hydrocortisone at concentrations of 10^{-6} to $10^{-8}M$, increased Ca^{2+} binding whereas insulin decreased Ca^{2+} binding. At hormone concentrations of 10^{-6} to $10^{-7}M$, glucagon had the greatest effect, but at $10^{-8}M$, hydrocortisone exerted the greatest effect. Glucagon and epinephrine both stimulated binding most at $10^{-7}M$, but the stimulation by hydrocortisone increases with decreasing hormone concentration. With hydrocortisone, a hydrophobic hormone, the physical state of dispersion may influence its effect on the membrane.

Adenosine triphosphate at a concentration of 0.3 mM abolished the effect of insulin, and decreased the effect of the other hormones.

Inasmuch as the concentration of each hormone is very small, the hormone effects cannot be stoichiometrically related to the Ca^{2+} binding. In these systems we calculate that at $10^{-8}M$ hydrocortisone, one molecule of hormone leads to a binding of 3000 atoms of calcium. This postulates that the hormones act catalytically or that they induce conformational changes over a large segment of the membrane, modifying the binding of many calcium ions.

The effect of insulin opposite to that of the other hormones provides additional evidence for the antagonistic action of insulin with glucagon and epinephrine. Insulin antagonizes the glucagon stimulation of adenylate cyclase in isolated plasma membranes of rat liver (*8, 10*).

With respect to the model of Rasmussen (*5*), our studies suggest that the hormone effects on calcium binding may also be related to calcium transport through the membrane. We demonstrated that hormones such as glucagon, epinephrine, and insulin have two independent effects on the membrane, one being to either activate or inhibit adenylate cyclase activity, and the other being to stimulate or inhibit calcium binding. Although the concentration of hormones that is required to influence calcium binding is higher than the physiological concentration (insulin and glucagon have concentrations in portal blood of 10^{-9} to $10^{-10}M$), the effects may have physiological significance as the isolated membranes might have lower biological sensitivity than do membranes on intact cells.

Inasmuch as the amount of calcium bound to the membrane influences the permeability of the membrane, our studies also may suggest that certain hormones influence membrane permeability by controlling the amount of bound metal ions. Thus, insulin may make the cell membrane more permeable to certain substrates by decreasing the amount of bound calcium ions.

The significance of the ATP effect on modifying the calcium binding due to hormones is not clear. Whether this is related to the chelating property of ATP, to ATP altering the membrane structure, or indirectly to the cyclic AMP formed from ATP is not known. In the membrane systems we used, cyclic AMP is bound very weakly to the membrane and stimulates Ca^{2+} binding only at very high concentrations (10^{-3} to $10^{-5}M$) of cyclic AMP.

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Norepinephrine and Dopamine: Assay by Mass Fragmentography in the Picomole Range

Abstract. *Gas chromatography–mass spectrometry makes possible the simultaneous measurement of norepinephrine and dopamine in concentrations of 0.1-milligram tissue samples. Specificity of the assay is confirmed both by the retention time of the compound and by the mass to charge ratio of the fragments recorded. The sensitivity is of the order of 0.5 picomole, and linearity of the response is maintained up to at least 200 picomoles.*

A number of simple biochemical models of adrenergic neurons predict that small pools of catecholamines are preferentially released by nerve impulses (*1*). Since only circumstantial evidence supports this prediction, these pools must now be identified, characterized, and measured if these multicompartment models are to guide future investigation on the molecular nature of the mechanisms regulating adrenergic function.

These clarifications could be obtained if a method were available to measure femtomole (10^{-15} mole) concentrations of norepinephrine (NE) and dopamine (DA) in small samples of tissue. Accordingly, we have directed our attention to the analytical technique of combined gas chromatography (GC) and mass spectrometry (MS) (*2*). Identification of an unknown substance by MS usually requires at least 10^{-6} mole of a compound to record its fragmentation pattern and establish its identity. When the fragmentation pattern of the compound is known, it is then possible to identify as little as 3×10^{-15} mole of the substance by the technique of mass fragmentography

Table 1. The GC-MS characteristics of the PFP derivatives of norepinephrine, dopamine, and their α-methyl analogs. The minus sign indicates that the intensity was negligible.

PFP-amine derivative	GC retention time (min)	Relative intensity (%) at m/e:						Peak ratio
		176*	190*	428†	442†	590†	604†	
α-MNE	2.08	–	100	–	–	–	10	10
NE	2.83	100	–	–	–	40	–	2.5
α-MDA	4.36	–	100	–	33	–	–	3.3
DA	5.83	40	–	100	–	–	–	2.5

* Cleavage between the α- and β-carbon atoms. The positive charge is retained on the nitrogen-containing fragment. † Cleavage between the nitrogen and the α-carbon atom. The positive charge is retained on the fragment containing the aromatic ring and one hydrogen atom is transferred onto the neutral moiety.

(*3*), a technique in which the mass spectrometer is used as a detector for the gas chromatograph. In this way the GC separates the compounds prior to MS analysis, and the GC retention time gives one measure of specificity to the analysis. The MS gives more exact specificity by measuring two or more abundant characteristic fragments (mass to charge, m/e) of the compound at its GC retention time. Our data show that it is possible to quantitate the ion density generated by these fragments, thereby providing an extremely sensitive and specific analytical method.

In our method, mass fragmentography is used to quantitatively assay NE and DA in tissue. For this quantitative assay, the choice of the internal standard is important. Ideally, the internal standard should be the same compound labeled with stable isotopes in a group included in each of the two fragments selected for the analysis (*4*). Since, at present, the availability of compounds labeled with stable isotopes is rather limited, we have used α-methylnorepinephrine (α-MNE) and α-methyldopamine (α-MDA) as internal standards for NE and DA, respectively. These internal standards allow for normalization of the data for losses due to sample manipulations and for fulfilling all the other requirements of a suitable internal standard.

In developing our method, the first problem we had to solve was that of forming volatile, stable derivatives of the four catecholamines. By reacting the four catecholamines with pentafluoropropionic anhydride (PFPA; Pierce Chemicals) volatile acylated derivatives were formed (*5*). When NE and DA were reacted with PFPA in the presence of ethyl acetate at 60°C for 30 minutes, four pentafluoropropionyl groups (PFP) were introduced in NE and α-MNE, while three PFP groups were introduced in DA and α-MDA (see Fig. 1). Using an LKB 9000 GC-MS equipped with the accelerating voltage alternator, we confirmed the structures of the four PFP amines by mass spectral analyses (Fig 1). For the GC we used a 12-foot (4-m) glass column (inside diameter, 2 mm) packed with OV 17 (3 percent) on Gas Chrom Q 100–120 mesh (Applied Science, College Park, Pa.), with a flash heater at 280°C, a column oven at 180°C, and helium flow at 20 ml/min. For the mass spectrometer we used a molecular separator at 240°C, an ion source at 290°C, electron energy at 80 ev, a trap current at 60 μa, and an electron multiplier of 3.7 kv.

Mass spectral analysis (Fig. 1) revealed that the catecholamine-PFP derivatives have two major fragmentation patterns: (i) cleavage of the bond between the α and β carbon atoms with retention of the positive charge on the nitrogen-containing fragment and (ii) cleavage of the bond between the α carbon and the nitrogen. The positive charge is retained on the catechol fragment and one hydrogen atom is transferred onto the neutral moiety. The specific fragments used in this analysis for each compound and their ratios are reported in Table 1. This information was relevant because, for mass fragmentography, the magnetic field of the MS was held constant at a m/e setting equivalent to the mass number of the most abundant fragment generated from the compound being eluted from the GC. Since the α-methyl analog derivatives have a fragmentation pattern similar to that of the amine derivatives, it seemed that they would serve as reliable internal standards. It is also necessary that the amines form PFP derivatives in amounts proportional to the initial concentration of catecholamines. Mass

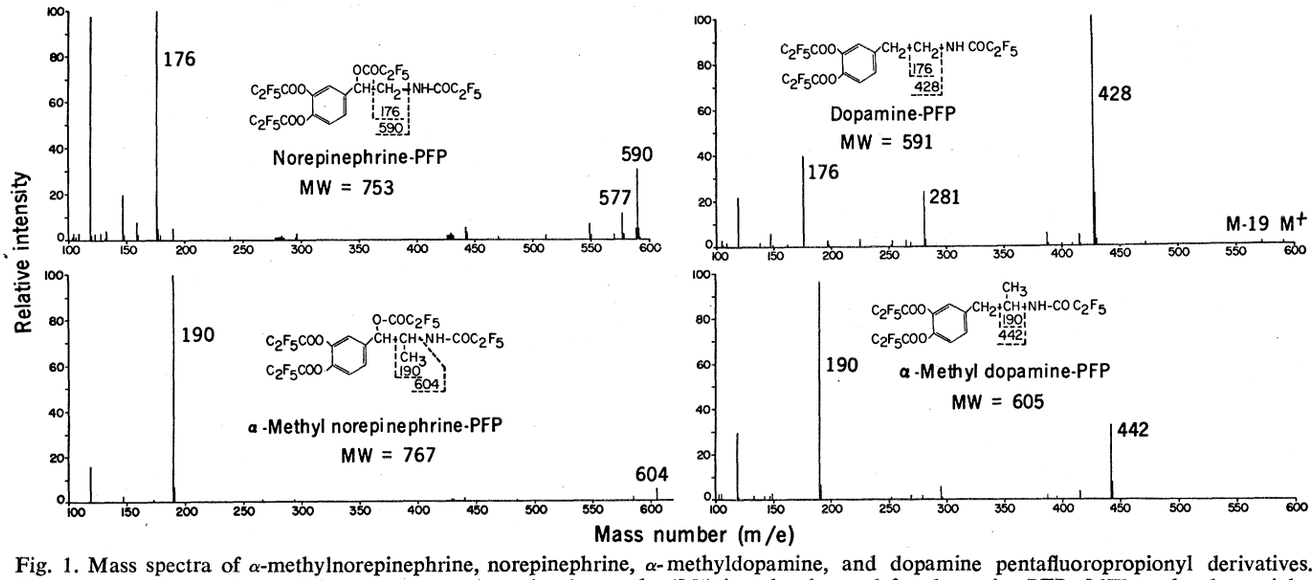

Fig. 1. Mass spectra of α-methylnorepinephrine, norepinephrine, α-methyldopamine, and dopamine pentafluoropropionyl derivatives. For mass spectrometric conditions, see text. A molecular peak (M^+) is only observed for dopamine-PFP; *MW*, molecular weight.

fragmentographic analysis showed that, when 0.5 to 200 pmole of each amine reacted with an excess of PFPA (100 μl) at 60°C in the presence of 20 μl of ethyl acetate, acylation of the catecholamines reached a steady state at 30 minutes. Using similar conditions, we have measured the amounts (5 to 50 nanomoles) of unreacted amines by a spectrofluorometric assay (*6*). In this procedure an aqueous solution of each amine was dried under nitrogen and reacted with PFPA in ethyl acetate at 60°C. At the end of 30 minutes the excess PFPA was blown off with nitrogen, and the dried acylated catecholamines were taken up into benzene. The unreacted amines, which were left in the reaction vessel, were taken up into acid and processed according to a spectrofluorometric assay (*6*). This procedure showed that, at the end of a 30-minute reaction, the amount of unreacted amines ranged from 5 to 15 percent of the original concentration. When this procedure was followed in the absence of PFPA, 95 percent of the catecholamines were recovered. These reaction characteristics applied to NE, DA, and the two internal standards. It was further determined by mass fragmentography that the amine-PFP derivatives once formed were stable for at least 24 hours if maintained in the presence of PFPA. The amine-PFP derivatives were less stable provided that the excess PFPA was removed and the residue was reconstituted in ethyl acetate for analysis. However, by recording the ion density of the most abundant fragment generated by each amine derivative, we found that the ratio of the ion density (measured as peak height) generated by the amine and its corresponding internal standard (NE-PEP with α-MNE-PFP; DA-PFP with α-MDA–PFP) remained constant for at least 10 minutes.

The PFP amine derivatives and their internal standards thus fulfill the basic requirement of having similar fragmentation patterns, react satisfactorily with pentafluoropropionic anhydride, and have a similar time constant for their stability. We tested the linearity of the reaction by holding the concentrations of the α-methyl analogs constant (20 pmole) and varying the concentration of NE and DA from 0.5 to 200 pmole. Each sample containing all four amines was processed separately and analyzed by mass fragmentography (Fig. 2A). The amine derivatives are identified by recording the ion density of a fragment at a specific *m/e* at its characteristic GC retention time (Table 1). For quantitation of the amines, the peak heights, which are proportional to the specific ion density of all four derivatives were measured. The ratio between NE-PFP (*m/e* 176) or DA-PFP (*m/e* 428) and their respective internal standard α-MNE–PFP (*m/e* 190) and α-MDA–PFP (*m/e* 442) was plotted on the ordinate with the absolute amine concentration (in picomoles) on the abscissa. In this way, a linear relation was obtained. This calibration was done in parallel with tissue assays, and the standard amine–internal standard line was used to determine the unknown amine concentration in the tissue.

Table 2. Mass fragmentographic assay of NE and DA in rat tissue. The number of analyses is given in parentheses. The results are given as the number of picomoles per sample.

Tissue	Wet weight (mg)	NE (pmole)	DA (pmole)
Superior cervical ganglion	1.4	100 ± 8.6 (7)	18 ± 2.3 (7)
Locus coeruleus	< 0.100	20 ± 1.6 (6)	3.2 ± 0.25 (6)
Striatum	27	54 ± 2 (3)	2200 ± 140* (3)
Cerebellum	250	270 ± 66† (4)	53 ± 10 (4)
Vas deferens	41	5300 ± 160‡ (5)	65 ± 8.2 (5)

Values obtained from the literature were converted to picomoles per sample to allow for direct comparison with the values in the table: * 1701 ± 89 (*9*); † 222 ± 19 (*10*); ‡ 4600 ± .41 (*11*).

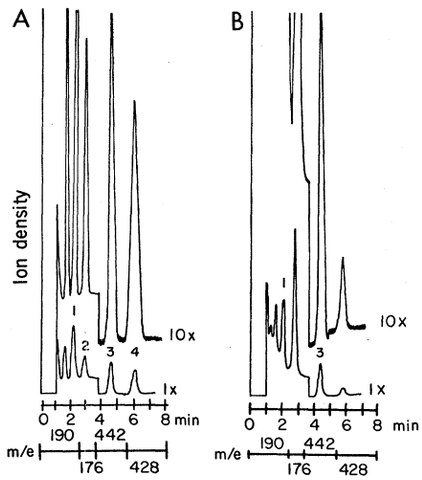

Fig. 2. (A) Mass fragmentogram of standard amine-PFP mixture. The derivatives in 10 μl of ethyl acetate were injected (2 μl) into the GC-MS, and the ion density of the most abundant fragment (*m/e*) of each compound recorded at its characteristic retention time; (1) α-MNE-PFP (20 pmole), 0 to 2.5 minutes at *m/e* 190; (2) NE-PFP (32 pmole), 2.5 to 3.6 minutes at *m/e* 176; (3) α-MDA-PFP (20 pmole), 3.6 to 5.1 minutes at *m/e* 442; and (4) DA-PFP (31 pmole), 5.1 to 7.0 minutes at *m/e* 428. (B) Mass fragmentogram obtained from analysis of 1/60 of a homogenate of a rat superior cervical ganglion. Internal standards (peaks 1 and 3) were added to the sample before processing. The peak between 1 and 3 has a retention time and fragmentation pattern which corresponds to NE-PFP, and the peak after 3 corresponds to DA-PFP.

To ascertain whether this linearity applied to tissue, we homogenized a superior cervical ganglion, a few milligrams of the corpus striatum, and a few milligrams of the vas deferens of a rat in 60 μl of 0.1*M* formic acid containing ascorbic acid (50 mmole). Samples (3 to 5 μl) of the homogenate were removed for protein analyses (*7*), and the homogenate was centrifuged ($1.2 \times 10^4 g$, 15 minutes, 4°C). Portions of the supernatants were transferred to vials containing 20 pmole each of the two internal standards (α-MNE and α-MDA); the samples were dried under nitrogen and processed with PFPA as described. A typical record for the sympathetic ganglion is shown in Fig. 2B.

The ratios between the ion densities generated by α-MNE–PFP (*m/e* 190), α-MDA–PFP (*m/e* 442), and by NE-PFP (*m/e* 176) in sympathetic ganglia and by DA-PFP (*m/e* 428) in striatum were measured and plotted on the ordinate against the tissue protein contained in each portion of the homogenate (abscissa), thus obtaining a straight line for each tissue.

The fragmentation patterns of NE-PFP and DA-PFP do not avail themselves to simultaneous multiple ion analysis with the LKB GC-MS 9000 (*8*). Mass fragmentography requires the analysis of at least two specific fragments at specific retention times. Specificity is then ascertained by comparing the ratios of the fragment intensities obtained from tissue analysis to those generated from pure standards. To satisfy this requirement, samples prepared from different tissue were first analyzed by focusing on the more abundant fragments of NE-PFP and DA-PFP, as stated above. A second analysis was then made; for NE-PFP the intensity of the fragment at *m/e* 590 was measured, and for DA-PFP the fragment at *m/e* 176 was measured (Table 1; Fig. 1). In both instances the

fragments recorded for α-MNE–PFP and α-MDA–PFP were the same: *m/e* 190 and *m/e* 428, respectively. Because of unavoidable differences between two analyses, it was necessary to use the internal standards to normalize the values for DA-PFP and NE-PFP. In this way, the ratio for two fragments analyzed for endogenous NE-PFP (*m/e* 176/590) and DA-PFP (*m/e* 428/176) compared with the ratios obtained from pure standards analyzed in the same way and with peak ratios calculated from the mass spectra (Table 1). Tissue samples were also processed without internal standards to ascertain that the fragments measured for the internal standards originated from the α-MNE and α-MDA added to the tissue sample and were not "biological background."

To demonstrate the practical significance of this method, we measured the NE and DA content in various tissues (Table 2). The data show that the concentrations of catecholamines detected with mass fragmentography compare with the reported available data. The true value of this method is that it can be applied to the study of catecholamines in very discrete brain structures weighing less than 100 μg (Table 2).

Thus the combined techniques of GC-MS can be used to quantitatively analyze 10^{-13} to 10^{-14} moles of endogenous amines. The compounds are separated by the GC and measured with the MS. The assay procedure is relatively rapid and we can process 15 to 20 samples in a day's work, including their dissection and the necessary standard curves. Considering the specificity given by the GC retention time and, more important, from the ion density recorded at a specific *m/e* setting during the elution of the various compounds from the GC, the specificity of this method surpasses and cannot be compared to any other existing quantitative method.

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Genetic Control of Lactate Dehydrogenase Expression in Mammalian Tissues

Abstract. *The amount of lactate dehydrogenase isozyme 4 in erythrocytes of mice is controlled by alleles at the* Ldr-1 *locus. The A subunits of lactate dehydrogenase from erythrocytes deficient in isozyme 4 cannot assemble in vitro with B subunits to form active isozyme. The inability to form hybrid enzyme is not due to a mutation in the structural gene for the A polypeptide. Rather, a factor that is bound to the A subunits of erythrocytes restricts free exchange with B subunits.*

The concentration of an enzyme in a mammalian cell is probably regulated at the level of synthesis or intracellular degradation (*1*). However, for those enzymes composed of more than one polypeptide chain, another level at which regulation could occur is the assembly of subunits to form the active enzyme. Rosenberg (*2*) showed that the two subunits of lactate dehydrogenase (LDH) in fish probably combine in vivo in a nonrandom fashion to form tissue-specific isozyme patterns, a result that suggests that the primary structures of the subunits are not the only determinants of the quaternary structure of the active enzyme. Fritz *et al.* (*3*) also proposed that the changes in rat heart LDH isozymes during development are regulated by events after synthesis of the polypeptide chains. If the quaternary structure of an enzyme is determined both by the primary structures of its polypeptides and by the environment in which these polypeptides assemble, then mutations that alter the environment could affect the expression of the enzyme. In this report we present evidence that the pattern of LDH isozymes in erythrocytes in mice is regulated by a factor (or factors), under genetic control, that affects the assembly of LDH subunits.

Mammalian LDH is a tetramer of two dissimilar subunits, A and B, which assemble in all possible combinations to form five isozymes. Isozymes LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5 have subunit compositions of B_4, B_3A_1, B_2A_2, B_1A_3, and A_4, respectively. These isozymes differ in charge and can be separated by electrophoresis (Figs. 1 to 3) or ion-exchange chromatography (*4*).

Shows and Ruddle (*5*) described a variant of the LDH pattern in mice. Erythrocytes of most inbred strains contained only LDH-5, but erythrocytes of strains SWR/J, DE/J, LP/J, and DW/J contained both LDH-5 and LDH-4. Thus, erythrocytes of the latter strains contained both A and B subunits, but erythrocytes of all other strains contained only A subunits. The tissue-specific isozyme patterns in all other tissues were identical among the strains. The erythrocyte pattern is inherited as a single Mendelian factor and is under the control of alleles at a locus *Ldr-1*, which is on linkage group XI (*6*).

Figure 1 shows the LDH isozymes from erythrocytes of mice homozygous for alternate alleles at the *Ldr-1* locus. Isozyme LDH-4 is deficient in the homozygous *Ldr-1*^{a} strain, C57BL/6J. When the LDH isozymes in erythrocytes were separated on diethylaminoethyl cellulose (DEAE-cellulose), LDH-4 was about 1 percent of the LDH activity in the C57BL/6J strain; in contrast, this isozyme was about 4 percent of the LDH activity in the homozygous *Ldr-1*^{b} strain, SWR/J. The tissue-specific isozyme contents of kidney, liver, and heart of the two strains was identical. Thus, the defect in LDH-4 expression is restricted to the erythrocyte.

A possible explanation for the tissue-specific effect of mutation at the *Ldr-1* locus is that this locus controls