shows that only dopamine and acetylcholine increased the turnover rate of the phospholipids in the snail, while in the rat these two substances in addition to noradrenaline and 5-HT produced this state[8]. However, it has been demonstrated[7] that the 4 substances tested affect the turn-over of phospholipids in different brain areas of the guinea-pig brain in various ways, suggesting that phospholipid turn-over is only influenced by substances which have a definite function in that tissue. This could explain the present results: firstly because the 'brain' of *Helix pomatia* consists of a number of ganglia, each of which varies in morphology and physiology[15], secondly because noradrenaline has a minor role in the CNS[1,2,4] and thirdly because 5-HT is the probable excitatory neurotransmitter in the snail[2,3,16,17]. Caution is required, however, before drawing direct conclusions from the data, since it is not certain how important the molar concentration of neurotransmitter is in its influence on the phospholipids[7,8]. In any event, the present results support the idea that neurotransmitter substances specifically affect the turnover rate of membrane phospholipids[18].

Zusammenfassung. Von den im Schneckenhirn (*Helix pomatia*) wahrscheinlich als Neurotransmitter wirkenden Substanzen führen Dopamin und Acetylcholin zu einem erhöhten Einbau von ^{32}P in Phospholipide; Serotonin zeigt eher einen gegenteiligen Effekt und Noradrenalin bleibt ohne Einfluss. Phosphatidylinositol weist die höchste Einbaurate auf. Die Resultate unterstreichen die Bedeutung von Dopamin, Acetylcholin und Serotonin als Neurotransmitter im Schneckenhirn und deren Einfluss auf den Metabolismus von Membranphospholipiden.

H.-H. ALTHAUS, V. NEUHOFF and N. N. OSBORNE

Max-Planck-Institut für experimentelle Medizin, Forschungsstelle Neurochemie, Hermann-Rein-Strasse 3, D-34 Göttingen (German Federal Republic, BRD), 1 October 1974.

[15] T. D. BULLOCK and G. A. HORRIDGE, *Structure and Function in the Nervous System of Invertebrates. II. Mollusca: Gastropoda*, (Freeman & Co., San Francisco-London 1965).
[16] N. N. OSBORNE, Br. J. Pharmac. *48*, 546 (1973).
[17] G. A. COTTRELL and J. B. MACON, J. Physiol. *236*, 435 (1974).
[18] We gratefully acknowledge the financial support of the Deutsche Forschungsgemeinschaft (SFB 33).

Detection of Arylhydroxylamines as Intermediates in the Metabolic Reduction of Nitro Compounds

The toxicity and carcinogenicity of aromatic nitro compounds apparently depends upon their metabolic activation to the corresponding hydroxylamine[1-3] by hepatic nitro reductases present in microsomes and cytosol[4,5]. The high reactivity and lability of these intermediates has, however, generally precluded their direct detection in biological systems. An electrochemical method for the determination of arylhydroxylamines based on their anodic oxidation at carbon paste electrodes has recently been reported[6]. This probe has been used as a sensor to monitor hydroxylamine turnover during the reductive metabolism of a series of aromatic nitro compounds in rabbit liver microsomal suspensions. The stability and fate of arylhydroxylamines under non-enzymatic conditions was also investigated.

Methods. 1-Hydroxyaminonaphthalene was synthesized by reduction of nitronaphthalene with zinc and ammonium chloride[7] and the hydroxylamine converted to the corresponding nitroso compound by oxidation with dichromate[8]. Liver microsomal suspensions were obtained from male New Zealand rabbits[9] and protein concentration was adjusted to 10 mg/ml. Incubation mixtures were prepared as described by KATO[10] and reactions were carried out for 15 to 60 min under an atmosphere of deoxygenated argon at 37 °C. Reaction vessels were equipped with a 3-electrode assembly consisting of a saturated calomel electrode (SCE), graphite rod counter electrode, and graphitenujol working electrode. Peak voltammograms were recorded on an X-Y recorder for all solutions at 2 min intervals during the course of incubations by applying a linearly varying potential of

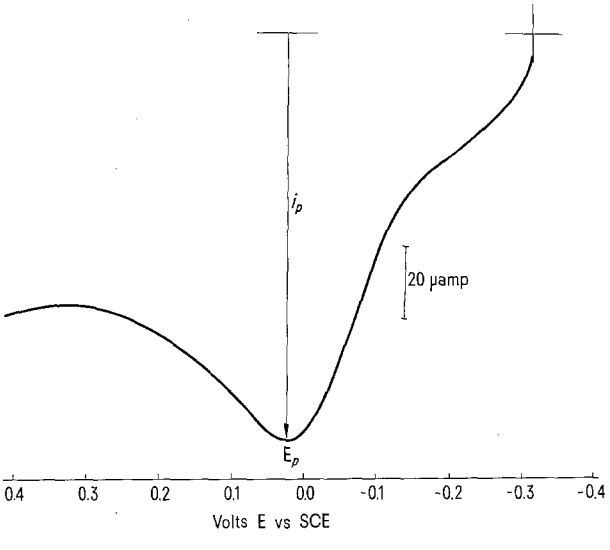

Fig. 1. Peak voltammogram for 4×10^{-5} *M* solution of 1-hydroxyaminonaphthalene in 0.1 *M* phosphate buffer (pH 7.4) at 37 °C (sweep rate: 0.10 V-sec^{-1}). Peak potential (E_p) and peak current (i_p) are labelled.

[1] J. R. GILLETTE, J. R. MITCHELL and B. B. BRODIE, A. Rev. Pharmac. *14*, 271 (1974).
[2] E. C. MILLER and J. A. MILLER, in *The Molecular Biology of Cancer* (Ed. H. BUSCH; Academic Press, New York 1974), p. 377.
[3] M. MITCHARD, Xenobiotica *1*, 469 (1971).
[4] R. KATO, A. TAKAHASHI and T. OSHIMA, Biochem. Pharmac. *19*, 45 (1970).
[5] Y. YOSHIDA and H. KUMAOKA, Proceedings of the First Symposium on Drug Metabolism Action (Ed. H. KITAGAWA; The Pharm. Soc., Japan, Tokyo 1970), p. 57.
[6] L. A. STERNSON, Analyt. Chem. *46*, 2228 (1974).
[7] E. E. SMISSMAN and M. D. CORBETT, J. org. Chem. *37*, 1847 (1972).
[8] A. I. VOGEL, *A Textbook of Practical Organic Chemistry* (Longman, London 1970), p. 630.
[9] R. A. WILEY, L. A. STERNSON, H. A. SASAME and J. R. GILLETTE, Biochem. Pharmac. *21*, 3235 (1972).
[10] R. KATO, T. OSHIMA and A. TAKANAKA, Molec. Pharmac. *5*, 487 (1969).

-0.30 to $+0.60$ V (sweep rate: 0.10 V-sec^{-1}) between the working and reference electrodes.

Results and discussion. Arylhydroxylamines are electroactive molecules that are readily oxidized [$E_{p/2}$ = -0.15 to $+0.10$ V (vs. SCE)] at carbon paste anodes (pH 7.4). These potentials are significantly different than those at which amino, nitroso and nitro group undergo oxidation, permitting the detection of arylhydroxylamines in the presence of their biochemical precursors and metabolic by-products. Figure 1 is a peak voltammogram recorded for the oxidation of a 0.1 mM solution of 1-hydroxyaminonaphthalene in 0.1 M phosphate buffer (pH 7.4). The peak potential (E_p) is qualitatively indicative of the compound electrolyzed and the magnitude of current (i_p) proportional to the concentration of electroactive species in solution.

1-Nitronaphthalene was incubated at 37 °C with viable rabbit liver microsomal suspensions and an NADPH-generating system in an oxygen-free atmosphere. The response of the electrode sensor after incubation had proceeded for 6 min is shown in Figure 2. The peak is derived from 1-hydroxyaminonaphthalene, formed during the metabolic reduction of 1-nitronaphthalene. Minor differences in the appearance of voltammograms recorded in aqueous solution and microsomes are a reflection of the environments in which the curves were obtained. The inhomogenous nature of the microsomal mixture shifts the oxidation potential slightly and somewhat flattens the curve. The magnitude of i_p was proportional to the concentration of nitro compound incubated, demonstrating the ability of the electrochemical probe to detect arylhydroxylamines during metabolic nitro reduction. When reactions were carried out in the presence of oxygen, no hydroxylamine was detected. These results are consistent with reports that oxygen totally destroys nitro reductase activity[11]. Peak current (i_p) was reduced 51% when incubations were run in a carbon monoxide (CO) atmosphere. Partial inhibition by CO indicates that microsomal reduction is mediated by cytochrome P-450, as has been reported by GILLETTE[12] and other workers[10]. The residual nitro reductase activity observed in a CO atmosphere suggests that hydroxylamine formation may also be mediated by a second enzyme system, e.g. NADPH-cytochrome c reductase, as proposed by YOSHIDA[5].

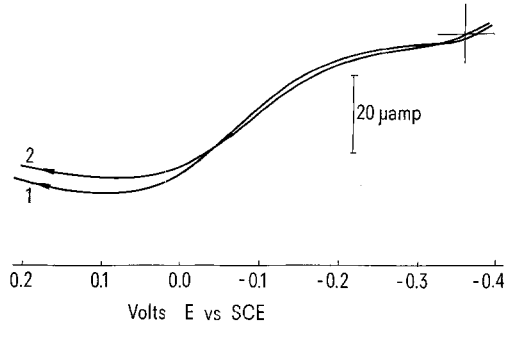

Fig. 2. Peak voltammogram of 1-hydroxyaminonaphthalene generated during the metabolic reduction of 1-nitronaphthalene. Voltammogram recorded directly in microsomal suspensions after incubation had proceeded for 6 min under anaerobic conditions. Two scans were recorded, 25 sec apart, to show the reproducibility of measurements.

Half Life of 1-hydroxyaminonaphthalene (5×10^{-5} M) in various chemical environments at 37 °C

Environment[a]	Half life (min)[b]
0.1 M phosphate buffer (pH 7.4)	45 ± 2
Buffer[c], Fe^{++} (1×10^{-4} M)/EDTA	43 ± 1
Buffer, Fe^{+++} (1×10^{-5} M)	15 ± 1
Buffer, viable microsomes	23 ± 2
Buffer, boiled microsomes[d]	13 ± 1

[a] Electrode assembly immersed in solution and peak voltammograms recorded at 2 min intervals. [b] Values represent mean $\pm$ S.D. for 4 determinations. [c] 0.1 M phosphate buffer (pH 7.4). [d] Microsomal suspensions were boiled for 20 min and rehomogenized before use.

The hydroxylamine, 1-hydroxyaminonaphthalene, was also incubated with liver microsomes and an NADPH-generating system. CO inhibited reduction to the amine by 82%, indicating the dependence of the reaction on cytochrome P-450. The major portion of the hydroxylamine was oxidized to nitroso compound, probably by a non-enzymatic route.

The nitrogen atom in the nitroso functional group is at an oxidation state between that found in the nitro compound and that present in the hydroxylamine, and may be presumed to be an intermediate in nitro reduction. 1-Nitrosonaphthalene was incubated anaerobically with rabbit microsomes in the presence of either NADPH or NADH. Peak voltammograms for the corresponding hydroxylamine were recorded after incubation had proceeded for 5 min. NADPH was a more efficient electron source; however, NADH also affected nitroso reduction. Conversion of nitroso compound to hydroxylamine also occurred non-enzymatically. NADPH and NADH served equally well as hydrogen donor when nitrosonaphthalene was reacted under an argon atmosphere with reduced nucleotide in phosphate buffer. The difference in reducing efficiency observed during enzymatic nitroso reduction when NADPH and NADH were present, supports the hypothesis that the reaction follows more than one mechanism; a non-enzymatic reaction employing NADPH and NADH with equal efficiency, and an enzymatic component which preferentially uses NADPH. These results from electrochemical measurements are in agreement with findings described by other investigators[13,14]. The nitroso metabolite arises not only from enzymatic reduction of the nitro group, but also from non-enzymatic oxidation of the hydroxylamine. The half life of 1-hydroxyaminonaphthalene was determined in several environments (Table) by recording peak voltammograms of hydroxylamine solutions at 2 min intervals and measuring peak current (i_p) intensities. Arylhydroxylamines are relatively unstable species, prone toward isomerization to aminophenols under acidic conditions and readily oxidized to nitroso compounds in the presence of oxygen or other oxidizing agents[6]. The increase in hydroxylamine turnover in the presence of boiled microsomes may reflect the exposure or release of protein bound oxidants (e.g. metal ions) during the denaturation process.

[11] J. R. FOUTS and B. B. BRODIE, J. Pharmac. exp. Ther. *119*, 197 (1957).
[12] J. R. GILLETTE, J. J. KAMM and H. A. SASAME, Molec. Pharmac. *4*, 541 (1968).
[13] M. KIESE, Ann. N.Y. Acad. Sci. *123*, 141 (1965).
[14] J. R. GILLETTE, Ann. N.Y. Acad. Sci. *123*, 154 (1965).

Similar qualitative results were obtained when nitrobenzene, nitrofluorene, and *p*-nitrocresol (or their nitroso and hydroxylamino derivatives) were carried through analogous investigations.

Zusammenfassung. Eine elektrochemische Sonde wurde entwickelt, um Arylhydroxylamin direkt in mikrosomalen Suspensionen festzustellen. Dieser Sensor ermittelte die Bildung von Arylhydroxylamin während der enzymatischen Reduktion von aromatischen Nitroverbindungen durch Mikrosomen der Kaninchenleber. Die Reduktion der Nitrogruppe wurde durch beide Enzyme, Cytochrom P-450-abhängige und P-450-unabhängige, katalysiert.

L. A. STERNSON[15]

Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens (Georgia 30602, USA), 13 November 1974.

[15] This work was supported in part by NIH grant No. CA 14158-02 from the National Cancer Institute (USA).

Release of Hydroxyproline from Rat Hearts Perfused with Collagenase

Various enzymes, i.e. collagenase, trypsin and hyaluronidase, have been used extensively for dissociating tissues. During preliminary experiments in this laboratory we found that perfusion of rat hearts with some preparations of hyaluronidase resulted in partial inactivation of 5'-nucleotidase; for example, 20 min of perfusion with Sigma Type I hyaluronidase caused a greater than $^1/_3$ loss of enzyme activity[1]. There have been a number of reports of other functional losses due to similar treatments of various tissues with dispersing enzymes[2–4]. We were therefore interested in identifying methods which effect dissociation of hearts while keeping functional losses to a minimum. As tissue dissociation was not readily quantitated by direct observation, a more objective result of enzyme activity was monitored. We measured the release of hydroxyproline, an amino acid which is contained almost exclusively in collagen and elastin. In this communication we report the rate of release of hydroxyproline by rat hearts exposed to collagenase alone or in combination with hyaluronidase and trypsin.

Materials and methods. Sources of enzymes: Collagenase Type III, hyaluronidase, Worthington; trypsin 1:250, Difco. Chemicals were reagent grade from various sources; C^{14}-hydroxyproline was purchased from Amersham.

Using the Langendorff technique, rat hearts were perfused for 5 min with complete Hank's solution to wash out blood and then perfused for 5 min with Hank's solution from which Ca^{++} was omitted. Recirculating perfusion with Ca^{++}-free Hank's solution containing enzymes was then started (30 °C) and 1 ml samples of this perfusate were taken for hydroxyproline assays as required. The hydroxyproline content of the samples was determined by the method of PROCKOP and UDENFRIEND[5]

[1] P. L. WOOD and K. NAKATSU, unpublished observations.
[2] D. B. MCNAMARA, Y. C. YATES, U. KROMER, N. L. STEPHANS and N. S. DHALLA, Proc. Can. Fedn biol. Soc. *15*, 71 (1972).
[3] C. S. GARAGLI and J. R. COOPER, Biochem. Pharmac. *23*, 911 (1974).
[4] I. A. MACCHI, F. ZEYTINOGLU and S. B. BEASER, Proc. Soc. exp. Biol. Med. *145*, 500 (1974).
[5] D. J. PROCKOP and S. UDENFRIEND, Analyt. Biochem. *1*, 228 (1960).

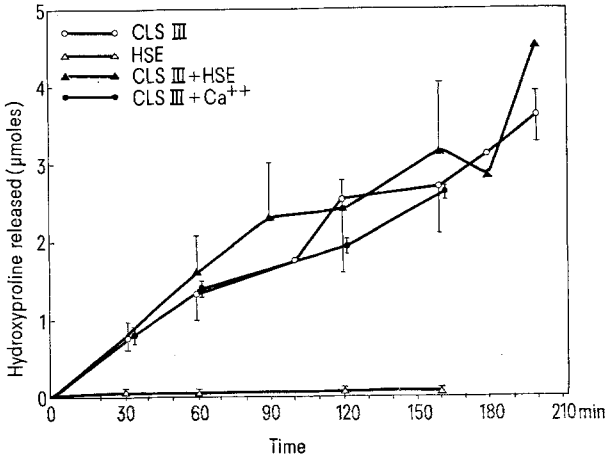

Fig. 1. Enzymatic release of hydroxyproline from perfused rat hearts. Rat hearts were perfused with collagenase (1 mg/ml CLS Worthington, Type III) and/or hyaluronidase (2 mg/ml HSE, Worthington) in Hank's solution from which Ca^{++} was omitted. The effect of 1.25 m*M* Ca^{++} is shown by the closed circles. A recirculating perfusion system was used and aliquots of the perfusate were taken for hydroxyproline determination at the times indicated. The data are expressed as μmoles of hydroxyproline released per heart at the perfusion times indicated and each point with a vertical bar represents the mean of 2 to 6 hearts ± S.D.

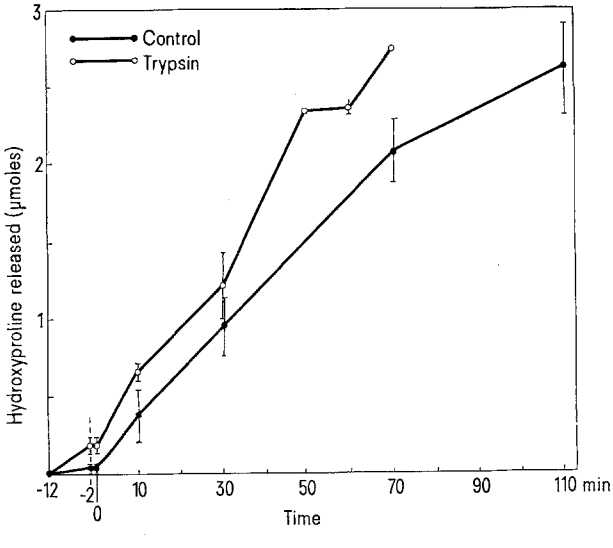

Fig. 2. Hydroxyproline relaese from perfused rat hearts after preperfusion with trypsin. Hearts were preperfused with trypsin (1 mg/ml) in Ca^{++}-free Hank's solution for 10 min and then the trypsin was washed out prior to perfusion with collagenase. Control hearts were preperfused with Ca^{++}-free Hank's solution. Other conditions same as for Figure 1.