Mechanism of the Respiratory Stimulation in Phagocytosing Leucocytes. The KCN-Insensitive Oxidation of $NADPH_2$

The present paper deals with the enzymatic basis of the respiration of phagocytosing polymorphonuclear (PMN) leucocytes, which is a disputed matter as regards the enzymes involved and their intracellular localization[1-10]. The results presented here provide evidence for a role played by the granule-bound $NADPH_2$-oxidase in stimulating the cyanide insensitive oxygen uptake during phagocytosis in PMN leucocytes.

Guinea-pig leucocytes (95% PMN) were obtained from peritoneal exudate[6]. Phagocytosis was induced by adding killed opsonized and washed bacteria (*Staphylococcus aureus* or *Bacillus subtilis*) to a leucocyte suspension in Krebs-Ringer phosphate solution (without Ca^{++}), incubated at 38 °C in shaken plastic tubes. Control tubes contained leucocytes without bacteria. After 10 min the suspensions were centrifuged and the leucocytes were washed once at 4 °C in the same medium, once, in 0.25 *M* cold sucrose, resuspended in 0.34 *M* sucrose and homogenized by a motor-driven Teflon pestle in a glass tube. After removal of the nuclear fraction and cell debris at low centrifugal force, each homogenate was divided into 2 aliquots, one (G + S) directly used for the metabolic assays, the other spun at 105,000 *g* (30 min) to obtain the supernatant (S) and granule (G) fractions. The latter was resuspended in the same volume of 0.34 *M* sucrose.

The oxygen uptake was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Ohio) at 28 °C. The medium contained 140 m*M* sucrose; 36 m*M* phosphate buffer, pH 7; 19 m*M* KCl; 0.5–0.7 m*M* $NADH_2$ or $NADPH_2$. When used, catalase was present at 2700 Sigma U/ml, pyruvate at 1 m*M* and KCN at 1 m*M*.

Since with the platinum surface polarized as a cathode, oxygen and hydrogen peroxide are indistinguishable[11], and since formation of hydrogen peroxide may take place in the oxidation of reduced coenzymes by leucocytic preparations[3-9], the problem arose of the efficiency of the endogenous catalase to decompose H_2O_2 in the presence of 1 m*M* KCN. It has been preliminarily shown that in the presence of 1 m*M* potassium cyanide the endogenous catalase decomposed added H_2O_2 and that the addition of catalase did not influence the rate of oxygen uptake of the leucocytic preparations, as recorded by the electrode with or without KCN.

During the measurement of oxygen uptake, simultaneous spectrophotometric assays were performed by taking suitable samples (50 μl) from the system and reading at 340 nm in a volume of 2 ml of 0.1 *M* KOH. One μatom of oxygen was consumed per μmole of $NAD(P)H_2$ oxidized. Protein was determined by the biuret reaction[12], after removal of residual bacteria in the G + S and G fractions of homogenates from phagocytosing cells. The swelling of granule preparations after addition of saponine (1 mg/ml) was followed by reading the optical density at 520 nm in a Beckman DU spectrophotometer. Materials: $NADH_2$, $NADPH_2$, and catalase from Sigma Chemical Co., and saponine, potassium cyanide and pyruvate from Merck, freshly prepared solutions.

The results summarized in Figure 1 show that in the presence of cyanide the oxygen uptake is strongly stimulated by the addition of either $NADH_2$ or $NADPH_2$ in the G and G + S fractions of homogenate from phagocytosing leucocytes, whereas little or no effect is observed in the same fractions from resting leucocytes as well as in the S fractions of either. It is evident that practically all the KCN-insensitive oxidase activities contained in the leucocyte homogenate (G + S) are recovered in the granule fraction (G).

The most relevant change induced by phagocytosis appears to be the enormous increase in the granule-bound $NADPH_2$-oxidase activity, which is in accord with our first conclusions[5-6]. When the experiments were repeated in the absence of cyanide, the oxygen uptake was significantly higher (30–40%) with either $NADPH_2$ or $NADH_2$ (in the G + S and G fractions). When the experiments were performed at pH 6, a small increase for both of the granular oxidase activities was observed.

It must be emphasized that in the conditions employed the oxidase activities of granule-containing fractions from resting leucocytes are hardly detectable, whereas those

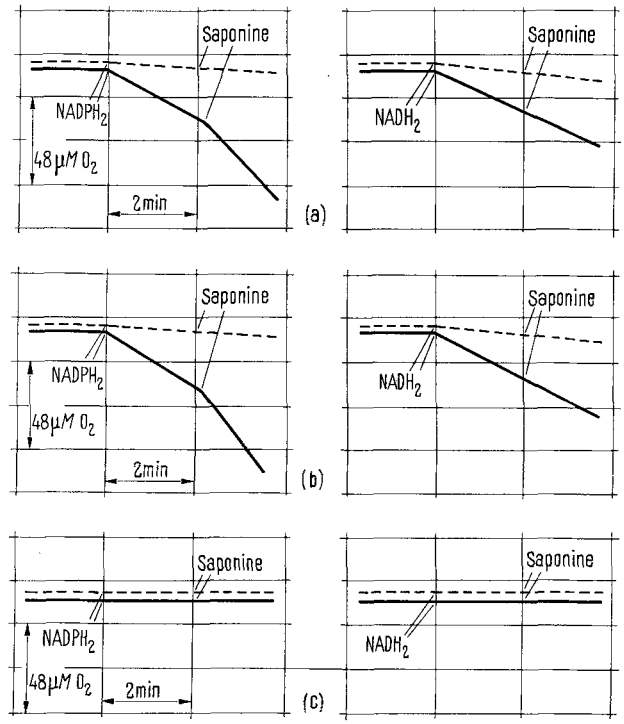

Fig. 1. Recording of the changes in oxygen tension (the downward deflection representing an increase of oxygen uptake) in a system of 1.5 ml containing 1 m*M* KCN. (a) Granules plus supernatant (5 mg of protein); (b) granules separated at 105,000 *g* and corresponding to the quantity present in (a); (c) supernatant (105,000 *g*) corresponding to the quantity present in (a). For composition of the system see text. – – – = Preparations from resting leucocytes; ——— = preparations from phagocytosing leucocytes.

[1] W. H. EVANS and M. L. KARNOVSKY, J. biol. Chem. *236*, PC 30 (1961).
[2] W. H. EVANS and M. L. KARNOVSKY, Fedn Proc. Am. Socs exp. Biol. *19*, 42 (1960).
[3] G. Y. N. IYER and J. M. QUASTEL, Can. J. Biochem. Physiol. *41*, 427 (1963).
[4] R. H. CAGAN and M. L. KARNOVSKY, Nature *204*, 255 (1964).
[5] F. ROSSI and M. ZATTI, Experientia *20*, 21 (1964).
[6] F. ROSSI and M. ZATTI, Br. J. expl Pathol. *45*, 548 (1964).
[7] M. ZATTI, F. ROSSI, and V. MENEGHELLI, Br. J. expl Pathol. *46*, 227 (1965).
[8] M. ZATTI and F. ROSSI, Biochim. biophys. Acta *99*, 557 (1965).
[9] F. ROSSI and M. ZATTI, Biochim. biophys. Acta *113*, 395 (1966).
[10] F. ROSSI and M. ZATTI, Biochim. biophys. Acta *121*, 110 (1966).
[11] B. CHANCE, in *Methods of Biochemical Analyses* (Ed. D. GLICK; Interscience, New York-London 1959), vol. 1, p. 408.
[12] A. G. GORNALL, C. J. BARDAWILL, and M. M. DAVID, J. biol. Chem. *177*, 751 (1949).

from phagocytosing leucocytes give rise to a very remarkable oxygen uptake. Such an enormous difference makes meaningless, with regard to the situation in phagocytosing cells, any speculation[1,2,4] based on experiments with oxidase activities and their sensitivity to inhibitors in homogenates from resting cells only.

The addition of saponine induces a swelling of the granules, with no difference between the decrease in optical density of granule suspensions from resting or phagocytosing leucocytes (Figure 2). During this swelling,

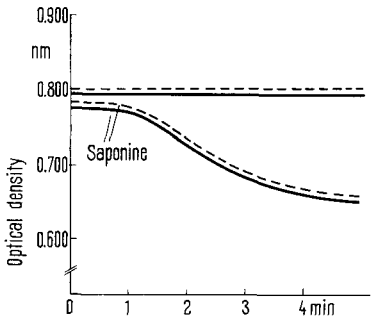

Fig. 2. Decrease in optical density of granules from resting (– – –) and phagocytosing (———) leucocytes. Upper curves: controls. Lower curves: effect of saponine, 1 mg/ml. Composition of the system as for Figure 1(a) except for the dilution of the preparations, adjusted to read approximately 0.800 at 520 nm.

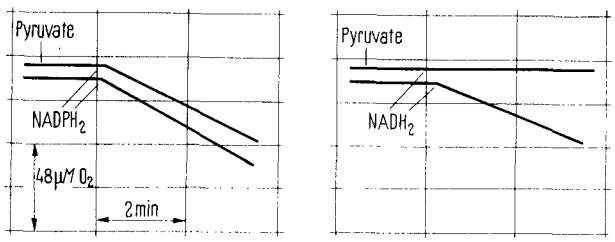

Fig. 3. Changes in oxygen uptake of a system containing 1 m*M* KCN and granules plus supernatant (see Figure 1(a)) from phagocytosing leucocytes. Upper curves: systems containing 1 m*M* pyruvate. Lower curves: controls without pyruvate.

the $NADPH_2$-oxidase from phagocytosing cells appears strongly stimulated whilst the $NADH_2$-oxidase is not (Figure 1). This shows that the attachment of the 2 enzymes to the granules, or their structure-linked latency, cannot be considered in the same way.

The experiments presented in Figure 1 also show that the addition of saponine does not stimulate the oxidase activities of granules of resting leucocytes, which is relevant to the hypothesis[7] that during phagocytosis the oxidase activities of swollen granules are more active because of their swelling. It is evident that the saponine-induced swelling is not comparable to that caused by phagocytosis and which is associated with changes in the availability of the granule-bound oxidases. Further studies are needed to elucidate these questions.

The results presented in Figure 3 show that the interrelationships between the granular $NADH_2$- and $NADPH_2$-oxidases and the $NADH_2$- and $NADPH_2$-linked activities of the lactate dehydrogenase are such as to prevent the $NADH_2$-oxidase from being active in the presence of pyruvate.

These findings indicate that in the phagocytosing PMN leucocyte the $NADPH_2$ can be oxidized by oxygen, whilst the $NADH_2$ is preferentially oxidized by pyruvate through lactate dehydrogenase.

Riassunto. Si è dimostrato che l'attività $NADH_2$ e $NADPH_2$ ossidasica dei granuli di leucociti polimorfonucleati, misurata come consumo di ossigeno con elettrodo di Clark in presenza di cianuro 1 m*M*, è enormemente aumentata nelle preparazioni da leucociti in fagocitosi, mentre l'attività del sopranatante è inapprezzabile. La competizione della lattico-deidrogenasi con le ossidasi sui due coenzimi ridotti è tale da consentire soltanto alla $NADPH_2$ ossidasi un ruolo rilevante nel sostenere il consumo d'ossigeno dei leucociti in fagocitosi. Questi risultati chiaramente dimostrano l'infondatezza di speculazioni basate su esperimenti esclusivamente condotti con omogenati da leucociti in riposo[1-3].

M. Zatti and F. Rossi

Unit Centre 'G. Vernoni' for the Study of Physiopathology and Institute of General Pathology, University of Padova (Italy), May 16, 1966.

On Short Adrenergic Neurons in the Accessory Male Genital Organs of the Bull

The vas deferens and the internal male genital glands of different mammals have recently proved to be innervated by short adrenergic neurons[1-5], which have a very dense terminal distribution in the various organs. Further, certain functional parameters of the isolated noradrenalin granules of the seminal vesicle (or, vesicular gland) and the vas deferens of the bull have in several respects been found to occupy an intermediary position between bovine splenic nerve granules and adrenomedullary granules[6,7]. In order to establish from which cell system these noradrenalin granules originate, it is necessary to obtain histochemical information on the catecholamine-containing structures of the internal male genital organs in this species.

Material and methods. Pieces from various internal male genital organs (proximal and distal parts of the seminal vesicle, ampulla of the vas deferens, and the prostate body) were removed from 5 adult bulls within 10 min

[1] N. O. Sjöstrand, Acta physiol. scand. *54*, 306 (1962).
[2] N. O. Sjöstrand, Acta physiol. scand. *55*, 376 (1962).
[3] B. Falck, Ch. Owman, and N. O. Sjöstrand, Experientia *21*, 98 (1965).
[4] Ch. Owman and N. O. Sjöstrand, Z. Zellforsch. *66*, 300 (1965).
[5] N. O. Sjöstrand, Acta physiol. scand. *65*, suppl. 257, 1 (1965).
[6] U. S. v. Euler and F. Lishajko, Life Sci. *5*, 687 (1966).
[7] L. Stjärne and F. Lishajko, J. Neurochem., in press (1966).