Acknowledgements—The skilful technical assistance of Margareta Viestam-Rains is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (No. 25X-2189), the Bank of Sweden Tercentenary Foundation and Karolinska Institutet.

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Biochemical Pharmacology, Vol. 35, No. 9, pp. 1611–1613, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Reserpine inhibition of lipid peroxidation and protein phosphorylation in rat brain

(*Received* 28 *November* 1984; *accepted* 24 *October* 1985)

Reserpine has been used as a pharmacological agent and as a tool to study turnover of monoamines in CNS for a long time. However, its mode of action at a molecular level is still not properly understood. Reserpine presumably acts on the membranes of the intraneuronal vesicles by a process which is ATP and Mg^{2+} dependent [1].

A number of psychotropic drugs, especially the phenothiazines, affect lipid peroxidation [2] as well as protein phosphorylation in cerebral preparations [3]. Here we report that reserpine is a very strong inhibitor of lipid peroxidation in rat brain homogenates and mitochondrial preparations and that it also affects protein phosphorylation in cerebral cortex slices as measured by ^{32}P incorporation.

Lipid peroxidation in homogenate or mitochondrial preparations of brain was determined as described earlier [4] in the presence or absence of reserpine. For *in vivo* experiments, rats were given reserpine (CIBA, India), 2 mg/kg, intraperitoneally for 1 day (acute) or 5 days (chronic). The effect of reserpine on some $O_2^{\cdot}$ (superoxide radical) mediated reactions was studied in a system containing epinephrine (3 mM) and EDTA (1 mM) in sodium carbonate buffer (0.05 M) at pH 10.2 as described [5]; the initial rate of formation of adrenochrome was followed at 480 nm. In another set of experiments, formation of diformazan from phenazine methosulfate (PMS) in the presence of nitroblue tetrazolium (NBT) and NADH was measured in the absence or presence of reserpine (10 μM). The final assay mixture contained 16 mM Tris–HCl buffer, pH 8.0, 73 μM NADH, pH 8.0, in Tris buffer, 5.2 μM PMS in water, 80 μM NBT in water with or without reserpine (10 μM). The absorbancy was read at 560 nm at 10 min after the onset of reaction [6]. Protein phosphorylation was measured exactly as described by Rodnight *et al.* [7] in rat cerebral cortex slices prepared according to Shankar and Quastel [8], except that the period of incubation was 1 hr. ^{32}P (15 μCi) was present right from the beginning of incubation. Reserpine (50 or 100 μM), when present, was added at the start of incubation. Protein bound phosphorus was determined by the method of Martin and Doty [9]. Protein was estimated by the method of Lowry *et al.* [10].

Results given in Table 1 show that reserpine at concentrations of 25, 50 and 100 μM effectively inhibited *in vitro* lipid peroxidation of brain homogenate. With 100 μM reserpine, the *in vitro* peroxidation was inhibited almost completely. With 25 or 50 μM reserpine, the *in vitro* peroxidation was reduced by more than 30 and 50% respectively. The extent of peroxidation was determined after 90 min of incubation at 37° in the presence or absence of the drug.

The time course of inhibition of mitochondrial lipid peroxidation by reserpine at 50 and 100 μM concentrations is shown in Fig. 1. Lipid peroxidation was initiated by 10 μM Fe^{2+}, and amounts of malonaldehyde formed at 0, 20, 40 and 60 min were measured. The inhibition was observed as early as at 20 min of incubation and was persistent throughout the period of incubation.

Chronic reserpinisation inhibited subsequent *in vitro* lipid peroxidation of the brain homogenate to a variable extent (20–40% compared to peroxidation of control samples after 90 min of incubation) in different sets of experiments (data not presented). Acute reserpinisation, i.e. a single injection of reserpine, did not seem to inhibit *in vitro* lipid peroxidation of brain homogenate as measured by malonaldehyde estimation (data not presented).

The effects of reserpine on $O_2^{\cdot}$ mediated reactions are presented in Table 2. Values from individual experiments

Table 1. Effect of reserpine on lipid peroxidation in brain homogenate

	Lipid peroxidation (nmoles malonaldehyde/g tissue)
Control	204 ± 22.56 (4)
With reserpine	
25 μM	124.5 ± 18.42* (4)
50 μM	98.5 ± 17.53* (4)
100 μM	9.86 ± 2.46* (4)

Values are the mean ± S.D. of the number of observations given in parentheses. The level of significance was tested by Student's *t*-test.

* Differs significantly from the control, $P < 0.001$.

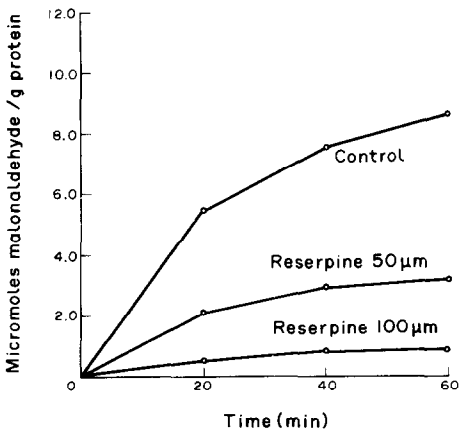

Fig. 1. Effect of reserpine on *in vitro* peroxidation of brain mitochondrial fractions. Mitochondrial fraction was isolated from adult rat brain and lipid peroxidation *in vitro* carried out without (control) or with the addition of reserpine (50 and 100 μM) as described in the text. Values given (μmoles malonaldehyde/g protein) at 0, 20, 40 and 60 min are from a single representative experiment which was repeated at least four times.

represent the change in extinction/min for the first reaction system (A) and the absorbancy change after 10 min for the second reaction system (B). Reserpine seemed to have no inhibitory effect on either reaction system.

Table 3 shows that reserpine also inhibited phosphorylation of proteins in incubated cerebral cortex slices, measured by ^{32}P incorporation into protein bound phosphate. Control experiments showed that the incorporation of ^{32}P in protein bound phosphorus increased with time. More than 60% inhibition could be observed at 50 μM reserpine. At higher concentrations of reserpine (100 μM), greater inhibition could be observed (80–90%; results not shown). The inhibition was evident whether the values were expressed per unit weight, protein, or protein bound phosphorus.

The inhibition of lipid peroxidation by reserpine has not been reported earlier. Drugs such as thiopental and chlorpromazine have been shown to possess antioxidant property, but a considerably higher dose is required to elicit significant effect [11, 12]. To elucidate the mechanism of this inhibition by reserpine, we looked for any scavenging action of reserpine on the initiating radicals of lipid peroxidation. However, at a 10 μM concentration reserpine failed to show any inhibition of $O_2^{\cdot}$ mediated reactions *in vitro*. Initiating radicals (e.g. $O_2^{\cdot}$, $OH^{\cdot}$, etc.) of lipid peroxidation are generated in the aqueous phase of the cell, whereas the propagating free-radicals are formed in the lipid phase of the membranes. It is known that the radical scavenging action of different agents and consequently their antioxidant property depend upon precise solubility and

Table 2. Effects of reserpine on $O_2^{\cdot}$ mediated reactions

$O_2^{\cdot}$ mediated autooxidation of epinephrine (change in extinction/min) at 480 nm		$O_2^{\cdot}$ mediated formation of diformazan (absorbancy after 10 min) at 560 nm	
Control (A)	Test (A) (10 μM reserpine)	Control (B)	Test (B) (10 μM reserpine)
1. 0.024	0.024	0.24	0.24
2. 0.023	0.023	0.26	0.24
3. 0.024	0.024	0.26	0.25
4. 0.022	0.023	0.26	0.27

Two different reaction systems (A and B) mediated by $O_2^{\cdot}$ radical were followed spectrophotometrically, without (control) or with (test) the addition of reserpine (10 μM) as described in the text. Values from individual experiments have been shown.

Table 3. Effect of reserpine on protein phosphorylation in rat brain cortical slices

Additions	cpm × 10^{-3}/100 mg tissue	cpm × 10^{-3}/100 mg protein	cpm/μg protein bound phosphate
Control (7)	16.71 ± 7.53	143.09 ± 56.81	813.86 ± 372.37
Reserpine, 50 μM (10)	6.75 ± 2.35*	51.47 ± 16.5*	317.92 ± 110.05*

Experiments were carried out as described in the text. Values are the mean ± S.D. of the number of observations given in parentheses. Statistical significance was tested by Student's *t*-test.

* $P < 0.01$.

intracellular distribution of the substance. It seems unlikely that reserpine which is very sparingly soluble in water at physiological pH could effectively scavenge the initiating free radicals of lipid peroxidation. On the other hand, it seems probable that, because of its high solubility in the lipid phase, reserpine interferes with the propagation phase of the peroxidative process.

Inhibition of phosphorylation by reserpine may be involved with the molecular mechanism of action of the drug. Phenothiazine derivatives, e.g. trifluperazine, have been shown to inhibit calmodulin-dependent synaptic protein phosphorylation [13]. Whether the effects of reserpine on protein phosphorylation in cortical slices are mediated by alterations in calmodulin- or cAMP-dependent protein phosphorylation is difficult to predict from the results of the present experiments. However, these observations are significant in view of the fact that both the protein phosphorylation and the lipid peroxidation can modify various membrane phenomena [14, 15].

In summary, results of our experiments show that reserpine is an effective inhibitor of both lipid peroxidation and protein phosphorylation in rat brain. Presumably, these effects are due to its action on the brain cell membrane.

Acknowledgements—This work was supported by Grant 9(112)/80-EMR II from the Council of Scientific and Industrial Research (India) and Grant F.23-67/83 (SR II) from the University Grants Commission (India).

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Biochemical Pharmacology, Vol. 35, No. 9, pp. 1613–1615, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Structural assignment of an *N*-glucuronide metabolite of the phenylethanolamine *N*-methyltransferase (PNMT) inhibitor 1,2,3,4-tetrahydroisoquinoline-7-sulfonamide by ^{15}N-NMR*

(*Received* 16 *July* 1985; *accepted* 5 *November* 1985)

The compound 1,2,3,4-tetrahydroisoquinoline-7-sulfonamide (SK&F 29661) has been shown to inhibit phenylethanolamine *N*-methyltransferase (PNMT) in animals [1]. After oral administration of [^{14}C]SK&F 29661 to the dog (15 mg/kg), about 90% of the radiolabel in urine is excreted as SK&F 29661 with 5–10% of the urinary radiolabel identified as a conjugate of SK&F 29661 [2]. Data from enzymatic hydrolysis and mass spectrometry indicated that the conjugate is a glucuronide of SK&F 29661. This conjugate is hydrolyzed by β-glucuronidase to SK&F 29661. Hence, it is an *N*-glucuronide and not a *C*-glucuronide, since *C*-glucuronides are chemically stable and remain intact after incubation with β-glucuronidase [3, 4]. However, the position of glucuronidation, whether at the ring nitrogen as in **1** or at the sulfonamide nitrogen as in **2**, could not be established from these studies (Fig. 1). Since we had only a limited amount of the *N*-glucuronide, we sought a nondestructive method to differentiate these two isomers. ^{1}H-NMR was attempted to detect and integrate the sulfonamide and amine protons of the *N*-glucuronide, but was not successful due to the interference of water peak and uncertainty of the chemical shifts of the amine protons. This report describes a unique application of ^{15}N-NMR to assign unequivocally the position of glucuronidation in drug metabolism studies.

[^{15}N]SK&F 29661 was synthesized according to published procedures [1, 5]. 2-Acetyl-1,2,3,4-tetrahydroisoquinoline was reacted with chlorosulfonic acid to give 2-acetyl-1,2,3,4-tetrahydroisoquinoline-7-sulfonyl chloride which was treated with $^{15}NH_4OH$ (99%) to form 2-acetyl-1,2,3,4-tetrahydroisoquinoline-7-sulfonamide. Hydrolysis of the sulfonamide with 18% hydrochloric acid gave [^{15}N]SK&F 29661 hydrochloride with ^{15}N-enrichment at the sulfonamide nitrogen.

Materials and methods

$^{15}NH_4OH$ (99%). This was purchased from MSD Isotopes, Merck Chemical Division.

[^{14}C]*SK&F* 29661. This was prepared and provided by the Chemical Technology Department of Smith Kline & French Laboratories according to literature procedures [6].

* Preliminary results were presented at the 1984 International Chemical Congress of Pacific Basin Societies, Honolulu, HI, December 16–21, 1984.