deferens elicited a contraction of the preparation within 1-3 sec. The cyclic nucleotide levels were determined by the methods of Gilman[12] and Steiner et al.[13] in preparations frozen 3-5 sec and 30 sec after electrical field stimulation. The cyclic AMP level was decreased by about 25% after 3-5 sec and remained lowered after 30 sec (figure 2). The cyclic GMP level was slightly increased after 3-5 sec, but after 30 sec the level was increased about 2-fold. We have also studied the cyclic nucleotide levels after norepinephrine stimulation. Norepinephrine (0.3 mM) elicited a contraction which lasted for about 60 sec. 5 sec after the norepinephrine addition, the cyclic AMP level decreased from 0.6±0.1 to 0.4±0.02 nmoles/g wet wt, but 30 sec after the addition the level had increased to 0.8±0.2 nmoles/g wet wt. A corresponding time study of the cyclic GMP level gave no significant changes. This is in accordance with earlier studies on vas deferens of rat[6], where no significant change of cyclic GMP level was demonstrated after 20 sec.

Our present data on guinea-pig vas deferens is not consistent with the recent suggestion[7] that cyclic GMP may act as feedback inhibitor of hormonally stimulated calcium influx. If cyclic GMP is a presumptive modulator of vas deferens contractility, our results agree better with the hypothesis that cyclic GMP may act as a co-mediator with calcium to promote contraction. However, in gallbladder it was recently demonstrated that cholecystokinin produced the same degree of contraction, even if the cyclic GMP production was blocked by indomethacin[14].

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A radioenzymatic method to measure picogram amounts of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) in small samples of brain tissue

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Summary. A radioenzymatic method for simultaneous determination of dopamine and DOPAC in small brain areas is described. By using this assay, 250 pg of dopamine and 150 pg of DOPAC can be estimated. The present method has been applied to compare the effect of different psychotropic drugs on the dopamine and DOPAC levels in the caudate nucleus, substantia nigra and medial basal hypothalamus.

Several radioenzymatic methods for measuring catecholamines in small tissue samples and biological fluids have recently been described[1-4]. These very sensitive methods are based on the estimation of labelled normetanephrine, metanephrine and 3-methoxytyramine (3-MT) formed from norepinephrine, epinephrine and dopamine (DA) respectively in the presence of catechol-O-methyl-transferase (COMT, E.C.2.1.1.6.) and ^{14}C- or ^{3}H-methyl-S-adenosyl-methionine (SAMe) as methyl donor. We described here a radioenzymatic procedure for the simultaneous determination of DA and 3,4-dihydroxyphenylacetic acid (DOPAC) in the same sample.

The present method is based on the O-methylation by COMT of DA and DOPAC to 3-MT and homovanillic acid (HVA), respectively, in the presence of ^{3}H-SAMe of high specific activity. ^{3}H-3-MT is then isolated by means of a small cation exchange chromatographic column of Dowex 50 W×4, while ^{3}H-HVA is isolated by Sephadex G-10, as described by Westerink and Korf[5]. The method described is the combination of 2 different assays: one for DOPAC by Argiolas et al.[6] and the other for DA by Zschaeck and Ramirez[7] with some modifications.

Material and methods. Reagents. All reagents used were 'pro analysis' and obtained from Merck. Dithiothreitol and 3-methoxytyramine-HCl were purchased from Calbiochem; S-adenosyl-methionine-methyl-^{3}H (8 to 12 Ci/mmole) from Amersham. Norepinephrine (NE), 3,4dihydroxymandelic acid (DOMA), vanilmandelic acid (VMA), 3,4-dihydroxyphenylethyleneglycol (DHPG), DOPAC, HVA, DA, L-DOPA from Sigma Chemical Company; Sephadex G-10 from Pharmacia Fine Chemicals; Dowex 50 W×4 from Bio Rad.

Purification of catechol-O-methyl-transferase. COMT was purified from rat liver according to Cuello et al.[3] up to ammonium sulphate back-wash step. Then the enzyme was dialyzed twice against 1000 vol. 0.1 M phosphate buffer pH 7, containing 10^{-4} M dithiothreitol, divided in small volumes and stored at −20 °C in small batches.

Animals and tissue preparation. Rats weighing 150-200 g were obtained from Charles River (Milan, Italy). All rats were housed at 24 °C and were under diurnal lighting conditions with light from 06.00 h to 18.00 h. The animals were killed by decapitation; caudate nucleus, substantia nigra and cerebellum were dissected as described by Spano et al.[8] and medial basal hypothalamus by Brown et al.[9]. The tissues were homogenized with a teflon microhomogenizer in about 50 vol. of ice-cold 0.1 M perchloric acid. Samples were centrifuged at 10,000× g for 10 min at 4 °C. A 50 µl aliquot of the clear supernatant was placed in 15 ml conical centrifuge glass tubes. Tissue blanks were prepared from cerebellum homogenates of normal rats. Standards were prepared by homogenizing the cerebellar tissue of similar weights as the brain area to be analyzed in ice-cold 0.1 M perchloric acid containing known concentrations of DA and DOPAC. These steps were carried out in a ice-cold bath.

Effect of different psychotropic drugs on dopamine and DOPAC levels in the caudate nucleus (CN), substantia nigra (SN) and medial basal hypothalamus (MBH)

Treatment (mg/kg)		Dopamine (μg/g)			DOPAC (μg/g)		
		CN	SN	MBH	CN	SN	MBH
Saline	-	11.90 ± 0.38	1.70 ± 0.09	0.71 ± 0.05	2.34 ± 0.13	1.76 ± 0.12	0.21 ± 0.02
Pargyline	70	16.70 ± 0.31*	2.39 ± 0.08*	0.98 ± 0.07*	0.06 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Reserpine	5.0	2.38 ± 0.09*	0.96 ± 0.06*	0.21 ± 0.02*	7.21 ± 0.25*	1.09 ± 0.07*	0.12 ± 0.01*
Haloperidol	0.5	10.77 ± 0.24	1.80 ± 0.10	0.73 ± 0.06	7.40 ± 0.30*	5.26 ± 0.23*	0.20 ± 0.02

Drugs were given i.p. 60 min prior to death. Each value is the mean ± SE of 6 experiments. * $p < 0.001$ in respect to control value.

DOPAC and DA assay. The following reaction mixture is freshly prepared before assays: 5 mg of the enzyme COMT, 1 μmole magnesium chloride, 1 μCi ^{3}H-SAMe, 60 μm Tris buffer, pH 9.1, and 0.1 mg dithiothreitol. This incubation mixture is added to each sample to reach a final volume of 100 μl. The pH of the final mixture is 9.1. The tubes containing the complete assay mixture are closed and incubated for 60 min at 37 °C in a Dubnoff metabolic shaker. The reaction is stopped by placing the tubes in ice-cold bath. A 50 μl aliquot of the reaction mixture is transferred in 15 ml glass stoppered tubes, containing 0.5 ml of 0.5 M borate buffer, pH 10. After vortex shake, 50 μg of 3-MT unlabelled in 10 μl of 0.01 N HCl are added as carrier, and this aliquot is used for ^{3}H-3-MT separation. 0.9 ml of ice-cold 0.4 N $HClO_4$ containing 0.5 μg nonradioactive HVA as carrier are added to the residue reaction mixture and used for ^{3}H-HVA isolation.

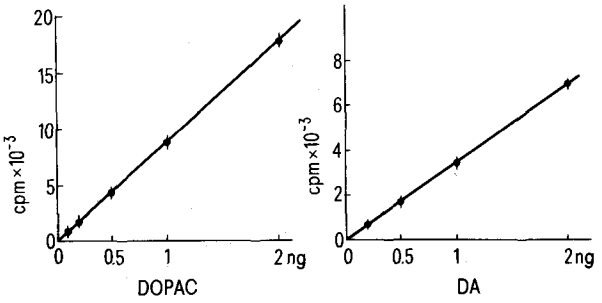
Extraction of ^{3}H-O-methylated products. ^{3}H-HVA isolation. The excess of perchlorate is precipitated by addition of 2 drops of KOH-formiate solution (made adding concentrated formic acid, 98%, into 10 N KOH until the concentration of the formic acid is 5 N). After centrifugation for 20 min at 4000 × g the clear supernatant is placed into columns of Sephadex G-10 (5 × 70 mm), which has previously been washed with 3 ml 0.01 N ammonia and 3 ml 0.01 N formic acid. After the supernatant has passed through the column, 2.5 ml 0.01 N formic acid and 1 ml 0.5 mM phosphate buffer pH 8.5 are added consecutively and the eluate discarded. ^{3}H-HVA is eluted with 2 ml of 0.5 mM phosphate buffer and collected in 15 ml glass stoppered centrifuge tubes containing 2 g of solid NaCl. The collected buffer is acidified with 0.1 ml of 1 N HCl and ^{3}H-HVA is extracted into 4 ml ethyl acetate. The organic phase is washed twice with 2.5 ml of 0.1 N HCl. After centrifugation, 3 ml of the organic phase are transferred into a scintillation counting vial. The radioactivity of the samples is counted, after adding 10 ml of Insta-Gel, by liquid scintillation spectrometry.

Because certain tissues contain sufficient amounts of acidic metabolites of norepinephrine to interfere with the estimation of DOPAC, a further separation by TLC may be necessary. The 3 ml of ethylacetate is transferred into a scintillation counting vial and evaporated under a stream of anhydrous nitrogen. The non-volatile product is taken up into 100 μl of ethanol containing HVA and VMA (50 μg of each compound) as additional carriers. 50 μl of the ethanol extract are spotted on chromatography sheets of silica gel F 254 (Merck) 500 μm in thickness, and run for 45 min at 24 °C. The solvent system used is: toluene: acetic acid: ethylacetate (5:4:1 by vol.). In this solvent system, VMA and HVA migrate with Rf values sufficently different to give a clear separation. The HVA and VMA spots are marked, cut out, scraped from the plate and extracted in counting vials with ethanol (1 ml). The radioactivity is counted, after adding 10 ml of Insta-Gel by liquid scintillation spectrometry.

^{3}H-3-MT isolation. ^{3}H-3-MT is extracted from borate buffer pH 10 into 6 ml of toluene-isoamyl alcohol (3 : 2 v/v). After centrifugation, 5.5 ml of the organic phase are transferred to another tube containing 1.1 ml of 0.1 N HCl. After shaking and centrifugating, 1 ml of the acid phase is transferred to a 5 × 70 mm Dowex 50 W × 4 cation exchange resin column, which has been activated according to Zschaeck and Ramirez[7] and previously washed with 20 ml of acetate buffer 0.1 M pH 4.8. After the acid phase has passed through the column, the latter is washed with 3 ml of water followed 3 ml of 2 N HCl, 12 ml of 3 N HCl and 4 ml of 0.5 N NaOH, ^{3}H-3-MT is then eluted with 2.2 ml of 0.5 N NaOH and collected into vials. The NaOH is neutralized with concentrated HCl and the radioactivity measured by liquid scintillation spectrometry after addition of 10 ml of Insta-Gel.

Results and discussion. The radioenzymatic procedure described allows the simultaneous determination of microquantities of DA and DOPAC in tissue extracts. The standard curves reported in the figure indicate that this method allows the determination of about 150 pg of DOPAC and 250 pg of DA. After blank subtraction, the linearity of the method ranges from 0.1 to 5 ng for DOPAC and DA. Concentrations of L-DOPA, DA, NE and DHPG up to 5 times that of DOPAC and DA, carried through the entire procedure, did not interfere with the assays of these substances. In fact the amount of radioactivity present in the last step did not exceed 1% that of DOPAC or DA. The assay procedure utilizing product purification by TLC is more specific. In fact TLC is necessary to separate ^{3}H-VMA, formed by DOMA, from HVA, because DOMA behaves like DOPAC in the various steps of the method and overlaps in the final estimation of DOPAC. However, since the amount of DOMA in the whole brain of normal rats has been found to be negligible[9], TLC usually is not needed. Therefore ^{3}H-HVA in the organic phase can be counted directly by liquid scintillation spectrometry.

We applied the above method to measure DA and DOPAC levels in the caudate nucleus, the substantia nigra and the medial basal hypothalamus, and the influence of different drugs on them. As the table shows, the concentration of DA and DOPAC in these brain structures are comparable with



Standard curves for DA and DOPAC assay in the same sample by COMT in the presence of ^{3}H-SAMe. Incubation conditions and extraction of ^{3}H-methylated products are described in methods. Each point is the mean ±SE of 6 determinations after subtraction of blank.

those obtained by using fluorimetric[5,11], gas chromatographic[12,13] and radioenzymatic[6,7,14] procedures. On the other hand, DOPAC values in the caudate nucleus were found to be about twice higher than those reported by others[5,13]. The difference might be due to the fact that we dissected an area restricted to the head of the caudate nucleus.

Pargyline, a MAO inhibitor, enhanced DA content and produced a disappearance of DOPAC levels in 3 brain structures studied within 60 min after treatment, indicating a rapid turnover of DOPAC in 3 brain areas. Reserpine caused a marked decrease of DA in these brain areas while surprisingly enhancing the DOPAC content only in the caudate nucleus. Haloperidol increased DOPAC levels in the caudate nucleus, substantia nigra, but failed to do so in the medial basal hypothalamus. Our results confirm previous observations[15] that different dopaminergic areas may respond to psychotropic drugs in a different manner.

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Effect of chronic alloxan diabetes and insulin administration on intestinal brush border enzymes

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Summary. Brush border sucrase and lactase activities are significantly elevated in alloxan-induced chronic diabetes and are restored to control levels after insulin treatment. Alkaline phosphatase and Mg-ATPase levels remain unchanged in diabetes, compared to a control group. Insulin treatment alone to control animals also led to enhanced activities of these enzymes.

Morphological and functional alterations in the intestine of diabetics have been well documented. Increase in the intestinal absorption of sugars and amino acids in diabetic animals has been described[1-4]. Similar changes in the activities of various disaccharidases in the intestine, following an acute dose of alloxan to rats, have been observed[5-6], which could be because of the specific induction of these enzymes in response to alloxan, or because of the metabolic disturbances associated with alloxan-induced diabetes. In order to differentiate between these 2 possible effects, and in view of the fact that there are no reports available on the effect of chronic diabetes on brush border enzymes, the present study was undertaken. In addition to its effects on brush border sucrase, lactase and alkaline phosphatase (AP), the effect of chronic alloxan diabetes on intestinal Mg-ATPase was also investigated.

Materials and methods. Male albino rats (120-140 g) bred in the Institute colony were used. The procedure for the induction of diabetes and insulin treatment of the animals was essentially the same as described by Chauhan and Sarkar[7]. Animals in control (A), diabetic (B), diabetic + insulin (C) and control + insulin (D) groups were observed for 120 days and sacrificed after giving ether anesthesia. Intestines were removed, washed with chilled normal saline, and brush border membranes prepared according to Schmitz et al.[8]. The membrane fragments were suspended in 10 nM sodium maleate, pH 6.8, containing 0.02% sodium azide. Sucrase and lactase activities were measured using glucose oxidase peroxidase system[9,10]. Alkaline phosphatase was determined as described by Eiccholz[11] Mg-ATPase activity was assayed in the intestinal homogenates as previously reported[12]. Blood sugar was measured by Somogyi's method[13]. Protein estimation was done according to Lowry et al.[14].

Results and discussion. Results on the effect of chronic alloxan diabetes and of insulin administration to rats are shown in table 1. There is a 2fold increase in the activities of both sucrase and lactase in alloxan-treated animals

Table 1. Effect of chronic alloxan diabetes and insulin administration on brush border disaccharidases

Group		Blood sugar at the time of sacrificing (mg/100 ml)	Sucrase μmoles glucose/min g protein at 37 °C	Lactase
A	Control	85 ± 11	318.3 ± 20.6	44.5 ± 1.5
B	Diabetic	357 ± 28	714.1 ± 27.6	86.3 ± 3.9
C	Diabetic + insulin	126 ± 12	347.3 ± 13.8	52.9 ± 5.3
D	Control + insulin	78 ± 10	536.2 ± 11.6	94.7 ± 4.6

Values are mean ± SD of 6-8 determinations.

Table 2. Effect of chronic alloxan diabetes and insulin administration on intestinal alkaline phosphatase and Mg-ATPase activities

Group		Alkaline phosphatase μmoles phenol/min g protein at 37 °C	Mg-ATPase μmoles Pi/min g protein at 37 °C
A	Control	13.37 ± 1.49	5.31 ± 0.45
B	Diabetic	13.29 ± 2.31	5.55 ± 0.58
C	Diabetic + insulin	14.42 ± 2.78	6.75 ± 0.84*
D	Control + insulin	20.43 ± 4.36	6.59 ± 0.53*

Values are mean ± SD, n = 8. * $p < 0.05$ compared to control.