# An opsonised microelectrode

## Observation of the respiratory burst of a single human neutrophil

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A 10 μm diameter gold microvoltammetric electrode, opsonised with human IgG, was used to study the respiratory burst of a single human neutrophil. The electrode oxidised superoxide produced near its surface by the neutrophil back to dioxygen. It is suggested that the current so detected is proportional to the rate of superoxide production by the NADPH oxidase of a single cell. In all cases the response consisted of a relatively rapid rise in current after cell addition, followed by a 2-phase decay. It is further suggested that this complex decay results from the production of superoxide being rate-limited initially by the NADPH concentration and later by the coupled metabolism of the hexose monophosphate shunt.

*Neutrophil* *NADPH oxidase* *Superoxide production* *Opsonised microelectrode* *Electrochemistry*

## 1. INTRODUCTION

The generation of potentially cytotoxic reduced oxygen species, namely the superoxide anion and consequentially hydrogen peroxide, is thought to play an important role in the destruction of invasive microorganisms by neutrophils. The evidence for the formation of the superoxide anion by stimulated neutrophils includes the superoxide dismutase-inhibitable reduction of ferricytochrome *c* [1] and nitroblue tetrazolium [2], spin trapping [3], and, more recently, the electrochemical oxidation of the superoxide anion at an opsonised electrode [4]. Although the superoxide anion is, itself, somewhat unreactive in aqueous media it does disproportionate readily to form hydrogen peroxide. Both $H_2O_2$ and $O_2^-$ may become involved in metal ion-catalysed reactions yielding the hydroxyl radical, $OH^{\cdot}$. $H_2O_2$ and $OH^{\cdot}$ are both powerful oxidants and consequently potential cytotoxic agents.

Neutrophils only generate these oxygen-derived metabolites in response to certain stimuli. The stimuli may be soluble, as in the case of phorbol myristate acetate [5] or insoluble, in which case the particle must be coated with an immunoglobulin such as IgG. It is believed that the stimulation of neutrophils involves a membrane perturbation. This response to stimuli is known as the respiratory burst on account of the rapid accompanying increase in oxygen uptake. The reduction of dioxygen to the superoxide anion, responsible for this rapid oxygen uptake, is thought to be catalysed by an NADPH oxidase [6]. The oxidation of NADPH to $NADP^+$ during the respiratory burst results in increased hexose monophosphate shunt (HMPS) activity in response to elevated $NADP^+$ levels. This has been shown by the release of radiolabelled $CO_2$ during the respiratory burst [7].

All investigations into the mechanism of the respiratory burst have been performed on large numbers of cells. This is a direct consequence of the fact that the methods employed are not sensitive enough to look at only one cell. We wish to report that, through the use of microscopically small electrodes, we are able to observe the respiratory burst of a single neutrophil.

In recent years the use in electrochemistry of

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microscopically small electrodes has become quite popular. Typically such electrodes are disc-shaped with a diameter of the order of 10 $\mu$m and can be constructed from carbon fibres or fine gold and platinum wires. These electrodes have various advantages in their applications over more conventional size electrodes of, say, 10 mm diameter. They were first demonstrated and originally designed by Wightman and co-workers [8,9] to minimise tissue damage upon implantation in the mammalian brain. The overall physical size of the electrode is the important criterion for their success in this case. The non-linear diffusion field which exists around a microelectrode results in simple steady-state voltammograms at conventional scan rates corresponding to rapid diffusional transport of material to its surface. This leads to considerable immunity of the electrodes to interfering homogeneous catalytic reactions which, in some cases, can be a useful advantage [10]. At higher scan rates, of up to $10^5$ V·s$^{-1}$ [11], such electrodes can be used to measure very fast heterogeneous rate constants or to study the electrochemistry of unstable intermediates. It is the small currents resulting from the small size of the electrode surface which give microelectrodes their favourable high-frequency response. These small currents also permit the use of microvoltammetric electrodes in more electrically resistive media without associated difficulties of uncompensated solution resistance. They can even be used successfully to carry out electrochemistry in organic solvents without supporting electrolyte [12]. The low current magnitude further allows experimental simplification to take place to a 2-electrode design cell with consequent improvement in the signal-to-noise ratio. There is clearly a further application of microelectrodes in the study of systems in which the size of the electrode is directly comparable to that of the species of interest. In our case, this is indeed true since the human neutrophil has a diameter of around 8 $\mu$m [13]. We believe that this will result, on average therefore, in the observation of the electrochemical response of a single cell. Not that the cells themselves are the electroactive species, but a microvoltammetric electrode might be expected to detect the local flux of electro-oxidisable superoxide associated with a single stimulated human neutrophil.

## 2. EXPERIMENTAL

Human neutrophils were prepared by a modification of the procedure of Boyum [14]. Fresh blood was collected by venous puncture into syringes containing heparin (Flow Laboratories) resulting in a final concentration of 10 units heparin/cm$^3$. Dextran T-500 (Pharmacia) was added to a final concentration of 1% (w/v) to increase the rate of erythrocyte sedimentation. After sedimentation was complete, the lymphocyte-rich supernatant was removed and layered on top of a histopaque (Sigma) solution and centrifuged at 400 $\times$ $g$ for 20 min. This yielded a cell pellet containing mainly neutrophils and a few contaminating erythrocytes. The contaminating erythrocytes were removed by hypotonic lysis in deionised water for 30 s, followed by adjustment to isotonicity by the addition of an equal volume of 1.8% (w/v) NaCl solution. Centrifugation of the suspension for 5 min at 400 $\times$ $g$ yielded a cell pellet containing better than 95% viable neutrophils. The cells were resuspended in Hanks' balanced salt solution. Cells were counted in a Neubauer chamber after a 20-fold dilution of stock suspension into a solution containing 0.1% (w/v) crystal violet and 0.1% (w/v) acetic acid.

A Clark type electrode (Rank Bros, Botisham, England) thermostatted at 37°C was used to determine the rate of oxygen consumption of the neutrophils upon stimulation. The stimuli employed were monodisperse polystyrene beads, mean diameter 1.0 $\mu$m (Polyscience), which had been preincubated with a solution of 15 mg·cm$^{-3}$ human IgG (Miles) for 30 min at 37°C. Each experiment contained $10^7$ neutrophils in 1 cm$^3$ Hanks' balanced salt solution. After a steady background rate of oxygen consumption had been recorded, an injection of 100 $\mu$l of a 1.25% (w/v) suspension of the opsonised polystyrene beads was used to initiate a respiratory burst.

A 2-electrode configuration was used for all electrochemical experiments. A 10 $\mu$m diameter gold microvoltammetric electrode was used throughout. Before each experiment it was polished lightly with an alumina (0.3 $\mu$m particle size, BDH)/water slurry on a Metron polishing cloth (Metaserv), rinsed thoroughly with deionised water, and dipped for ~2 min into a solution of 30 mg·cm$^{-3}$ human IgG in Hanks buffer at 37°C

to opsonise it. It was then rinsed very thoroughly with deionised water and connected to the cell. This consisted of a small plastic sample tube which contained 150 $\mu$l Hanks' medium and which was thermostatted at 37°C by a circulating water bath (Howe) whose pipes were lagged thoroughly with cotton wool to minimise thermal fluctuations. The secondary electrode was a simple loop of silver wire, lightly anodised in HCl to give a coating of AgCl, which rested at the bottom of the tube around its periphery. The cell was positioned inside a metal box to act as a Faraday cage to minimise electrical noise pick-up. The fixed potential source, which was battery driven (Oxford Electrodes), and current amplifier (Keithley model 427) were also located inside the Farady cage. The reference potential of the Ag/AgCl electrode in Hanks' medium was measured at intervals with respect to the saturated calomel electrode. A potential was applied to the secondary electrode, since the working electrode is kept at pseudo-ground, to maintain the working electrode at 50 mV vs SCE [4]. A gain of $10^{10}$ with a rise time of 300 ms was used in current amplification. This signal was then taken to an external 1 Hz low-pass filter and finally recorded against time on a Gould 60000 series X-Y recorder running in the time-base mode.

After connection to the electronics, the response from the microvoltammetric electrode was allowed to stabilise for 1–2 min. The Faraday cage was quickly opened, a 75 $\mu$l aliquot of a suspension of neutrophils was rapidly added from a Pasteur pipette to the 150 $\mu$l of Hanks' medium already in the cell, and then the box closed. Control experiments, such as that shown in fig.1a, confirm that these operations do not give any significant current response.

## 3. RESULTS

The result derived from addition of human neutrophils to an electrochemical cell containing an opsonised gold microvoltammetric electrode is shown in fig.1, traces b–d being representative examples of those obtained. All the current-time profiles showed a rise in oxidation current immediately after addition of the cells. There was no observable lag. A large number of results, such as those shown in fig.1b and c, showed a rapid rise giving

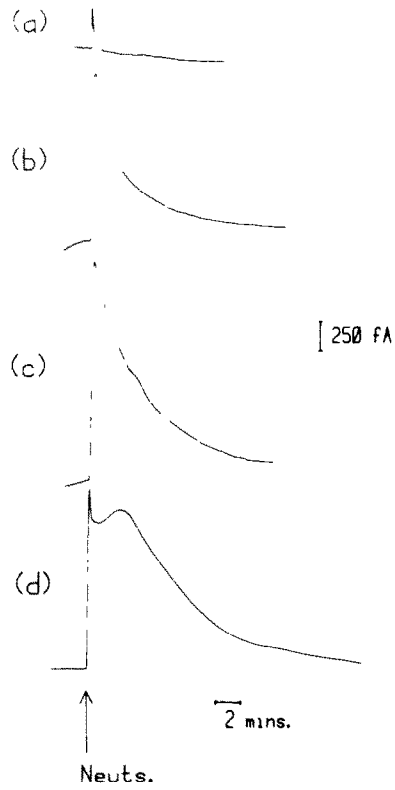


Fig.1. Typical current-time profiles recorded at an opsonised gold microelectrode (10 $\mu$m diameter) after addition, as indicated, of a 75 $\mu$l aliquot of (a) buffer, (b–d) neutrophils ($7.5 \times 10^6$ cells) to 150 $\mu$l Hanks' medium. Potential applied equivalent to 50 mV vs SCE. Temperature, 37°C.

maximum current response in 20–25 s followed by a decay with time. Some experiments, however, gave current-time responses like that shown in fig.1d; there was an initial spike followed by a much slower rise in current, taking up to 3 min, again followed by a decay. We suggest that the initial spike corresponds simply to a short-lived capacitative change of the type observed on addition of buffer only (fig.1a). Such a feature is hidden in the early parts of fig.1b and c. The sizes of the maximum currents recorded were, in all cases, largely comparable and generally found to lie within the range 1.05–1.85 pA. Similarly, the current decay which followed always exhibited non-simple behaviour. A first rapid decay phase was followed by a second slower phase, with a transition point always being observed. The current

decays could not be fitted to a single exponential. This is discussed fully later. In some cases the transition point actually showed a small 'bump' which occurred consistently around 2–3 min after the addition of cells (figs 1c and 2a), but it was not always observed.

Experiments were carried out to define better the type of electrode process being observed. Fig.2a shows a current-time profile, of the fig.1c type, in which there was a rapid rise in oxidation current to a maximum value of 1.5 pA followed by an initial rapid decay. There is a bump at 2 min and subsequent slower decay. The sulphydryl reagent *N*-ethylmaleimide (NEM) (100 $\mu$M) was then added. The current fell rapidly to an approximately zero level which remained steady thereafter. The superoxide-producing enzyme NADPH oxidase is strongly inhibited by such sulphydryl reagents [15]. Fig.2b shows the result of the addition of active cells to a medium already containing NEM. There was only a very small oxidation current transient which quickly gave way to a steady background current. Fig.2c shows a current-time transient obtained when neutrophils were added to medium containing 100 $\mu g \cdot cm^{-3}$ human Cu-Zn superoxide dismutase (SOD). The oxidation current response was very much smaller with a maximum current in this case of near 0.5 pA, followed by a rapid decay. There is evidence of a bump around 3–4 min after addition of cells followed by a slower decay. The entire response lasted for almost precisely the same time as those experiments conducted in the absence of SOD, such as those illustrated in fig.1b–d. The only known substrate for SOD is the superoxide anion.

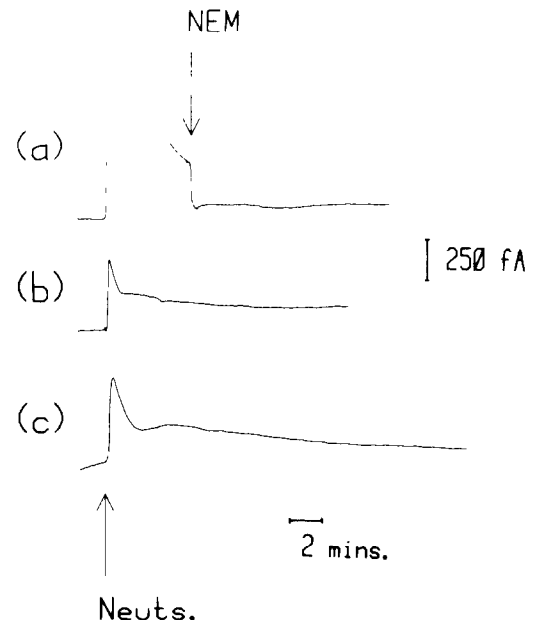


Fig.2. Current-time profiles recorded at an opsonised gold microelectrode on addition of neutrophils as indicated. Conditions as in fig.1 plus: (a) *N*-ethylmaleimide added to 100 $\mu$M as indicated; (b) *N*-ethylmaleimide (100 $\mu$M) present in medium before addition of cells; (c) human Cu-Zn superoxide dismutase (100 $\mu g \cdot cm^{-3}$) present in medium before addition of cells.

## 4. DISCUSSION

It is clear from the results of the experiments described that, to observe an oxidation-current transient such as those shown in fig.1b–d, it is necessary to have viable human neutrophils present. Inhibition of, for example, the superoxide-producing NADPH oxidase by the sulphydryl reagent NEM causes a rapid and complete loss of oxidation current (fig.2a). Furthermore, the very extensive inhibition of the current response by SOD points again towards superoxide as the electrode-oxidisable species. These results, obtained at the opsonised gold microvoltammetric electrode, are in complete accord with our earlier results obtained at a macroscopic opsonised graphite electrode [4]. In this case a rotating graphite disc electrode of diameter 6 mm was employed rather than the 10 $\mu$m gold wire used in the present study. Maximum currents of the order of 90 nA were observed compared to around 1.5 pA at the gold microelectrode. Such currents were, however, again strongly inhibited by SOD present in the solution when neutrophils were added. NEM was similarly found to be a potent inhibitor of the observed oxidation current response. The study also proved that surface modification by IgG was a prerequisite for the observation of the superoxide-oxidation current; neither a clean electrode, gold or graphite, nor one coated with a protein such as bovine serum albumin was sufficient to elicit a response.

We suggest that the IgG-coated electrode surface causes the activation of human neutrophils by

binding of Fc receptors upon their membrane surface. This in turn activates the membrane-bound NADPH oxidase to produce superoxide. Since the coated surface is that of an electrode, then the superoxide so produced may be electrochemically reoxidised back to dioxygen. The combination of activating and detecting species in the form of an opsonised electrode is crucial to the success of the technique. The electrode is able to reoxidise a fair proportion of the superoxide generated on it before spontaneous dismutation. However, the presence of the enzyme SOD in the solution at the electrode-cell interface causes a much greater rate of dismutation and so greatly lowers the flux of superoxide anion reaching the electrode surface. Hence the much smaller result of fig.2c compared with fig.1c.

It is interesting to make a comparison of the magnitude of such superoxide-reoxidation currents with the rate of oxygen consumption measured for the neutrophils in a conventional oxygen electrode experiment. Since spontaneous dismutation of all $O_2^-$ leads, in the latter case, to the production of 1 mol hydrogen peroxide per mol dioxygen consumed, then clearly a 2-electron reduction of such dioxygen is eventually taking place. A factor of 2 is therefore important in converting an observed dioxygen consumption rate of $1.3 \times 10^{-5}$ $mol \cdot min^{-1} \cdot cell^{-1}$ into an effective electron flux output of 4.2 $pA \cdot cell^{-1}$. This compares with electrochemically observed currents of the order of 1.5 pA. If the latter indeed corresponds to a measured flux of superoxide from no more than one cell then the percentage collection efficiency of around 35% is quite fair. This compares with an estimate of 7% in our macroscopic opsonised electrode experiments [4]. It may well be that our approximated packing density for cells on the surface of such an electrode was too optimistic since there is no reason to assume an intrinsically greater collection efficiency per cell at a gold microelectrode compared with a graphite macroelectrode. The two should be very nearly the same. Clearly then, a lower surface density of activated cells on the opsonised macroelectrode easily reconciles the apparent discrepancy between the 2 figures.

The variability of results obtained is illustrated in fig.1. Such apparent irreproducibility is not unexpected if the experiment is indeed measuring a fixed proportion of the superoxide flux associated with a single stimulated neutrophil: the addition of neutrophils to the electrochemical cell will not always lead to the binding of a neutrophil completely over the 10 $\mu$m diameter electroactive area. This will lead to a smaller measured current. Similarly, the cell activation may not be rapid in all cases leading to a much longer time to the peak of cell-derived current, as in fig.1d. A heterogeneous population of neutrophils is to be expected such that the observed respiratory burst of a single cell is unlikely to be truly identical to that of another. Such variability is normally lost in conventional experiments which record an ensemble response or envelope of individual respiratory bursts. The opsonised macroelectrode is just such an experiment.

The observed current-time profiles all show a superoxide oxidation current decaying with time after an initial rapid rise. None of the decay phases are simple smooth curves. All show a complex time dependence. Fig.1b shows a transition from fast to slower decay at $t = 3$ min after addition of cells. As noted earlier, fig.1c shows the same decay rate change but also a transient increase in current at the same time as the slope change. We suggest that it may be possible to relate such observations to the metabolic pathways involved in superoxide production. There is known to be a pool of NADPH present in resting neutrophils which is available as a source of reducing equivalents for the NADPH oxidase to use in the production of superoxide upon activation [16]. It is clearly tempting to suggest that the initial rapid decay of the observed current corresponds to a first-order depletion from the pool of NADPH as substrate for the superoxide-producing enzyme. However, as fig.3 shows, a plot of the logarithm of the current decay of fig.1b against time shows a clear break at $t =$ 3 min. Either the rate constant for such depletion of NADPH changes dramatically at this time or else some other process is also becoming important in determining the NADPH concentration and hence the superoxide flux/observed current. The frequent observation in a series of experiments of a transient increase in current around this same time indeed suggests the very likely presence of an NADPH-producing process which has a transiently increasing magnitude. We suggest that this is the metabolism of glucose via the hexose monophosphate shunt leading to the production of NADPH and thence superoxide. The observed

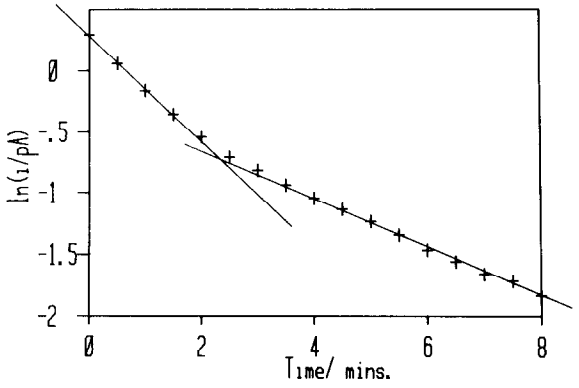


Fig.3. Plot of the natural logarithm of the superoxide-oxidation current observed in fig.1b against time, commencing just after peak cell current observation.

2-phase decay may then be due to a baton-changing mechanism much as in a relay race: the second runner starts going before the first has stopped. The glucose metabolism which produces NADPH is initiated as the NADPH pool is depleted, but before it is fully depleted. Glucose-6-phosphate dehydrogenase activity is known to be controlled by $NADP^+$ levels [17]. This idea gives qualitatively the correct forms of current-time decay as shown in fig.4a. Here a simple exponential decay, corresponding to pool depletion, is summed with a transiently increasing function followed by a slower exponential decay. This latter decay may correspond to the ending of the respiratory burst. Neutrophils are not expected to consume oxygen indefinitely in the presence of glucose. Fig.4b shows a similar summation but in this case just for 2 simple exponential functions, the one with the slower rate constant having a lower amplitude. This too gives qualitatively 2 decay phases similar to the experimental result of fig.1b. However, no variation of parameters in this case can ever produce the often observed transient increase, as in fig.1c. Indeed, metabolism favours the baton-changing mechanism as modelled by fig.4a. The increasing $NADP^+$ concentration due to superoxide formation causes an increased rate of $NADP^+$ reduction by the HMPS. The second function of fig.4a, with a rising initial part followed by a slow decay, is necessary to achieve this, a simple exponential decay (fig.4b) being inadequate. The time to maximum for this function is critical in determining whether the net transient in-

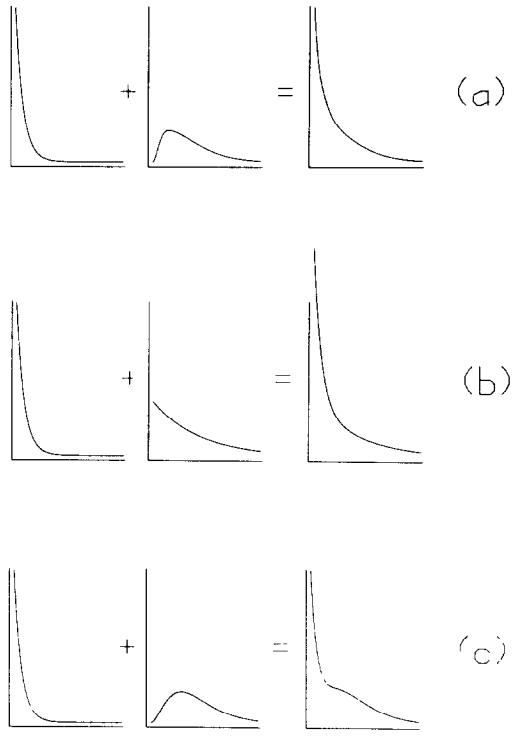


Fig.4. Summation of mathematical functions to simulate the decay phases of experimentally observed results. In all cases the left-hand function is based on $\exp(-3t)$. The second function added to this is: (a) a normalised $t^2 \exp(-3t/2)$ function using the rapid growth phase of $t^2 \exp(-3t)$; (b) $\exp(-3t/2)$; (c) a normalised $t^2 \exp(-3t/2)$ function. The scales are arbitrary.

crease is observed. If the peak occurs early during the 'pool decay' then it will be masked. If, however, the time to maximum is slightly longer then its effect can clearly be observed in the total function (fig.4c). This time to maximum is expected to depend upon the efficiency with which the metabolic pathways responsible for NADPH production are activated in response to an increasing $NADP^+$ concentration. This may well show some variability for different individual cells.

In conclusion we believe our results correspond to the first observation of the respiratory burst of a single human neutrophil. Furthermore, they may shed some light on the kinetics of superoxide production by NADPH oxidase as limited, we propose, by NADPH concentration, and, later, by further coupled metabolism.

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