and DBF. NOBF did not interfere with respiratory processes in Ehrlich cells, even at quite high concentration. BF and DBF exert an almost identical inhibitory effect on exogenous respiration, the least effective being NOBF (Ehrlich cells). The decrease in the respiratory rates in cancer cells might be due to the effects of benfluron and its metabolites on the cell membrane. P388 murine leukemia cells are less "sensitive" than Ehrlich ascites cells.

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Depolarization produces an acidification of adrenal gland perfusates

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Neuronal cell activity leads to considerable changes in the extracellular ionic composition in the vicinity of the cell membrane surface following the opening of ion channels. Changes in extracellular H^+ concentration can also be expected and have been detected by the use of pH sensitive electrodes, in the central nervous system, *in vivo* [1, 2], *in vitro* [3] or in brain slices [4, 5], and also in the peripheral nervous system ([6], see Ref. 7 for a review). External pH changes in the course of stimulation are characterized by an initial alkaline shift of short duration followed by a long lasting acid transient. However, the origin of these pH changes remains obscure. It has been proposed that the initial alkaline shift could result from a channel mediated transmembrane flux of proton equivalents [6]. The long lasting extracellular acid transient peaks at the end of stimulation, and returns to the original basal pH level. It could originate from the cellular release of metabolic products [1, 6], or from the exocytotic secretion of the highly acidic (pH 5.5) content of synaptic vesicles [5].

The bovine adrenal medulla has served as the most useful model tissue for the investigation of the exocytotic secretory processes [8, 9]. Much of the earlier progress on cholinergic receptor regulation of catecholamine secretion was accomplished by using perfused adrenal glands [10]. Stimulation of the adrenal gland with cholinergic agonists leads to the exocytosis of the vesicular content. By using various agonists and media of various buffering capacity, we have found that stimulation resulted in an acidification of the perfusion medium of a magnitude similar to that found in other systems. However, firstly the acidification could not be directly correlated with the amount of catecholamine released and then the time-course of both phenomena was different, suggesting that the acidification did not only result from the exocytosis of the acidic content of the chromaffin granules.

Materials and Methods

Bovine adrenal glands obtained from the slaughterhouse were removed within 20–30 min after the death of the animal and were kept in Locke solution during transportation to the laboratory. Retrograde perfusion were carried out essentially as described by Chubb and Smith [11] with some modifications. The composition of the perfusion fluids was (in mM): (a) regular Locke solution: 145 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 10 dextrose, 0.1 ascorbic acid, and 5 Hepes* buffer, pH 7.4; in some experiments, Hepes buffers at 0.5 mM and 1 mM were used. (b) High potassium solution was of the same composition as standard Locke solution except that NaCl was partially (56 mM) replaced with an equivalent concentration of KCl. (c) Ca^{2+}-free Locke solution was similar to the solution mentioned except that $CaCl_2$ was omitted. In the experiments in which acetylcholine (ACh) and nicotine were tested, they

* Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid).

were solubilized in normal or Ca^{2+}-free Locke solutions. The perfusion medium was buffered with Hepes rather than bicarbonate. The lower buffering capacity of Hepes permitted more clear cut pH variations (see Ref. 3 for a discussion). Glands were perfused at 37° at a flow rate of 5 mL/min. Perfusion began with Ca^{2+}-free Locke solution for 45 min, then stimulation was evoked by perfusion for 2 min with Locke solution containing 2.2 mM $CaCl_2$ and the stimulant.

pH was recorded with a contact pH electrode (Ingold) in contact with the perfusate leaking from the gland. A Beckman pH meter model 3500 connected to a recorder was used to follow the perfusion medium pH changes. Samples were collected for 1 min intervals, kept at 4° and centrifuged to remove any particulate matter and analysed for catecholamines, protein and enzymatic activities. Total catecholamines were measured according to Anton and Sayre [12] with no further purification on alumina. Protein was estimated by the method of Bradford [13] with bovine serum albumin as a standard. Acetylcholinesterase was assayed as described by Ellman *et al.* [14] in the presence of 10^{-5} M tetraisopropylpyrophosphoramide to inhibit non-specific cholinesterase. Lactate dehydrogenase was measured by the method of Kornberg [15].

Results

Stimulations were always started after an initial 45 min period of perfusion of the glands: during the first 30 min, a rapid washing out of protein, acetylcholinesterase and lactate dehydrogenase was observed, which then reached a low and constant basal level [11].

Figure 1 shows the pH changes and the secretion of catecholamine and AChE when glands were perfused with a 5 mM Hepes–Locke buffer and stimulated with various concentrations of ACh. A dose-dependent release of catecholamine was observed. Concomitantly, an acidification of the perfusate during the 2 min of stimulation occurred, followed by a slow return (3–5 min) of the pH to the basal level. When stimulation was performed with nicotine or with high K^+ (Fig. 1), a similar pH decrease accompanied the secretion of catecholamine. The cytoplasmic enzyme lactate dehydrogenase was constant in the perfusate during the perfusion period, indicating that the stimulation-induced increase in catecholamine and AChE secretion did not result from cell leakage. However, the intragranular protein AChE [16–19] was released during the stimulation.

In order to study the influence of the buffering capacity of the perfusion medium on the extent of the acidification, perfusion was performed with a 0.5 mM Locke–Hepes buffer. As expected, under these conditions the extent of the pH drop was enhanced (Fig. 2). Increasing ACh concentrations led to higher catecholamine release whereas the acidification was similar for the three concentrations of ACh tested, suggesting that no direct correlation existed between the acidification and the amount of granule contents secreted. This was further confirmed when K^+ was used to stimulate the gland: the amount of catecholamine secreted was twice that observed with 10^{-4} M ACh, while the pH decreases were similar in both cases. No catecholamine release occurred when 10^{-4} M ACh was added in the absence of Ca^{2+} and the slight acidification observed could be due to the hydrolysis of ACh by membrane bound AChE.

Table 1 summarizes the maximum pH changes obtained with Locke solutions containing different Hepes concentrations under various stimulation conditions. As already shown in Fig. 2, a significant acidification could be observed in media of low buffering capacity (0.5 mM Hepes). Increasing the buffer concentration reduces the

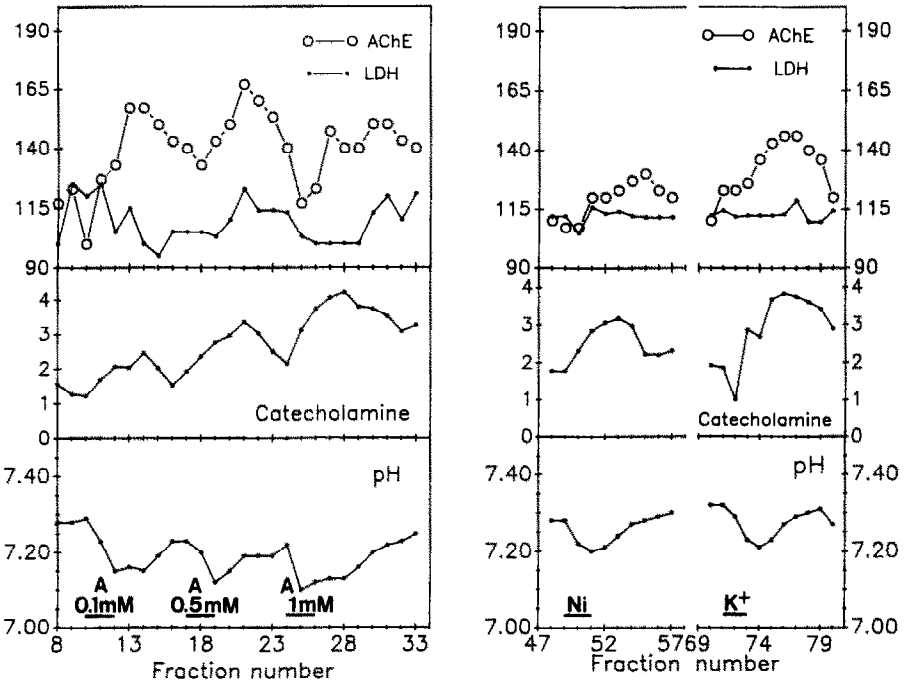

Fig. 1. pH variations in the perfusate of adrenal glands perfused with a Locke–Hepes medium (Hepes at 5 mM, pH 7.3). Adrenal glands were perfused at 5 mL/min with a Locke–Hepes medium without Ca^{2+}, and stimulated for 2 min in the presence of Ca^{2+} as indicated (A: ACh; Ni: nicotine 10^{-5} M). Collection time was 1 min for each fraction. Catecholamine (μg/mL), lactate dehydrogenase (LDH) and AChE were also measured. LDH and AChE are expressed as the percentage activity of the basal level. (Basal levels were 0.33 nmol/min/mL for LDH and 0.5 nmol/min/mL for AChE.) This experiment is representative of three experiments.

Table 1. pH changes in the perfusates of adrenal glands buffered with Hepes at various concentrations

	Hepes (mM) 0.5 (N = 2)	1.0 (N = 4)	5.0 (N = 2)
$CaCl_2$	0.17 ± 0.03 (0.049)	0.12 ± 0.01 (0.065)	0.01 (0.026)
ACh (10^{-3} M)	0.20 ± 0.07 (0.057)	0.13 ± 0.05 (0.070)	0.02 ± 0.01 (0.053)
ACh (10^{-4} M) + $CaCl_2$	0.78 ± 0.16 (0.186)	0.45 ± 0.16 (0.214)	0.16 ± 0.04 (0.399)
K^+ + $CaCl_2$	0.67 ± 0.13 (0.168)	0.29 ± 0.10 (0.148)	0.12 ± 0.01 (0.304)

Adrenal glands were perfused with Locke–Hepes (Hepes at various concentrations), pH 7.6. The table shows the maximum pH decrease from the baseline when the stimulants indicated were added for 2 min in the perfusion medium. The concentrations (in mM) of H^+ produced by the pH drop are given in brackets. This concentration was deduced from the variation of the concentration of protonated Hepes during the pH decrease, using the classical formula: $pH = pK_a + 1\,g\,(A^-/AH)$, where A^- and AH are the concentrations of unprotonated and protonated Hepes, respectively, and taking a pK_a of 7.55; N indicates the number of independent experiments.

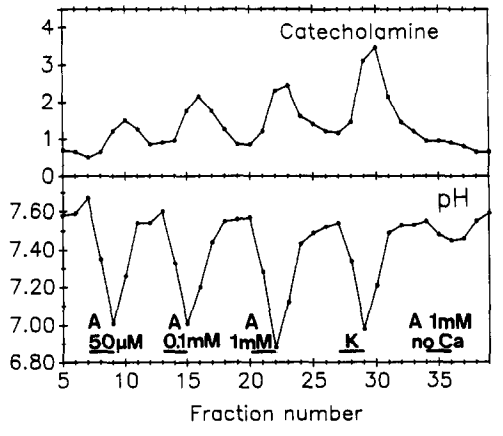

Fig. 2. pH variations and catecholamine (µg) in the perfusate of adrenal glands perfused with a Locke–Hepes buffer. The experimental conditions are the same as in Fig. 1 except that the Hepes buffer was at 0.5 mM. This experiment is representative of three experiments.

extent of the acidification. The pH drop which could be observed in the presence of Ca^{2+} alone or 10^{-3} M ACh without Ca^{2+} at low buffer concentration was completely abolished with 5 mM Hepes. ACh added in the presence of Ca^{2+} resulted in higher pH changes than the sum of ACh and Ca^{2+} individually. A similar change was obtained when high K^+ plus Ca^{2+} was tested.

Discussion

The present study shows that, as has been found in other secretory tissues [1–6], an acidification of the extracellular medium can be detected in the adrenal gland on stimulation. The extent of the acidification was dependent on the buffering capacity of the perfusion medium [3, 20]. However, in our case no initial alkaline transient could be observed in the adrenal gland. If it occurred, it may be below the detection limits of our system.

The pH decrease was dependent on the presence of Ca^{2+} and occurred mainly under conditions of membrane depolarization. However, addition of Ca^{2+} provoked by itself a slight pH drop. As neuronal membranes carry a net negative charge (pH_i 4.1) at neutral pH, perfusion of the adrenal gland with Ca^{2+}-free Locke solution will lead to some protonation of the negative surface charges. The acidification observed after addition of Ca^{2+} to the perfusion medium may thus originate from the chase by Ca^{2+} of protons from the protonated membrane surface. The slight pH drop observed with ACh in the absence of Ca^{2+} could result from the acetic acid produced by hydrolysis of acetylcholine.

However, the acidification of the perfusate cannot result from the summation of the slight pH drops provoked individually by ACh and Ca^{2+}, since with 0.5 mM Hepes this summation would represent 0.38 pH units, whereas stimulation with ACh in the presence of Ca^{2+} induced an acidification of 0.78 (see Table 1). Furthermore, high K^+ or nicotine also produced significant acidification. Thus, the perfusate acidification must have several origins [7].

It has been assumed [5] that the pH drop of the external medium on stimulation could arise from the exocytotic release of the highly acidic content of synaptic vesicles. Perfused adrenal gland is a well suited model to test this assumption because the secretion of catecholamines in the perfusion medium can be taken as an indication of the vesicular content release. If this assumption is valid, it would be expected that the acidification of the perfusion medium would closely parallel the secretion of catecholamines, in duration and intensity. However, in the present study we found that there was always a 1 min time-lag between the maxima of acidification and catecholamine release. Furthermore, the degree of acidification could not be correlated to the amount of catecholamine secreted (compare stimulation with either 5×10^{-5} M ACh, 10^{-4} M ACh and K^+; Fig. 2). Therefore, these results suggest that the stimulus-induced acidification of the external medium has an additional origin other than the simple release of the granular protons. Chromogranins are acidic proteins (pH_i *ca.* 4.5) due to their high number of acidic amino acids and are abundant in chromaffin granules [21]. After release, proteins reach a neutral pH in the extracellular medium and protons will be lost from these acidic groups. However, the contribution of these proteins to pH reduction during release cannot be predicted. Contributions from other sources can also be considered.

Intracellular acidification has been detected during electrical activity in molluscan neurons [22], but recovery from the pH changes was found to require long periods (10 min); then it was shown that depolarized snail neurons lose protons during depolarization [23] leading to an

acidification of the neuron external surface [20]. If similar intracellular acidification and outward proton currents occurred during membrane depolarization of chromaffin cells, the pH of the extracellular medium would indeed decrease and this would last only for the time-course of stimulation, as was found in the present study.

Previously detected extracellular acidification of nerve cells were considered to result from the release of metabolic acids [1, 6] because it was found that NaF, an inhibitor of the metabolic degradation of glucose to pyruvate inhibited the extracellular pH decrease [1]. However, in these studies, intense electrical stimulation was used to observe pH decreases, which were detected with pH sensitive microelectrodes implanted in the brain. Extracellular acidification was preceded by a rapid (1–10 sec) alkaline transient and peaked after the end of stimulation before returning slowly (2–3 min) to the basal level [1]. In an *in vitro* study using isolated rat vagus nerves [6], the pH decrease was even longer lasting (4–5 min after the end of stimulation) and was inhibited by ouabain, suggesting the involvement of the Na^+–K^+ pump. The discrepancies between the characteristics (time-course and magnitude) of the detected extracellular pH changes reported in the present study and those reported by others [1, 6] could be due to different preparations used and also to differences in technological approach.

The pH change observed in the perfusate of the adrenal gland is easy to measure and has practical advantages. On stimulation, the pH always decreased slightly before catecholamine secretion and returned to the baseline level after completion of catecholamine secretion. Thus, on-line measurement of pH during perfusion may be useful: (a) to estimate the void volume of the perfusion system; (b) to follow the time-course of chromaffin granule secretion; and (c) to determine the decay in secretory response to prolonged exposure to high K^+, or to repetitive nicotinic stimulations.

In summary, stimulation of adrenal glands with a variety of agonists or high potassium produced an acidification of the perfusion medium. The magnitude of the transient pH decrease was similar to that found in other nervous structures, and depended on the buffering capacity of the perfusion medium. However, no alkaline transient could be detected in this tissue. This acidification required Ca^{2+} and occurred under conditions producing catecholamine release. Since firstly the acidification could not be directly correlated with the amount of catecholamine released and secondly the time-course of both phenomena was different, suggesting that the acidification did not only result from the exocytosis of the acidic content of the chromaffin vesicles. The extracellular acidification may in addition originate: (a) partly from the proton release from acidic proteins, (b) from an output of acid equivalents from the cytoplasmic medium, either by the Na^+/H^+ antiporter present in all animal cells [24], or another mechanism yet to be determined.

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DNA breakage caused by hydrogen peroxide produced during the metabolism of 2-methyl-1,4-naphthoquinone (menadione) does not contribute to the cytotoxic action of the quinone

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Menadione is a redox active quinone that increases intracellular levels of superoxide anion free radical ($O_2^{\cdot-}$) and hydrogen peroxide [1]. Although a causal role for H_2O_2 and $O_2^{\cdot-}$ has been suggested [1, 2], the relative contribution of these reactive species in menadione-induced cell damage has not been definitely established. It is known that $O_2^{\cdot-}$ is characterized by a low reactivity, as compared to the high reactivity of hydroxyl radicals ($OH^{\cdot}$) formed by H_2O_2 and redox active metals and therefore it could be speculated that the toxicity of $O_2^{\cdot-}$ is mediated by its conversion (catalysed by the enzyme superoxide dismutase) to hydrogen peroxide. On the other hand, the extremely high reactivity of the $OH^{\cdot}$, and its very short half life, could be limiting factors, as far as toxicity is concerned, since this species may react with almost every biological macromolecule (critical and non-critical targets). The low reactivity of $O_2^{\cdot-}$ may be an important factor for allowing these species to reach critical targets, such as the DNA, where lethal lesions could be produced. Thus, a possibility exists that $O_2^{\cdot-}$ may mediate at least part of the cytotoxic response elicited by menadione. Indeed, recent evidence indicates that $O_2^{\cdot-}$ is capable of producing DNA damage and cytotoxicity [3].

In this study we have investigated the role played by hydrogen peroxide in the induction of DNA damage and cytotoxicity following treatment of cultured mammalian cells with low concentrations of menadione. Evidence is presented which suggests that most of the DNA breaks detectable following treatment with the quinone are produced by a Fenton reaction. These DNA lesions, however, do not significantly contribute to menadione cytotoxicity.

Materials and Methods

Materials. Radiolabelled compounds were purchased from New England Nuclear (Boston, MA, U.S.A.). Free acid EDTA, disodium EDTA, tetrasodium EDTA, sodium dodecyl sulfate, 1,10-phenanthroline and menadione (2-methyl-1,4-naphthoquinone) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Tetraethylammonium hydroxide was purchased from Merck-Schuchardt (Munchen, F.R.G.). Polycarbonate filters were from Nuclepore (Pleasanton, CA, U.S.A.). McCoy's 5a medium, foetal bovine serum and trypsin were from Gibco (Grand Island, NY, U.S.A.).

Cells and radioactive labelling. Wild type (AA8) Chinese Hamster Ovary (CHO) cells were routinely grown in McCoy's 5a medium supplemented with 15% foetal bovine serum, 2 mM L-glutamine and 1% penicillin–streptomycin in an atmosphere of 5% CO_2 in air, at 37°. All experiments were performed with log-phase cells (1×10^6/60 mm dish). Experimental cultures for alkaline elution were plated in 60 mm tissue culture dishes, labelled overnight with [*methyl*-^{14}C]thymidine (0.05 μCi/mL) and then chased for 6 hr in a label-free medium.

Alkaline elution assay. Cells containing [^{14}C]DNA were exposed for 20 min to increasing concentrations of menadione at 37°, in the absence or presence of 1,10-phenanthroline, and then analysed for DNA breakage. At the end of treatments, cells were removed from the dishes by trypsinization (1% trypsin for 5 min at ice temperature). The filter elution assay was carried out by a procedure virtually identical to that described by Kohn *et al.* [4] with minor modifications [5]. Briefly, 5×10^5 cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters and then rinsed twice with 10 mL of ice-cold Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose) containing 5 mM EDTA (disodium salt). Cells were then lysed with 5 mL of 2% sodium dodecyl sulfate, 0.025 M EDTA (tetrasodium salt), pH 10.1. Lysates were rinsed with 7 mL of 0.02 M EDTA (tetrasodium salt) and the DNA was eluted overnight in the dark with 1.5% tetraethyl ammonium hydroxide/0.02 M EDTA (free acid)/0.1% sodium dodecyl sulfate (pH 12.1), at a flow rate of *ca.* 30 μL/min. Fractions of approximately 3 mL were collected and counted in 7 mL of Lumagel containing 0.7% glacial acetic acid. DNA remaining on the filters was recovered by heating for 1 hr at 60° in 0.4 mL of 1 N HCl followed by the addition of 0.4 N NaOH (2.5 mL) and was again determined by scintillation counting. DNA was also recovered from the interior of the membrane holders after vigorous flushing with 3 mL of 0.4 N NaOH. This solution was processed for scintillation counting as described above.

Cytotoxicity assay. Cells were seeded at a density of 5×10^5 cells/60 mm dish and, after 24 hr, were treated for 20 min with various concentrations of menadione in complete medium either in the absence or presence of 1,10-phenanthroline. Cell monolayers were then rinsed twice with Saline A and incubated for 48 hr in a drug-free medium. Cell number was estimated after trypsinization with a hemocytometer.

Superoxide assay. Superoxide production was measured as the reduction of acetylated cytochrome *c* using the wavelength pair 550–540 nm [6]. Addition of superoxide