Proton-induced dismutation of superoxide in aprotic media by bile pigments

J. M. Ribó, J.-A. Farrera and J. Claret*

*Departament de Química Orgànica, * Departament de Química Física, Facultat de Química, Universitat de Barcelona, c/ Martí i Franquès 1, E-08028 Barcelona, Catalunya (Spain)*
Received 17 March 1989; accepted 27 June 1989

Summary. Cyclic voltammetry of molecular oxygen in aprotic media (dimethylformamide) and in the presence of bilirubin and other bile pigments shows that superoxide anion ($O_2^{\cdot-}$) undergoes proton-induced dismutation. Lactam hydrogens of bile pigments are sufficiently acid to induce $O_2^{\cdot-}$ disproportionation to O_2 and H_2O_2. Because of its characteristic lipophilic behavior, a biological role for natural bilirubin similar to that of other non-enzymatic lipophilic scavengers of $O_2^{\cdot-}$ is suggested.
Key words. Bile pigments; bilirubin; superoxide dismutation; superoxide scavenger.

In normal intermediary metabolism molecular oxygen is reduced to superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)[1]. These oxygen derivatives have the potential for generating other reactive oxygen species. The biological consequences of uncontrolled production of such oxygen species are mutations, cytotoxicity, carcinogenesis, cellular degeneration, etc[2-4]. Aerobic organisms have antioxidant processes which defend them. These processes can be enzymatic (action of superoxide dismutase and catalase) or non-enzymatic (e.g. reaction with tocopherols, and ascorbic acid)[5].

In a paper[6] based on the general hypothesis that end products of degradative metabolic pathways may play an important role as protective agents, the in vitro behavior of BR as a chain-breaking antioxidant in lipid autoxidation was shown[6,7]. Here we study the chemical interaction of bilirubin, and of bile pigments in general, with $O_2^{\cdot-}$, the primary biological reactive species formed by one electron transfer to dioxygen. Some authors[8-12] have already reported chemical or biological interaction between $O_2^{\cdot-}$ and bile pigments, but the chemical process involved was not clarified and, moreover, these papers are dispersed over a wide scientific field and there are no cross-references among them.

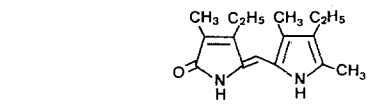
Material and methods

$O_2^{\cdot-}$ is unstable in protic media but can be easily obtained in an aprotic solvent, e.g. in dimethylformamide (DMF), where its behavior is well known[13]. The electrochemical behavior of bile pigments has also been extensively studied[14-19].

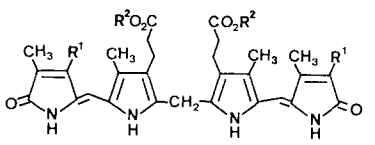
Bilirubin IXα (BR) was of commercial origin (Sigma), its purity was controlled by HPLC. Bilirubin IXα dimethyl ester (BR-DME) was obtained from BR following McDonagh[20c], and mesobilirubin IXα (MBR) from BR following Kuenzle et al.[21]. Mesobiliverdin IXα dimethyl ester (BV-DME) was obtained following McDonagh[20c] from mesobiliverdin IXα, which was obtained from MBR according to Landen et al.[22]. (*Z*)-3,8-diethyl-2,7,9-trimethyl dipyrrin-1 (10 *H*)-one (DPN) was obtained according to Falk et al.[23]. Voltammetry: a hanging mercury drop electrode (HMDE) was used: area 1.56 mm^2, sweep range 2–200 mV s^{-1}; potential values are given with reference to s.c.e.. For more experimental details see Claret et al.[18]. Dioxygen solutions were obtained by bubbling fixed volumes of air through Ar degassed solutions: intensity reproducibility of the peak corresponding to the $O_2^{\cdot-}$ formation was better than ± 10%. Dioxygen concentrations in the solution were estimated from the peak intensities: ≅ 15 μA at 100 mV s^{-1} for an O_2 saturated DMF solution (against air) (0.4 mM at 1 bar)[24]. Each substrate was tested at three different bile pigment concentrations (1×10^{-4} M, 2.5×10^{-4} M, and 5×10^{-4} M) and at least at four O_2 concentrations between 1×10^{-5} M and saturation. A supporting electrolyte which does not interact with $O_2^{\cdot-}$, such as tetrabutyl ammonium perchlorate (TBAP), must be used; in similar experiments performed using $LiClO_4$ as supporting electrolyte the same splitting effect was observed on addition of bile pigments, but the formation of peak 2, corresponding to one electron dioxygen reduction, was changed to an irreversible process and new peaks at more negative potentials were built up (stabilizing the effect of lithium as $O_2^{\cdot-}$ ligand[13]).

Results and discussion

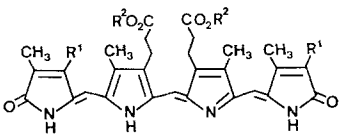
The corresponding blank solutions (O_2 in anhydrous DMF solutions, 0.1 M in TBAP as supporting electrolyte) show, as has already been described[13,25], a reversible diffusion-controlled peak at about −0.8 V (s.c.e.) (see fig. 3a: peak 2 in the figures), corresponding to the one electron reduction of dioxygen to $O_2^{\cdot-}$. In the presence of bile pigment (see formula scheme) a new voltammetric reduction peak (peak 1) is observed at more positive potentials (≅ 200–100 mV: table and figures show electroanalytical data of representative results). The peak 1 appears at −0.72 – −0.73 V for DPN, MBV-DME and BR-DME, and at −0.64 – −0.65 V for the tested bile pigments with free carboxylic acid groups, i.e. MBR and BR. Neutralization of the free carboxylic acid groups of BR with 1,1,3,3-tetramethylguanidine (TMG) displaces peak 1 to a potential value similar to that of



DPN



	R^1	R^2
BR	C_2H_3	H
MBR	C_2H_5	H
BR-DME	C_2H_3	CH_3



	R^1	R^2
BV	C_2H_3	H
MBV-DME	C_2H_5	CH_3

Potential values (s.c.e) of voltammetric peaks (HMDE, 100 $mV \cdot s^{-1}$)[a] corresponding to the process $O_2 + e^- \rightleftarrows O_2^-$, in presence of bile pigments (2.5×10^{-4} M), 0.1 M TBAP, 1×10^{-4} M O_2.

	U_{p1} (V)[b]	U_{p2} (V)[c]
Blank solution	----	− 0.84
DPN	− 0.73	− 0.84
BR-DME	− 0.73	− 0.83
MBV-DME	− 0.72	− 0.83
MBR	− 0.65	− 0.84
BR	− 0.64	− 0.83
Blank solution with TMG ($\cong 6\ 10^{-4}$ M)	----	− 0.88
BR with TMG	− 0.73	− 0.87

[a] for experimental details see [17, 18]; [b] irreversible peak; [c] reversible peak.

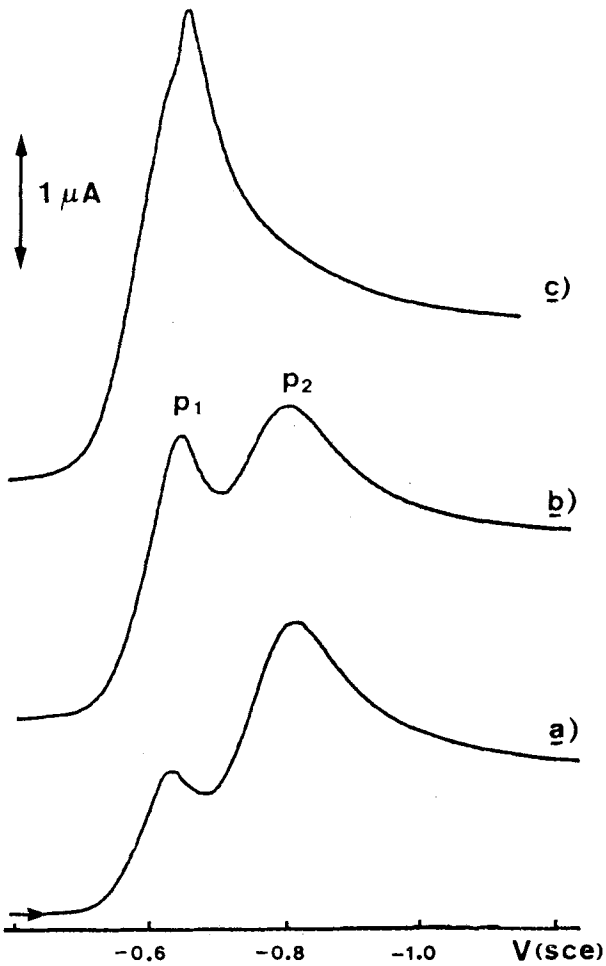


Figure 1. Voltammetry (100 $mV \cdot s^{-1}$) of O_2 1×10^{-4} M in DMF, 0.1 M TBAP at several MBR concentrations: *a* 1.25×10^{-4} M. *b* 2.5×10^{-4} M. *c* 5×10^{-4} M.

BR-DME (peak 1 at − 0.73 V). At constant O_2 concentration the peak current of peak 1 increases with bile pigment concentration at the expense of peak 2. After reaching a constant value when the peak current of peak 2 has fallen to zero, the voltammogram pattern remains unchanged by further addition of bile pigment (fig. 1).

At very low O_2 concentrations only peak 1 is observed in the voltammetric pattern. However, if the O_2 concentration increases whilst the bile pigment concentration is kept constant, the peak current of peak 1 reaches a constant value; in these conditions, peak 2 begins to appear and its peak current increases with O_2 concentration (fig. 2). The peak potential of peak 2 seems to be concentration-independent, but by increasing the oxygen concentration the peak potential of peak 1 is shifted to more positive values (fig. 2). By the addition of acetic acid to a blank solution the same splitting (peak 1 at 0.15 V more positive potential than peak 2) was obtained. Traces of water also give a small peak 1 at similar potential values (fig. 3 a).

An important difference between the two reduction peaks is observed by cyclic voltammetry. Peak 2 is reversible and it corresponds to the generation of stable O_2^- in the aprotic DMF solution, but peak 1 is clearly irreversible (fig. 3). As is shown in the example of figure 3 c, irreversibility of peak 1 is even observed when both peaks appear at very similar potentials.

Furthermore, in the presence of O_2 the typical reduction peak pattern of the corresponding bile pigment in DMF is changed, with new species appearing, together with those represented by the normal voltammogram. In the absence of dioxygen such changes in the voltammogram can be reproduced by the addition of a strong base such as TMG. Consequently, it appears that the changes are due to the formation of a bile pigment anion by interaction with O_2^-. This deprotonation of the bile pigment by O_2^- was confirmed by adding the bile pigments to O_2^- solutions in DMF (obtained by potentiostatic electrolysis of O_2), and by recording their UV/Vis spectra. These spectra were comparable to those of the bile pigment anions (NH deprotonation: i.e. DPN^-, $MBV\text{-}DME^-$, $BR\text{-}DME^-$, MBR^{3-}, and BR^{3-}) obtained by the addition of an excess of TMG to DMF solutions. Both types of experiments were reversible by acid neutralization.

All these results can be accounted for by the well-known process of the reduction of O_2 to O_2^- in aprotic solvents and its proton-induced dismutation to O_2 and H_2O_2 in the presence of weak acid substrates [13, 25–28]. It may be observed by cyclic voltammetry in aprotic solvents that

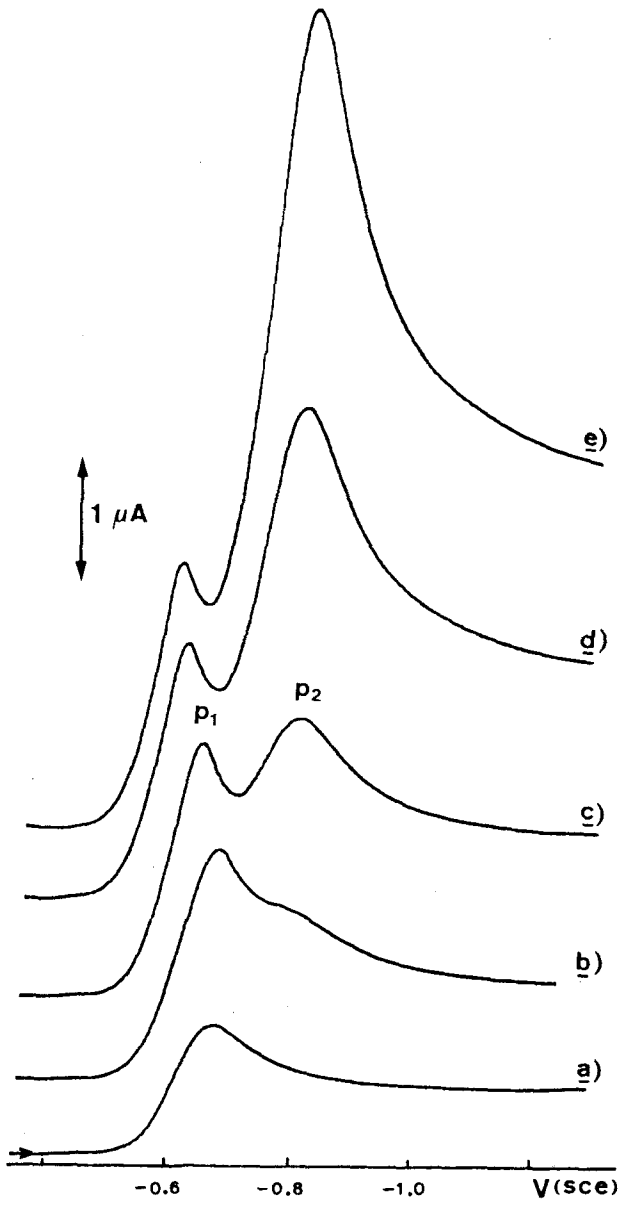


Figure 2. Voltammetry (100 $mV \cdot s^{-1}$) of O_2 in DMF, 0.1 M TBAP, and 2.5×10^{-4} M MBR. *a* 1×10^{-5} M. *b* 2×10^{-5} M. *c* 7×10^{-5} M. *d* 1.5×10^{-4} M. *e* 2×10^{-4} M.

the originally reversible monoelectronic peak (peak 2), corresponding to $O_2 + e^- \rightleftarrows O_2^{\cdot-}$, becomes an irreversible process of overall stoichiometry when weak acid substrates are added (in our case peak 1),

$$O_2 + 2e^- + 2HB \rightarrow O_2H_2 + 2B^-$$

This new process is due to the dismutation of $O_2^{\cdot-}$ according to the overall process,

$$2O_2^{\cdot-} + 2HB \rightarrow O_2 + O_2H_2 + 2B^-$$

This process determines that, despite the fact that $O_2H\cdot$ only has a $pK_a = 4.69$, the proton-driven disproportionation causes $O_2^{\cdot-}$ to have an apparently unique strong basicity in aprotic media[13]. It is relevant here that it has been stated that the lactam proton of bile pigments has pK_as values between 14 and 20 (bilirubins: $pK_a \cong 15$)[29,30], i.e. values of the order of those found for many substrates which generate proton-induced dismutation, e.g. nitromethane[25].

The dismutation process can be explained by several mechanisms. Sawyer[17,19] concluded, from his electroanalytical results and on the basis of drive force arguments, that the mechanism was, in the case of weak acid substrates in DMF, the protonation of $O_2^{\cdot-}$ and later recombination of the $O_2H\cdot$ formed radicals to give H_2O_2 and O_2. However, recently Savéant[25], using kinetic analysis of double potential step chronoamperometry, concluded that the mechanism which is at work in the presence of weak acids, such as phenols, is the corresponding one to one electron transfer from $O_2^{\cdot-}$ to $O_2H\cdot$,

$$O_2^{\cdot-} + HB \rightleftarrows O_2H\cdot + B^-$$
$$O_2^{\cdot-} + O_2H\cdot \rightarrow HO_2^- + O_2$$
$$HO_2^- + HB \rightleftarrows H_2O_2 + B^-$$

The chemical reaction between $O_2^{\cdot-}$ and BV and its dimethyl ester has been studied in DMSO and DMSO/water solutions[31,32] ($O_2^{\cdot-}$ from KO_2 and from xanthine-xanthine oxidase system), and the formation of the radical anion of the bile pigment has been reported. The results reported here clarify the nature of the chemical interaction between $O_2^{\cdot-}$ and BR. The results reported in the literature[31,32] can be explained through the formation of BR^{3-} (or BR^{4-}?), which can then – more easily than BR^{2-} or BR – be oxidized by the H_2O_2 obtained in the $O_2^{\cdot-}$ dismutation. Moreover, these results show that, in vitro, BR may be as good an $O_2^{\cdot-}$ scavenger as α-tocopherol[33].

Chemists and biologists are still puzzled by the metabolic pathway in mammals reducing BV to BR. In mammals, in contrast to birds, reptiles and amphibians, biliverdin reductase, in most of the organs studied, is more active than heme oxidase, which accounts for the fact that BV is rarely detected in mammalian plasma or tissues[20a,b]. It is not clear why BR is the end product of heme catabolism in mammals; in contrast to BV, BR is not readily excreted, and requires many transport and conjugation processes for its removal and elimination ($\cong$ 300 mg BR/day in humans)[34]. In addition, it is cytotoxic. BR considered from a chemical point of view, despite the fact that it has two free carboxylic acid groups, shows surprisingly strong lipophilic characteristics and solubility behavior when it is compared to BV[20c,35]. Consequently, BR may interact with $O_2^{\cdot-}$ in lipophilic surroundings in living organisms, acting as a biological non-enzymatic $O_2^{\cdot-}$ scavenger. Other facts reported in the literature support this hypothesis: a) molybdenum-flavin oxidases which have O_2 as electron acceptor are characteristic enzymes of mammals; they

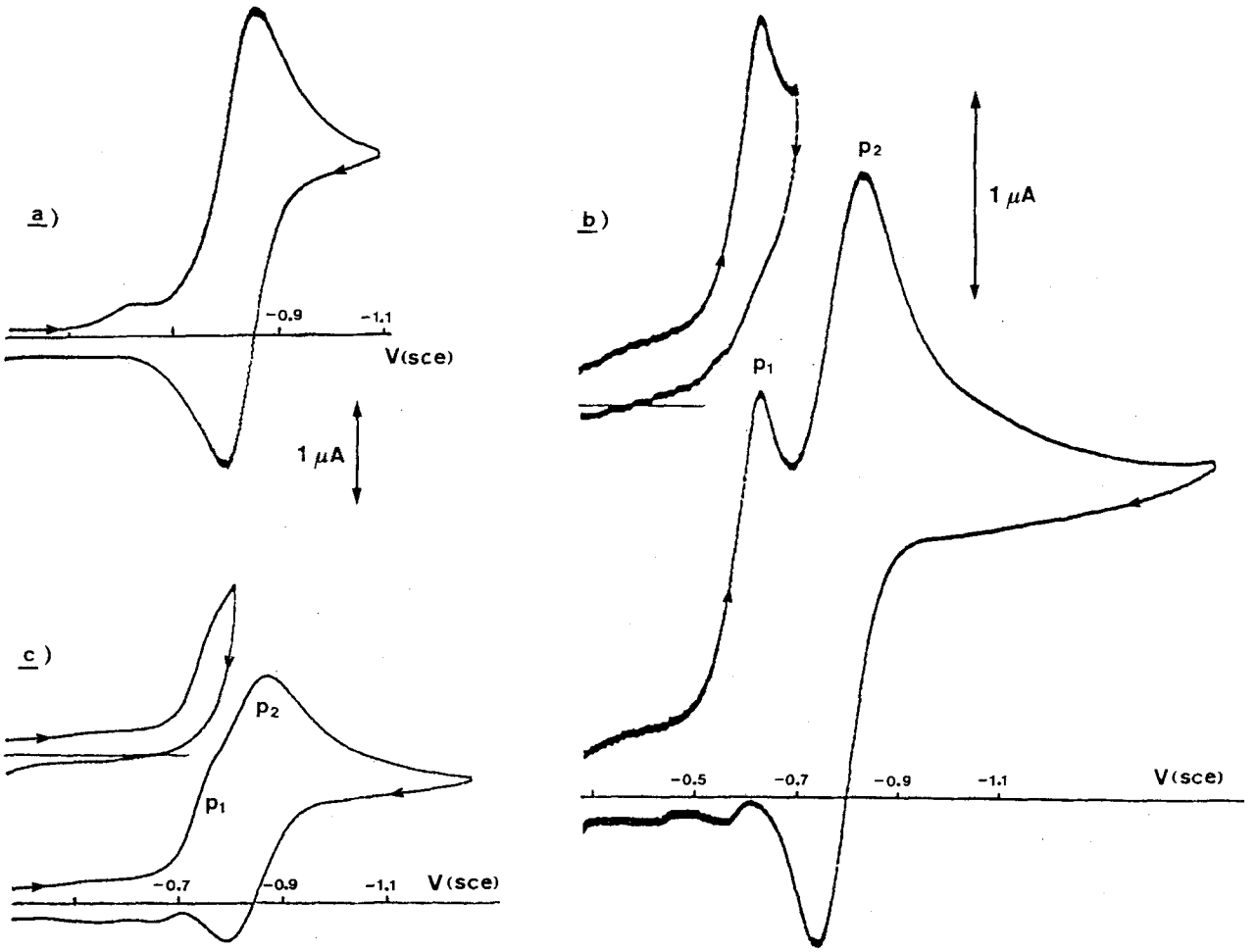


Figure 3. Cyclic voltammetry of O_2 in DMF, 0.1 M TBAP, showing the reversibility of the normal process $O_2 + e^- \rightleftarrows O_2^-$ (peak 2), and the irreversibility of peak 1, which appears on bile pigment addition. *a* blank solution 1×10^{-4} M O_2: the small peak ($\cong -0.6$ V) is due to the presence of traces of water (bubbled air in this case was not dried). *b* 1.2×10^{-4} M O_2 and 2.5×10^{-4} M BR. *c* 7×10^{-5} M O_2 and 2.2×10^{-4} M MBV-DME.

are widespread, but are especially abundant in liver and small intestine tissues and in milk[36], b) an inverse relationship has been reported between superoxide dismutase activity and hyperbilirubinemia in newborns[37], and c) vitamin E level is lower in newborns than in adults[38].

Acknowledgment. This research was supported by the CAICYT (project 459-84).

1 Chance, B., Sies, H., and Boveris, A., Physiol. Rev. *59* (1979) 527.
2 Fridovich, I., A. Rev. Biochem. *44* (1975) 147.
3 Cerutti, P. A., Science *227* (1985) 375.
4 Ando, W., and Mor-Oka, Y., (Eds) The Role of Oxygen in Chemistry and Biology. Elsevier 1988.
5 see bibliographic review in Stocker et al.[7].
6 Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N., and Ames, B. N., Science *235* (1987) 1043.
7 Stocker, R., Glazer, A. N., and Ames, B. N., Proc. natl Acad. Sci. USA *84* (1987) 5918.
8 Robertson, P., and Fridovich, I., Archs Biochem. Biophys. *213* (1982) 353.
9 Kaul, R., Kaul, H. K., Bajpai, P. C., and Murti, C. R. K., J. Biosci. (India) *1* (1979) 377.
10 Nakamura, H., Uetani, Y., Komura, M., Takada, S. Sano, K., and Matsuo, T., Biol. Neonate *52* (1987) 273.
11 Kaul, R., Kaul, H., and Murti, C. R. K., FEBS Lett. *111* (1980) 240.
12 Wolfram, I., Vegh, M., and Horvath, I., Acta biochim. biophys. Hung. *21* (1986) 307: CA *106*: 191335c.
13 Sawyer, D. T., and Valentine, J. S., Acc. Chem. Res. *14* (1981) 393.
14 van Norman, J. D., Analyt. Chem. *45* (1973) 173.
15 Slifstein, Ch., and Ariel, M., J. electroanal. Chem. *75* (1974) 551.
16 Longhi, P., Mannitto, P., Monti, D., Mussini, T., and Rondinini, S., Electrochim. Acta *26* (1981) 541.
17 Claret, J., Feliu, J. M., Muller, C., Ribó, J. M., and Serra, X., Tetrahedron *41* (1985) 1713.
18 Claret, J., Feliu, J. M., Muller, M., Ribó, J. M., and Serra, X., Bioelectrochem. Bioenerg. (J. electroanal. Chem. *211*) *15* (1986) 125.
19 Longhi, P., Mussini, T., Rondinini, S., Dada, G., Manitto, P., and Monti, D., Bioelectrochem. Bioenerg. (J. electroanal. Chem. *231*) *17* (1987) 101.
20 a) Schmid, R., and McDonagh, A., The Porphyrins, vol. VI, p. 257. Ed. D. Dolphin. Academic Press, 1978. b) James, B. R., *ibid.* vol. V, part C, p. 205. c) McDonagh, A., *ibid.* vol. VI, p. 293.
21 Kuenzle, C. C., Wiebel, M. H., and Pelloni, R., Biochem. J. *133* (1973) 357.
22 Landen, D. G., Park, Y.-T., and Lightner, D. A., Tetrahedron *39* (1983) 1893.
23 Falk, H., Grubmayr, K., Höllbacher, G., Hofer, O., Leodolter, A., Neufingerl, F., and Ribo, J. M., Mh. Chem. *108* (1977) 1113.
24 Dymond, J. H., J. Chem. Phys. *71* (1967) 1892.
25 Andrieux, C. P., Hapiot, P., and Savéant, J.-M., J. Am. chem. Soc. *109* (1987) 3768.
26 Chin, D. H., Chiericato, G., Nanni, E. J., and Sawyer, D. T., J. Am. chem. Soc. *104* (1982) 1296.
27 Sawyer, D. T., Chiericato, G., Angelis, Ch. T., Nanni, E. J., and Tsuchiya, T., Analyt. Chem. *54* (1982) 1720.

28 Cofre, P., and Sawyer, D. T., Analyt. Chem. *58* (1986) 1057.
29 Falk, H., and Leodolter, L., Mh. Chem. *109* (1978) 883.
30 Falk, H., and Zruneck, V., Mh. Chem. *114* (1983) 1107.
31 Galliani, G., Monti, D., Speranza, G., and Manitto, P., Tetrahedron Lett. *25* (1984) 6037.
32 Galliani, G., Monti, D., Speranza, G., and Manitto, P., Experientia *41* (1985) 1559.
33 Nanni, E. J., Stallings, M. D., and Sawyer, D. T., J. Am. chem. Soc. *102* (1980) 4481.
34 Heirwegh, K. D. M., and Brown, S. B., (Eds) Bilirubin (CRC, 1982) vols 1 and 2.
35 Lightner, D. A., and McDonagh, A. F., Acc. Chem. Res. *17* (1984) 417.
36 Krenitsky, T. A., Tuttle, J. V., Cattau, E. L., and Wang, P., Comp. Biochem. Physiol. *49B* (1974) 687.
37 Bracci, R., Buonocuore, G., Talluri, B., and Berni, S. G., Acta paediatr. scand. *77* (1988) 349.
38 Bracci, R., Ciccoli, L., Faciani, G., De Donno, M., and Dettori, M., Boll.-Soc. Ital. Biol. Sper. *54* (1978) 879.

0014-4754/90/010063-05$1.50 + 0.20/0

Glucose-evoked recovery of hepatic thyroxine 5'-deiodinase independent of de novo protein synthesis in fasted rat

M. Nishida and C. S. Pittman

Birmingham V.A. Medical Center and University of Alabama School of Medicine, University Station, Birmingham (Alabama 35294, USA)
Received 7 April 1989; accepted 28 August 1989

Summary. The glucose-evoked recovery of Type I thyroxine 5'-deiodinase activity in the hepatic microsomes of fasted rat was not inhibited by either cycloheximide, puromycin or actinomycin D during 3 h after glucose feeding; however, [^{3}H]-leucine uptake by the liver or the hepatic microsomal fraction was significantly inhibited by cycloheximide and puromycin but not by actinomycin D. These results indicate that the glucose-evoked recovery of deiodinase activity may be independent of de novo protein synthesis.
Key words. Thyroxine 5'-deiodinase; starvation; caloric ingestion; cycloheximide; puromycin; actionomycin D.

Dietary intake is one of the modulatory factors of thyroid hormone metabolism in the peripheral tissues[1]. Starvation reduces the conversion of hepatic thyroxine (T_4) to 3,5,3'-triiodothyronine (T_3) and results in a decrease of serum T_3 level[2], whereas refeeding a starved subject with a simple carbohydrate such as glucose returns both the enzyme activity and hormonal concentration to normal levels[3]. Since serum insulin level is also elevated by refeeding, one explanation for the glucose-evoked increase of 5'-deiodinase activity can be a de novo synthesis of the enzyme protein via an insulin-mediated pathway of energy supply in the tissue[4]. To examine this hypothetical explanation, the present study was designed to determine whether inhibitors of protein synthesis also suppress the elevation of glucose-dependent activity of thyroxine 5'-deiodinase in fasted rats.

Materials and methods

125-I labeled T_3 (> 1200 μCi/ng) and T_4 (> 1200 μCi/ng) were purchased from Amersham Chemical Co. (Arlington Heights, IL, USA). Tritiated leucine ([^{3}H]-Leu, 146.5 Ci/nM) was acquired from New England Nuclear (Boston, MA, USA). Dithioerythritol (DTE), T_3, T_4, bovine serum albumin (BSA), cycloheximide (CH) and actinomycin D (AD) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Puromycin (PM) was purchased from Nutritional Chemical Co. (Cleveland, OH, USA).

Male Sprague-Dawley rats, weighing 220 g, were maintained on Purina chow pellets (Wayne Lab. USA) and tap water ad libitum. The dosages of protein inhibitors were chosen as the highest dose which the test animals survived 48 h after injection. The animals received a daily subcutaneous injection of T_4 at 0.75 μg/100 g b.wt for five consecutive days before fasting[5]. The T_4 supplement was continued during fasting until the day before sacrifice. The animals were divided into three groups and each fasted for 48 h before experiment. The control group was given a bolus of water through an intragastric tube. The second and third groups were refed with a bolus of 50% glucose (1 ml/100 g b.wt) in the same way. The second group was given a protein synthesis inhibitor by intraperitoneal injection (i.p.) as described in the legends while the third group was given an i.p. injection containing 0.9% NaCl solution only. All animals were sacrificed 3 h after refeeding. Under anesthesia, blood was withdrawn from the abdominal vein; then the liver was perfused with 0.9% NaCl solution containing 10 μ/ml heparin, excised and minced. Homogenization took place with 0.05 M Tris-HCL containing 5 mM DTE, 10 mM EDTA, 0.25 M sucrose at pH 7.2. The microsomal fraction was harvested according to the method of Hogeboom[6]. The resulting pellet after centrifugation at