninger, 1970).

This hypothesis is based on the findings of 2 kinds of work. (i) Work generally carried out by means of Warburg manometry, with tissue slices, isolated mitochondria and other tissue fragments. In this kind of work, the exact topographic sites of metabolic events become unavoidably randomized during the preparative or measuring steps. (ii) Exceedingly ingenious microfluorimetric and spectrophotometric work, carried out either with single isolated cells or samples of isolated organelles (Chance, 1952, 1962; Chance & Williams, 1956; Kohen, 1964; Kohen, Kohen, Legallais & Kun, 1966). In this kind of work great precision in the determination of molecular sites is instantly obtainable, as well as a moderate degree of morphological resolution. Unfortunately, large populations of cells cannot be scanned rapidly; homogeneity of the cell sample has to be assumed. To this day, it is not known with any degree of certainty whether the clear-cut mechanism of control postulated by the currently accepted biochemical hypothesis is equally applicable to the majority of intact cells; how metabolic differences between organelles of a single cell, and different cells of a given population, are controlled (David, de Almeida, Castro & Brown, 1962; David & Brown, 1968); or what significance should be attached to any observed metabolic differences in terms of specific functions carried out by cells.

The work described in this paper was undertaken in an attempt to throw light on some aspects of metabolic control and its relation to cell function in a favourable cell system: polymorphonuclear neutrophil leucocytes from the blood of the urodele *Amphiuma tridactylum*. Neutrophils are thought to form a homogeneous cellular class; they can be obtained in large numbers in favourable condition for quantitative cytochemical work simply by preparing blood smears. Neutrophils are of particular interest because: (i) they can either function as ‘vegetative’ cells (when their intermediate metabolism might be considered to be that which is necessary for the preservation of cellular integrity), or become mobilized into amoeboid and phagocytic activity (reviews by Downey, 1938; Andrew, 1965); and (ii) they are among the few cell types in which cellular aerobic oxidation does not inhibit glycolysis (review by
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Seitz, 1969). *Amphiuma* neutrophils are particularly convenient cells, since they are at least 5 times the diameter of corresponding mammalian cells. We studied a single enzyme system, menadione reductase (NAD[P]H₂:2-methyl-1,4-naphthoquinone oxidoreductase, E.C. 1.6.5.2). Menadione reductase is a suitable flavoprotein (fp, equation 1) for this study, since it is exceedingly active in urodele neutrophils, and its activity can be readily and unequivocally assessed in cytochemistry by the application of improved tetrazolium-reduction methods.

We sought answers to the following questions, (i) Is menadione reductase activity (hence, respiration) controlled by the concentration of ATP in neutrophils (through feedback inhibition) or by the concentration of ADP (through feedback reinforcement)? (ii) To what extent are the coupling of oxidative phosphorylation and a functional respiratory chain necessary for effective rate-control? (iii) Are the level of reductase activity, its subcellular localization, and regulatory mechanisms identical in all neutrophils? (iv) If they are not identical, can the observed differences be related to known functional differences? In practice, the effects of adding varying concentrations of ATP, ADP, 2,4-dinitrophenol, and azide, alone and in combination, on the reduction of a tetrazolium salt by neutrophils incubated in optimum concentrations of NADH₂ and menadione, were measured by scanning microspectrophotometry, and assessed morphologically by amplitude-contrast microscopy.

**MATERIALS AND METHODS**

**Stabilized blood smears**

Specimens of *Amphiuma tridactylum*, were anaesthetized in 3 % (w/v) ethyl carbamate. Coagulation was prevented by the intra-arterial injection of 400 i.u. heparin. Auricular blood was collected in chilled and siliconized Pasteur pipettes. Wet coverglass-smears were stabilized in 0.2 % formaldehyde vapour at 4 °C. Preliminary experiments (Accola, Williamson & David, 1970; David & Accola, 1970; Williamson & David, 1970; G. B. David, in preparation) indicated that the activity of menadione reductase was the same in untreated blood cells, stabilized smears, and lyophilized cells and smears. Stabilized smears consistently yielded a greater number of morphologically undamaged neutrophils than the other methods. The working solution (BPA/BSS, Table 1) was developed empirically for enzymic work with amphibian blood cells.

**Enzymic technique**

Smears were incubated in Columbia dishes in the media listed in Table 2. In preliminary experiments, smears were also incubated in smaller aliquots of solutions made up as those given in Table 2, but containing NADPH₄ instead of NADH₂. Since the 2 coenzymes produced exactly the same results, NADH₂ was used as the substrate in the bulk of the experiments. Solutions made up with either NADH₂ or NADPH₂ as substrate, but with cytochrome c instead of menadione as intermediate acceptor, yielded a different localization of the reaction product when NADPH₄ was the substrate. This provides confirmatory evidence that the enzyme studied here was menadione reductase, E.C. 1.6.5.2, rather than the related flavoproteins E.C. 1.6.2.1 and 3 (the cytochrome c reductases).

The first set of experiments (pp. 725-727) was designed to find out if ATP inhibited menadione reductase activity, and to obtain an idea of the relationship between length of incubation, morphological distribution of the end-product, and optical density measured microspectrophotometrically. Sets of smears were transferred directly from the working solution (Table 1) to each of the following media listed in Table 2: (1), (2) and (3). One set in each medium was incubated for 15, 30, 70, 120 and 160 min at 4 °C. At the completion of the enzymic incubation,
Table 1. Composition of the basic working solution* (BPA/BSS)

<table>
<thead>
<tr>
<th></th>
<th>g/l.</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>3.74</td>
<td>66.7</td>
</tr>
<tr>
<td>KCl</td>
<td>0.32</td>
<td>4.5</td>
</tr>
<tr>
<td>MgSO₄</td>
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<td>0.6</td>
</tr>
<tr>
<td>CaCl₂</td>
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<td>1.5</td>
</tr>
<tr>
<td>NaHCO₃</td>
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<td>20.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>8.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Albumin†</td>
<td>65.00</td>
<td>—</td>
</tr>
</tbody>
</table>

* Dissolved in a 5 mM Sorensen buffer, pH 7.1 (1.34 g/l. Na₂HPO₄·7 H₂O + 0.69 g/l. NaHjPCVHjO).
† Crystallized bovine plasma albumin (Sigma).

Table 2. Concentration of reactants, mM in the incubation media used*

<table>
<thead>
<tr>
<th>Medium</th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
<th>(7)</th>
<th>(8)</th>
<th>(9)</th>
<th>(10)</th>
</tr>
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<tbody>
<tr>
<td>NADH₂</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Menadione**</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>TNBT†</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DNP†</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<td>1</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Dissolved in 10-ml aliquots of BPA/BSS (Table 1), buffered to pH 7.1, and used at 4 °C.
** 2-methyl-1,4-naphthoquinone bisulphide (Sigma).
† 2,2',5,5'-tetra-p-nitrophenyl-3-3'-(3-3'-dimethoxy-4-4'-phenylene) ditetrazolium chloride (Sigma).

The coverglasses were washed in the working solution, briefly rinsed in the same solution but without albumin, dipped momentarily in H₂O, and mounted on ordinary slides with Uvinert (G. T. Gurr) aqueous mounting medium. The finished slides were examined within 24 h, and later scanned with the microspectrophotometer. Such preparations were not permanent, since they were essentially unfixed. The first signs of deterioration were nuclear swelling and the gradual appearance of Novikoff artifacts (Novikoff, 1959, 1963), crystalline precipitates at lipid-aqueous interfaces.

The second set of experiments (pp. 727–733) was designed to permit a detailed comparison of the effects of inactivating the electron-transport chain by blocking with azide the transfer of electrons to oxygen (equation 1), uncoupling oxidative phosphorylation with 2,4-dinitrophenol, in the presence and absence of ADP and ATP (ADP, ATP and dinitrophenol are collectively described in this paper as 'adjuvants'). Sets of smears were pretreated for 15 min at 4 °C in media prepared as media (1), (2), (4), (5), (6), (8), (9), (10) of Table 2, but without NADH₂, menadione or TNBT. They were then incubated in the appropriate full media given in Table 2, for 22 min at 4 °C. The after-treatments and methods of observation were the same as in the first set. The entire second set of experiments was replicated once, and parts of it several times.

As additional controls, some stabilized smears were mounted in Uvinert directly from the working solution, or after incubation for 22 min at 4 °C in 10⁻³ M 2,4-dinitrophenol. They were
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then examined microspectrophotometrically. At the relevant wavelength ranges, the measured absorbances of the untreated stabilized smears did not exceed 0.015; those of the smears treated in dinitrophenol, 0.020. Therefore, light-losses due to refractive effects and dinitrophenol staining did not contribute significantly to the absorbance values recorded in the main experiments (though they were taken into account).

The preparative procedures described above represent improvements over those used previously for the study of other amphibian blood-cells (Accola et al. 1970; David & Accola, 1970; Williamson & David, 1970), and altogether different cell types (David, 1964; Fahimi & Amanasingham, 1964; Fahimi & Karnovsky, 1966; David & Brown, 1968). The use of large amounts of crystallized albumin, and low incubation temperatures in a 'protective' medium, were introduced nearly a decade ago (David et al. 1962; see also discussion in David & Brown, 1968). Both were found to be essential for morphologically adequate results.

May–Grünwald/Giemsa smears

Since leucocytes are ordinarily examined in blood films stained in Romanovsky dyes, a number of air-dried coverglass smears were treated by the May–Grünwald/Giemsa method. The procedure described by Tooze & Davies (1967) was followed exactly.

Instrumental

Morphological observations and photomicrography were carried out by amplitude-contrast microscopy (David & Williamson, 1971), with a special instrument utilizing flat-field apochromat objectives (David & Nomarski, 1970). Amplitude-contrast microscopy was found to be invaluable for this work because of the super-modulation characteristics of the instrument when used for the examination of optically 'noisy' objects (see David & Williamson, 1971), and its exceptionally shallow depth of field — permitting effective optical sectioning. The photomicrographs illustrating this paper were taken with the oil-immersion planapochromat objective, N.A. 1.3, at full aperture (immersed condenser), in light of 436, 546 or 577 nm, isolated from a stabilized Osram HBO 100 W/2 arc by an interference wedge (Veril S-200, Schott/Mainz).

Microspectrophotometry was usually carried out at 560 nm, near the absorption peak of the bis-formazan of TNBT, with a calibrated Vickers M85 scanning microspectrophotometer. Integrated scanning, area, and 'plug' (Mendelsohn, 1966) measurements were recorded. Care was taken to compensate for glare, scattered light, and depth of field (see below). These measurements provided the bulk of the data needed for adequate statistical analysis. 'Plug' measurements at high magnifications were also carried out with a modified Zeiss MPM 1 microphotometer. Microspectrophotometric measurements in this paper are expressed as 'mean cytoplasmic absorbances', at 560 nm, $E_{\text{cyt}}$. In principle,

$$ E_{\text{cyt}} = \log T^{-1} $$

and

$$ T = I/I_0, $$

where $T =$ transmittance, $I_0 =$ the intensity of the light transmitted by the cytoplasm of a neutrophil, and $I_0 =$ the intensity of the light incident on the cell. A careful 'plug' measurement potentially yields a reasonable approximation of $E_{\text{cyt}}$ (Mendelsohn, 1966). In a scanning integrating microspectrophotometer, such as the Vickers M 85, $E_{\text{cyt}}$ is related to the indicated value, $E$ thus:

$$ E_{\text{cyt}} = E k_1 k_2 (k_3 A)^{-1}, $$

where $k_1 =$ a constant, accounting for the absorbance × projected area of the nucleus, the Schwarzschild-Villiger effect, and light lost through scattering and refraction; $k_2 =$ the absorbance proportionality constant of the instrument; $k_3 =$ the area proportionality constant; $A =$ the area measurement in arbitrary units appearing in the instrument readout. Using the x 10, N.A. 0.25 objective of the M 85, and a flying spot 4 μm in diameter, the regression of $E_{\text{cyt}}$ over $E$ was found to be linear, within ± 1%, when $k_1 = 1.34$, $k_2 = 76.1$, and $k_3 = 446$. The area of the neutrophils, in mm², was given by $k_3 A$. 
RESULTS

General remarks

In stabilized smears of *Amphiuma* blood, polymorphonuclear neutrophil leucocytes were diskoidal; their average diameter, based on flying-spot measurements of 320 cells, was 38.7 ± 1.2 μm, and they were about 4 μm thick. Their average integrated projected area was 1172 ± 116 μm². These dimensions were independent of the method of treatment; the variability of dimensions between neutrophils in a single smear was greater than those between smears incubated in different media.

In smears stained by the May-Grünwald/Giemsa method (Figs. 8–9), both the nucleus and the granular endoplasmic reticulum were intensely basiphil. The appearance of the latter was reminiscent of Nissl bodies in Purkinje neurons of vertebrates (David, 1964). Specific granules were very lightly and metachromatically stained (Fig. 9). These results are somewhat at variance with those described by Tooze & Davies (1968) who, using exactly the same technique, found the cytoplasm to be totally unstained. We have seen unstained neutrophils, but these invariably were damaged cells. Neutrophils that we considered to have been well preserved (e.g. Figs. 8, 9), closely resembled Tooze & Davies' (1968) electron micrographs of neutrophils of *Triturus cristatus*.

Three stages of neutrophil maturation could be identified in the smears: cells which might be classified as early metamyelocytes, in which the nucleus was sausage-shaped, frequently arcuate, but not convoluted; possibly late metamyelocytes, in which the nuclear 'sausage' was quite twisted and convoluted, but of nearly even diameter throughout its length; and 'segmented' neutrophils, in which the nucleus was convoluted and pinched into discrete segments, interconnected by a tenuous tube of karyoplasm (Jordan, 1938). Since no differences in cell size, enzymic localization, general level of enzymic activity, or response to the adjuvants and azide, could be discerned between the 3 stages of maturation, they were grouped together for analysis.

Neutrophils could be identified easily in stabilized smears after the menadione reductase reaction, since they were the most active cells of the myeloid series. Furthermore, their unique nuclear morphology and distribution of cytoplasmic organelles remained incontrovertibly recognizable.

In electron micrographs of neutrophils of urodeles (Tooze & Davies, 1968), the cytoplasm is seen to be filled by inclusions of many types: abundant smooth and rough endoplasmic reticulum and ribosomes, small mitochondria, characteristic ‘specific granules’, a few compound globules, bundles of microtubules and filaments, and a prominent idiozome. The idiozome consists of a spherical region containing a pair of centrioles and many microtubules, surrounded by a palisade of mitochondria and packed elements of smooth endoplasmic reticulum. These features are very similar to those seen in neutrophils of mammals (Policard & Bessis, 1955; Fawcett, 1966, p. 53). In high-resolution amplitude-contrast micrographs of neutrophils in smears (e.g. Figs. 8–19), there was no difficulty in recognizing the endoplasmic reticulum (Figs. 8–9, 11–17), mitochondria (Figs. 10–19), specific granules (Figs. 9, 16), com-
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pound globules, and the idiozome (Figs. 11-17). Structures that might possibly represent bundles of microtubules or filaments, were also occasionally seen. The idiozome is the most prominent cytoplasmic structure; in optical sections, the relative location of, and close association between mitochondria and endoplasmic reticulum, are seen with particular clarity. Mitochondria were found to be filamentous, and smaller than those in erythrocytes and thrombocytes of the same species: most were about 150 nm in diameter, and shorter than 4 μm; they were frequently coiled. It was not possible to distinguish between granular and smooth endoplasmic reticulum by amplitude-contrast microscopy, other than by their localization within the cytoplasm. Furthermore, it was not always easy to decide whether a particular structure was endoplasmic reticulum or packed mitochondria. Positive identification required careful optical sectioning by amplitude-contrast microscopy. Mitochondria disappear when the focus of the objective is changed by about 0.5 μm; membranes of the endoplasmic reticulum, when oriented parallel to the optical axis of the microscope, remain in apparent focus (Figs. 12-15, 16-17). When the membranes are oriented at right angles to the optical axis (i.e. surface view) they are invisible. Specific granules were found to be uniformly distributed in the cytoplasmic ground-substance; their shape varied from subspherical to rod-like, with an average diameter of 0.1-0.5 μm (Tooze & Davies, 1968; David & Williamson, 1971, figs. 7-8). A few compound globules, 0.6-1.0 μm in diameter, were also sometimes seen.

After the menadione reductase reaction, the enzymic activity was predominantly mitochondrial in approximately one third of the neutrophils. In the remaining two thirds, the endoplasmic reticulum and idiozome were as reactive as or more reactive than the mitochondria. Neither all mitochondria nor all aggregates of the endoplasmic reticulum were equally active. The cortex of compound globules was sometimes active. No activity was detectable in the centrioles, structures presumed to be microtubules and filaments, specific granules, or nucleus. There were large differences in the level of activity between different neutrophils, even in the same smear. This pattern of localization of menadione reductase activity differs from that in other blood cells of Amphiuma (Accola et al. 1970; David & Williamson, 1971) or other cells of mammals, such as muscle (Fahimi & Amarasingham, 1964; Fahimi & Karnovsky, 1966), but resembles that in nervous tissues of vertebrates (David et al. 1962; David, 1964).

Quantitative effects of ATP

The results of the first set of experiments are summarized in Fig. 1 and Table 3. In a general way, these results confirm the accepted hypothesis about the rate-limiting effects of ATP through feedback inhibition. ATP feedback inhibition is probably more intense than indicated in Fig. 1 and Table 3. After the longer incubation periods, some neutrophils had absorbances in excess of 1.3, beyond the range of linear measurement of our microspectrophotometer; of necessity, a disproportionately large number of the less heavily stained cells were selected for measurement in smears incubated in 10^-3 M ATP or no ATP. At the other end of the time scale, the heavily stained cells probably received disproportionate representation, since they were more readily identifiable under the microspectrophotometer than the least reactive neutro-
Fig. 1. Effect of added ATP on menadione reductase activity in *A. tridactylum* neutrophils. Each point represents the mean of measurements on 10 cells. Ordinate: absorbance at 560 nm. Abscissa: time of incubation. Δ—Δ, no ATP added; ×—×, 10⁻³ M ATP; ○—○, 10⁻² M ATP.

Table 3. Absorbance at 560 nm of neutrophils: means of 10 cells, ± 0.04, as a function of concentration of ATP in the incubation medium, and time of incubation at 4 °C

<table>
<thead>
<tr>
<th>Min of incubation</th>
<th>Concentration of ATP, mM</th>
<th>Means ± 0.02</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>15</td>
<td>0.36</td>
<td>0.30</td>
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<tr>
<td>30</td>
<td>0.59</td>
<td>0.47</td>
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<tr>
<td>70</td>
<td>0.93</td>
<td>0.70</td>
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<tr>
<td>120</td>
<td>0.99</td>
<td>0.85</td>
</tr>
<tr>
<td>160</td>
<td>1.24</td>
<td>1.20</td>
</tr>
<tr>
<td>Means, ± 0.016</td>
<td>0.80</td>
<td>0.70</td>
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</table>

Analysis of variance

<table>
<thead>
<tr>
<th>D.F.</th>
<th>Mean square</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between concentrations of ATP</td>
<td>2</td>
<td>3.459</td>
<td>25.86</td>
</tr>
<tr>
<td>Between times of incubation</td>
<td>4</td>
<td>2.4074</td>
<td>179.7</td>
</tr>
<tr>
<td>Interaction</td>
<td>8</td>
<td>0.1792</td>
<td>13.4</td>
</tr>
<tr>
<td>Residual</td>
<td>135</td>
<td>0.0134</td>
<td>—</td>
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</table>
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The statistical significance of the results was assessed by an analysis of variance (Fisher, 1950, 1951).

Menadione reductase activity was proportional to the time of incubation and inversely proportional to the concentration of ATP; furthermore, there were inconsistencies (interaction) in the effects of ATP after different times of incubation. All differences were significant at the 0.1% level of confidence. The standard deviation of a single measurement corresponded to a coefficient of variation of 18.7%. The coefficient of variation of the absorbances given in the body of Table 3 was 5.6%. The observed interaction indicates a methodological difficulty. Inconsistencies might be attributed to the comparatively inefficient inhibition by ATP after 15 min incubation. Presumably, the very high inherent activity of menadione reductase in neutrophils, even at 4 °C, resulted in the formation of substantial amounts of reaction product before the intracellular concentration of ATP became equilibrated with that of the medium. For this reason, in the second set of experiments (pp. 727-733), neutrophils were equilibrated with the adjuvants before being placed in the enzymic incubation media.

A more interesting anomaly can be seen when the analysis is extended. It follows from the mathematical premisses of the analysis of variance (Johnson, 1949) that the absorbance of an individual neutrophil differs significantly from that of the corresponding group mean (for a given level of ATP and time of incubation) if the difference between them exceeds a criterion, $d$:

$$d = \pm t_{(0.05)} \cdot \sqrt{s^2 \cdot \sqrt{1 + (1/n)}}$$

where $t_{(0.05)}$ = the 5% value of the $t$ distribution for 135 degrees of freedom (D.F.) (Fisher & Yates, 1953, table 111), $s^2$ = the residual variance given in Table 3, and $n$ = the number of cells on which the estimate of the group mean is based (10 for a given level of ATP and time of incubation).

When this criterion was applied to the data it became obvious that approximately 10% of the neutrophils were very much more active and 10% much less active than could be explained by sampling fluctuations within a homogeneous population. For instance, a neutrophil had an absorbance of 0.82 after 30 min incubation without ATP (group mean = 0.59); in another neutrophil, $E = 0.38$ after 120 min incubation with $10^{-3}$ M ATP (group mean = 0.85). Furthermore, amplitude-contrast microscopy indicated that in the cells with low activity, the activity was predominantly mitochondrial, whilst in the remaining neutrophils, the idiozome and endoplasmic reticulum were also active. These results suggest the existence of 3 metabolic varieties of neutrophils.

Comparisons of the effects of adjuvants

The results of the second set of experiments are summarized in Tables 4–6. The effects of terminal respiratory blocking by azide were analysed separately (Table 6) because the variance of the distribution of absorbances after its use was substantially different from those obtained after ATP, ADP, dinitrophenol and their combinations. Each value in the body of Table 4 is the mean of 100 cells. The analysis of variance
Table 4. Effects of ATP, ADP and DNP on the activity of menadione reductase in neutrophils: mean absorbances at 560 nm of 100 cells, ± 0.01, after incubation for 22 min at 4 °C in 3 mm NADH₂, 1 mm menadione, 1 mm TNBT

<table>
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<tr>
<th>Adjuvants</th>
<th>E, ± 0.01</th>
<th>% change, ± 1.7 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.49</td>
<td>0</td>
</tr>
<tr>
<td>1 mM ATP</td>
<td>0.40</td>
<td>-18</td>
</tr>
<tr>
<td>1 mM ADP</td>
<td>0.59</td>
<td>+21</td>
</tr>
<tr>
<td>1 mM DNP*</td>
<td>0.43</td>
<td>-12</td>
</tr>
<tr>
<td>1 mM ATP +</td>
<td>0.48</td>
<td>-2</td>
</tr>
<tr>
<td>1 mM DNP</td>
<td>0.62</td>
<td>+26</td>
</tr>
<tr>
<td>Mean, ± 0.004</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

Analysis of variance

<table>
<thead>
<tr>
<th>D.F.</th>
<th>Mean square</th>
<th>$e^{11}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between adjuvants</td>
<td>5</td>
<td>0.7296</td>
<td>956.1</td>
</tr>
<tr>
<td>Residual</td>
<td>594</td>
<td>0.0076</td>
<td>—</td>
</tr>
</tbody>
</table>

*2,4-dinitrophenol.

indicated that a single neutrophil had a coefficient of variation of 17.4%, and that the difference in the effects of the adjuvants was significant at the 0.1% level of confidence. The percentage inhibition (−) or activation (+) of menadione reductase activity produced by each adjuvant is given in the right-hand column of Table 4. Confidence limits for these values were established by using the criterion $d$ (equation 5). On this basis, all the mean absorbances were different from the grand mean and from one another, with the exception of that corresponding to the combined effects of ATP and dinitrophenol (DNP).

At a first glance, then, ATP and dinitrophenol, used alone, very significantly inhibited menadione reductase activity, and ADP very significantly activated it. This is well in agreement with established biochemical knowledge. However, when ATP and dinitrophenol were used together, their effects seemingly cancelled each other out.

These results, interesting though they are, represent only statistical effects: they represent the average mechanisms of control of electron transport integrated over large numbers of neutrophils. Therefore, they are the cytochemical equivalent of results obtained with biochemical homogenates. A somewhat different picture emerges when the reactions of individual neutrophils are considered.

Activity curves were plotted as histograms of the percentage distribution of neutrophils having absorbances between 0.00 and 1.20, in steps of 0.05, after incubation for the demonstration of menadione reductase, with and without adjuvants and their combinations. They are given in Figs. 2–7.

Fig. 2 indicates that, in the absence of adjuvants, the range of reductase activity in different neutrophils can vary by a whole logarithmic order, and that it is a multimodal distribution. Glancing at Figs. 3–7 we see that different adjuvants operated at different portions of the activity curve. For instance, ATP (Fig. 3) and dinitrophenol
Fig. 2. Histogram showing the distribution of menadione reductase activity in neutrophils incubated without adjuvants.

Fig. 3. Histogram showing the distribution of menadione reductase activity in neutrophils incubated in the presence of 10^{-4} M ATP.

(Fig. 5) inhibited the most active neutrophils; ADP (Fig. 4) only activated the less-active cells. The results of the first set of experiments (pp. 725–727) suggested the desirability of defining metabolic classes of neutrophils. This could be done on the basis of the analysis of variance (Table 4) and the histogram of the menadione reductase activity in the absence of adjuvants (Fig. 2). Let \( E \) = the absorbance of a single cell; \( \bar{E} \) = the mean absorbance of all the neutrophils analysed (0.50, Table 4); \( t_{(0.01)} \) and \( t_{(0.05)} \) = the 1% and 5% points in Student’s \( t \)-distribution (Fisher & Yates, 1953, table III), corresponding to D.F.\( \to \infty \); \( s^2 \) = the residual variance of the entire distribution (0.0076, Table 4); \( n \) = the number of neutrophils making up the estimate.
Fig. 4. Histogram showing the distribution of menadione reductase activity in neutrophils incubated in the presence of $10^{-5}$ M ADP.

Fig. 5. Histogram showing the distribution of menadione reductase activity in neutrophils incubated in the presence of $10^{-4}$ M dinitrophenol.

of $E^*$; $E(I)$, $E(II)$ and $E(III)$ = the absorbance ranges characterizing metabolic classes I, II and III. We define:

Class I,

$$E(I) < E^* - [t_{(0.05)} \cdot \sqrt{s^2 \cdot \sqrt{1 + (1/n)}}];$$

Class II,

$$E(II) = E^* \pm [t_{(0.05)} \cdot \sqrt{s^2 \cdot \sqrt{1 + (1/n)}}];$$

Class III,

$$E(III) \geq E^* + [t_{(0.05)} \cdot \sqrt{s^2 \cdot \sqrt{1 + (1/n)}}].$$

(6)
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Fig. 6. Histogram showing the distribution of menadione reductase activity in neutrophils incubated in the presence of $10^{-3}$ M ATP and $10^{-3}$ M dinitrophenol.

Fig. 7. Histogram showing the distribution of menadione reductase activity in neutrophils incubated in the presence of $10^{-3}$ M ADP and $10^{-3}$ M dinitrophenol.

Class I is assumed to contain those neutrophils the menadione activity of which was so low ($E < 0.25$) as probably to place them in a separate population from that of the remaining neutrophils. Class III ($E \geq 0.75$), conversely, accounts for a hypothetical hyperactive population. Class II ($0.35 \leq E \leq 0.64$) comprises a third population. Neutrophils with activities intermediate between Class I and II, or II and III, presumably represent limiting values of the main classes.

Now, we can re-group the results of the second set of experiments in terms of absorbance classes – hence, metabolic classes – (Table 5), and reinterpret the histograms (Figs. 2–7). The percentages of neutrophils falling within each of the classes defined by equation (6), and their mean absorbances, are given in Table 5.
Table 5. Percentage neutrophils with absorbances at 560 nm between given limits, and mean absorbances (between parentheses), after incubation for 22 min at 4 °C in 3 mM NADH, 1 mM menadione, 1 mM TNBT and adjuvants

<table>
<thead>
<tr>
<th>Metabolic class</th>
<th>Absorbances</th>
<th>0</th>
<th>1 mM ATP</th>
<th>1 mM ADP</th>
<th>1 mM DNP</th>
<th>1 mM ATP + 1 mM ADP</th>
<th>1 mM ATP + 1 mM DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>E &lt; 0.25</td>
<td>11</td>
<td>3</td>
<td>25</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.25–0.34</td>
<td>(0.18)</td>
<td>(0.19)</td>
<td>(0.16)</td>
<td>(0.21)</td>
<td>(0.21)</td>
<td>(0.21)</td>
</tr>
<tr>
<td>II</td>
<td>0.35–0.64</td>
<td>60</td>
<td>57</td>
<td>83</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.65–0.74</td>
<td>(0.48)</td>
<td>(0.51)</td>
<td>(0.47)</td>
<td>(0.48)</td>
<td>(0.55)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>E ≥ 0.75</td>
<td>15</td>
<td>4</td>
<td>7</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.87)</td>
<td>(0.82)</td>
<td>(0.82)</td>
<td>(0.81)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Effects of NaN₃ on the activity of menadione reductase in neutrophils; mean absorbances at 560 nm, of 50 cells, ±0.007, after incubation for 22 min at 4 °C in 3 mM NADH, 1 mM menadione, 1 mM TNBT, 100 mM NaN₃, and adjuvants

<table>
<thead>
<tr>
<th>Adjuvants</th>
<th>E, ± 0.07</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>1 mM ATP</td>
<td>0.09</td>
</tr>
<tr>
<td>1 mM ADP</td>
<td>0.11</td>
</tr>
<tr>
<td>Mean</td>
<td>0.116 ± 0.004</td>
</tr>
</tbody>
</table>

Analysis of variance

<table>
<thead>
<tr>
<th>D.F.</th>
<th>Mean square</th>
<th>ε²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.0466</td>
<td>19.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>147</td>
<td>0.0024</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

We see from the first column of Table 5 that there are 3 symmetrical distributions of neutrophils: Classes I, II, and III. Neutrophils of Class I were activated by ADP, ADP combined with dinitrophenol, and ATP combined with dinitrophenol, into Class II (+ ~ 200%); by ATP alone, half-way to Class II (+61%); and they were slightly inhibited (—11%) by dinitrophenol alone. Class II neutrophils were activated by ADP, alone or combined with dinitrophenol, half-way to Class III (+12–15%); they were essentially unaffected by ATP, dinitrophenol, and ATP combined with dinitrophenol. Class III neutrophils were inhibited by ATP and dinitrophenol down to Class II (—45 to —47%), and essentially unaffected by ADP, alone or combined.
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with dinitrophenol; they were not activated by any of the adjuvants. Considering these findings in conjunction with Figs. 2–7, it becomes clear that the system of classifying neutrophils into 3 metabolic classes reflects qualitative differences in control mechanisms. Further, adjuvants activated or inhibited stochastically, not continuously. Individual neutrophils were shifted by multiples of one-half class interval, not differentially.

Turning to amplitude-contrast microscopy (Figs. 10–19), we see that the localization of menadione reductase activity differed in the 3 metabolic classes. In Class I neutrophils (Fig. 10) the activity was predominantly mitochondrial; the idiozome and endoplasmic reticulum were not very active. In Classes II (Figs. 12–15) and III (Figs. 11, 16–17) the mitochondria were more elongated; the endoplasmic reticulum and, more especially, the idiozome, became exceedingly active. The filamentous character of mitochondria was well preserved by all the incubation media, except no. 7 (Table 2) – which contained $10^{-9}$ M dinitrophenol, and neither ATP nor ADP. Here, the mitochondria were slightly swollen, and sometimes fragmented. The differences in localization between classes were the same in neutrophils incubated with and without adjuvants.

Effects of terminal blocking

The effects of terminal blocking of the electron-transport chain with $10^{-4}$ M azide, on the activity of menadione reductase, are summarized in Table 6 (p. 732). By and large, the reductase activity decreased to rather less than one quarter of that demonstrable when the electron-transport chain was intact. The analysis of variance (Table 6) indicated that there were statistically significant differences in the level of reductase activity between cells treated in azide alone, and in azide with the adjuvants, ATP and ADP. Furthermore, the reductase activity of cells treated in ATP and ADP was not significantly different from the mean activity of azide-treated cells, or from each other. Utilizing the criterion $d$ (equation 5), we see that the comparatively high activity of neutrophils treated in azide alone was attributable to about 10% of the cells, which had absorbances of the order of 0-24–0-26. These neutrophils probably were of Class III, judging from Fig. 2, from their further inhibition by ATP (Table 6), and lack of activation by ADP. The appearance of azide-treated neutrophils is illustrated by Figs. 18 and 19. It will be seen that the residual activity is almost entirely mitochondrial, and that some mitochondria are very much more active than others. The interpretation of the morphological results is somewhat complicated because azide, in contrast to the adjuvants, induced both mitochondrial swelling and fragmentation.

DISCUSSION

General validity

The results of the experiments described in this paper indicate that the quantitative cytochemical study of menadione reductase activity in stabilized urodele neutrophils by microspectrophotometry is a valid method of approaching the question of control of cellular respiration. Known activators, inhibitors and poisons (e.g. ADP, ATP and azide) produced predictable and consistent results. In contrast to, say, manometric
methods, no assumptions need be made concerning homogeneity of the sample of cells studied – indeed, homogeneity was impugned by the results. In contrast to the elegant microfluorimetric methods developed by the Johnson Foundation (Chance & Williams, 1956; Chance, 1962; Kohen, 1964; Kohen et al. 1966), scanning microspectrophotometry after the tetrazolium reaction permits the rapid accumulation of measurements in hundreds of cells. Furthermore, the combined use of amplitude-contrast microscopy and microspectrophotometry has the advantage of allowing a comparatively high-resolution assessment of the localization of the enzymic activity.

However, the relationship between measurable absorbance per unit time after tetrazolium-reduction, the amount of NADH oxidized by the enzyme, and the Michaelis-Menten constant of the reaction, is not known precisely (Eadie, Tyrer, Kukums & Hooper, 1970). Indeed, with currently available methods, it cannot be determined precisely. Therefore, the absorbance values given in this paper must be interpreted cautiously. Though they are quantitatively valid and reliable for comparisons between the groups of cells examined, these absorbances should not be taken to mean absolute units of enzymic activity. Work is in progress in this laboratory to design a new system of scanning ultraviolet microspectrophotometry and fluorimetry which combines rapidity of collection of information on large populations of cells, and possibility of directly measuring kinetic constants.

Control in randomized populations of neutrophils

The results given in Tables 3, 4 and 6, considered separately from the remainder of the experimental data, are in essential agreement with the textbook findings of classical biochemistry (Lehninger, 1965, 1967, 1970; Loewy & Siekevitz, 1969). Neglecting differences between classes of cells, we can reaffirm that in Amphiuma neutrophils, menadione reductase activity – hence, respiration – is controlled both by the concentration of ADP (feedback reinforcement) and ATP (feedback inhibition). Tables 4 and 6 also indicated that a functional respiratory chain is a more important requirement for effective rate-control than coupling of oxidative phosphorylation. However, even when respiration was blocked by azide, and menadione reductase activity was slight, some control functions could still be demonstrated. These confirmatory results increase the confidence that might be placed on the further findings of this study, findings which cannot be compared directly with those obtainable from classical biochemical work.

The effects of dinitrophenol require special comment. Dinitrophenol was added to ADP (and used alone) as a means of distinguishing between the effects of concentration of ADP per se, and those of coupling oxidative phosphorylation. Clearly (Table 4), it is the concentration of ADP and not the rate of phosphorylation which is effective in the control of respiration. Yet, dinitrophenol apparently had inhibitory properties of its own (Tables 4 and 5). This might be attributed at least in part to its swelling or 'beading' effects on mitochondria, with consequent loss of soluble constituents; when swelling was prevented by the simultaneous addition of ATP or ADP, inhibition was largely reversed. Some direct or non-metabolic inhibition of menadione reductase through the attachment of dinitrophenol to amino groups in the active centre of the
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The enzyme cannot be discounted. The slight potentiation of ADP by dinitrophenol is not surprising. When a large concentration of ADP is present, oxidative phosphorylation is activated, thereby rapidly increasing the intracellular concentration of ATP, which, in turn, limits the rate of electron transport by feedback inhibition. When dinitrophenol is added, oxidative phosphorylation is uncoupled; no new ATP is formed: there is no feedback inhibition. It is also probable that the endogenous concentration of ATP may be then further reduced through activation of mitochondrial ATPase by dinitrophenol (Loewy & Siekevitz, 1969).

The 3 metabolic classes of neutrophils

The intracellular localization and level of menadione reductase activity, the apparent mechanism of control of this activity, were not identical in all neutrophils. On the basis of level of enzymic activity, neutrophils could be subdivided into 3 classes, I, II and III, of progressively higher activity. The activity was mitochondrial in Class I neutrophils; the endoplasmic reticulum and idiozome were also active in Classes II and III. This proposed classification of neutrophils, and presumed functional significance of the metabolic classes are, to us, the most exciting conclusions of this study.

Class I neutrophils might be tentatively assumed to form a reserve of vegetative cells. Their intermediate metabolism might be only adequate to preserve cellular integrity. There is comparatively little metabolism to inhibit. All adjuvants (except dinitrophenol used alone) enormously activated electron transport in these neutrophils. Furthermore, activation was seen to be an all-or-none phenomenon, increasing menadione reductase activity by either about 60% (ATP) or 200% (ADP). Neutrophils of Class I, when activated, became morphologically and enzymically indistinguishable from those of Class II. The interpretation of their partial activation by ATP seems obscure. Neglecting the effects of ATP, the assumption that 'vegetative' neutrophils can be 'mobilized' into Class II neutrophils in the presence of high concentrations of ADP seems consistent with the results.

Electron transport in Class II neutrophils is still controlled by feedback reinforcement, and not by feedback inhibition: it is activated by ADP (with or without dinitrophenol), and hardly affected by ATP and dinitrophenol (alone or in combination). Now, however, the idiozome region and the endoplasmic reticulum are also active.

Class III neutrophils are probably fully primed; their electron transport is controlled by feedback inhibition (ATP), and it is strongly inhibited by dinitrophenol. Inhibition by ATP or dinitrophenol again is an all-or-none phenomenon, with no intermediate levels. Inhibited Class III neutrophils become indistinguishable from Class II cells. Feedback reinforcement with ADP is inoperative. The most likely functional explanation is that Class III cells are those neutrophils which have been metabolically primed to engage in amoeboid movement and phagocytosis. The idiozome, with its complement of microtubules, presumably must be activated before streaming, amoeboid movement and eventually diapedesis can occur (Bernhard & de Harven, 1958; Porter, 1965). This mobilization process is necessarily endergonic:
it seems probable that the appearance of a micro-environment of unusually high metabolic activity in the region of the idiozome is the means of supplying the additional energy needed for mobilization in the micro-domain where it is most needed. It is very probable that Class III neutrophils, once they leave the bloodstream by diapedesis, must subsist under essentially anaerobic conditions. Their intermediary metabolism might, therefore, be glycolytic rather than oxidative. The possibility must be entertained that the demonstrated persistence of glycolysis in large populations of neutrophils under aerobic conditions (Seitz, 1969) does not indicate a reversal of the Pasteur effect in all neutrophils, but rather that primed neutrophils (Class III) are glycolytic whilst still in the blood stream, whilst ordinary neutrophils are not.

Another exciting possibility is that the activation of the endoplasmic reticulum observed in cells of Classes II and III, implies the activation of a second respiratory pathway (e.g. NADH$_2$:cytochrome b$_6$ oxidoreductase), localized in the respiratory assemblies of the endoplasmic reticulum.

The work described in this paper was supported by grants from the Research Division of the American Optical Corporation and the National Science Foundation. One of us (J. M. M.) acknowledges support from the National Science Foundation Summer Research Program, Grant URP. GY-8748, a scholarship from the Scholars' Programme of the College of Arts and Sciences, University of Denver, and an undergraduate student assistantship funded by the American Optical Corporation. We are grateful to Dr H. G. Davies of the MRC Biophysics Research Unit, King's College, London, for the gift of a sample of the particular batch of May–Grünwald stain used by Tooze & Davies (1967, 1968). We are grateful to Miss Glenda Miller, Mr P. Marccll, Mr R. Broderick and Mrs Carole Brass for technical assistance.

REFERENCES


Control of respiration in leucocytes


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Fig. 8. May–Grünwald/Giemsa smear, amplitude-contrast microscopy, photographed at 577 nm to emphasize basophil constituents. Segmented neutrophil, showing characteristic nuclear morphology, and Nissl-type aggregates of granular endoplasmic reticulum (erg). × 2500.

Fig. 9. Part of another neutrophil in the same smear as that in Fig. 8, but photographed by amplitude-contrast microscopy at 546 nm to emphasize the metachromatic specific granules (sgr). × 2500.
Control of respiration in leucocytes
Fig. 10. Neutrophil of Class I, menadione reductase reaction, amplitude-contrast microscopy at 436 nm, × 2500. The maximum visibility of cytoplasmic details is obtained when this micrograph is viewed along the axis of the unlabelled arrow. Note the filamentous mitochondria (mit).

Fig. 11. Neutrophil of Class III, menadione reductase reaction, 10⁻⁸ M ADP, 10⁻⁸ M dinitrophenol, photographed by amplitude-contrast microscopy at 577 nm, × 2500. Note the intense activity, prominent idiozome (idi), presumably smooth (eri) and granular (erg) endoplasmic reticulum, and mitochondria (mit). Compare with Figs. 16, 17.
Control of respiration in leucocytes
Figs. 12–15. Optical sections through a neutrophil of Class II, menadione reductase reaction, $10^{-4}$ M ADP, amplitude-contrast microscopy, $\times 2500$; abbreviations as in Figs. 8–11. Note the very dark idiozome region, and the changes in appearance with focusing of the aggregate of rough endoplasmic reticulum in the lower right of the cell ($\text{erg}^\circledast$). Filamentous mitochondria ($\text{mit}$) of different levels of activity and smooth endoplasmic reticulum ($\text{ers}$) are also seen.
Control of respiration in leucocytes
Figs. 16, 17. Optical sections through portions of the neutrophil of Class III shown in Fig. 11, photographed by amplitude-contrast microscopy at 436 nm, × 3200. Note the complicated structure of the idiozome, and the relative spatial distribution of mitochondria and endoplasmic reticulum. The specific granules (tgr) remain unstained, and are seen by negative contrast.
Control of respiration in leucocytes
Figs. 18, 19. Neutrophils of Class III, menadione reductase reaction in the presence of $10^{-4}$ M sodium azide, amplitude-contrast microscopy at 546 nm, $\times 2000$. Note the swollen and sometimes fragmented mitochondria; compare with Figs. 16–15. Also note the good preservation of the mitochondria in the erythrocyte (rbc) in the lower right-hand corner of Fig. 19.
Control of respiration in leucocytes

18

19

mit

mit

mit

mit

10 μm

rbc

rbc