Ralph Lowell Laboratories
McLean Hospital
Belmont, MA 02178, and
Department of Biological Chemistry
Harvard Medical School
Boston, MA 02115, U.S.A.

ANURADHA S. PAPPU
GEORGE HAUSER*

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* Address all correspondence to: Dr. George Hauser, Ralph Lowell Laboratories, McLean Hospital, 115 Mill St., Belmont, MA 02178, U.S.A.

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Quantitative correlation between secretion and cellular content of catecholamines and dopamine-β-hydroxylase in cultures of adrenal medulla cells*

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Essentially all of the catecholamines (CA) and dopamine-β-hydroxylase (DBH) of the bovine adrenal medulla is present in the chromaffin vesicles [1–3]. Osmotic lysis of the vesicles results in the total release of the CA and solubilization of about 50 per cent of the DBH activity [4]; the remainder of the DBH is tightly bound to the membrane and requires a detergent for solubilization [5]. The concurrent release of CA and DBH upon nicotinic stimulation of isolated perfused adrenal glands has been demonstrated in several laboratories [6–10] and has been used to support the hypothesis that secretion occurs by exocytosis. However, there is disagreement about the quantitative relationship between the relative amounts of CA and DBH secreted and that present in the cell. Viveros *et al.* [6] using isolated bovine adrenal glands found a parallel increase in the amounts of CA and DBH released; the ratio of DBH:CA in the perfusate was similar to the DBH:CA ratio in subcellular fractions of the gland. Similarly, Ito *et al.* [7] using perfused guinea pig adrenal glands found that a variety of secretagogues caused the release of DBH and CA in very nearly the same ratio as that found in the soluble fraction of chromaffin vesicles. Aunis *et al.* [8] and Serck-Hanssen *et al.* [9] also found a parallel increase in the amounts of CA and DBH released upon stimulation, but the ratios of DBH:CA in the perfusates were considerably less than that of the soluble content of isolated chromaffin vesicles. They attributed this to both a release of CA and DBH from vesicles having a lower than average DBH:CA ratio and to partial inactivation of the DBH that was released. On the other hand, Dixon *et al.* [10] found a poor correlation between the amounts of CA and DBH released in stimulated perfused adrenal glands; the ratio of DBH:CA in the perfusate was variable and much less than that of the soluble content of isolated chromaffin vesicles. They concluded that the release of CA was quantal but that the release of DBH was not necessarily coupled to it.

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Studies on the *in vivo* release of CA and DBH provide strong indirect evidence that concurrent release of both DBH and CA occurs from chromaffin vesicles and that their release is quantal in nature in both rabbit [11] and rat [12]. Following treatment of rabbits with insulin, Viveros *et al.* [11] found a decrease in CA, soluble DBH and intact chromaffin vesicles, indicating that the chromaffin vesicles had released their entire soluble content in a quantal "all or none" manner. Similar findings were reported by Slotkin and Kirshner [12] in the rat, while Fenwick *et al.* [13] have reported that stimulation of adrenal medulla cells with acetylcholine results in the release of CA and DBH in ratios similar to that present in the soluble fractions of lysed cells. We have used stable cultures of isolated adrenal medulla cells to obtain additional information on the release of CA and DBH in response to various stimuli and in different media.

Methods

Isolated bovine adrenal medulla cells were prepared as previously described [14] and cultured in 6 well (35 mm) cluster dishes (Costar 3506) at a density of 1.2×10^6 cells per well. The cells were allowed to remain in culture at least 3 days before use. Cells were stimulated at room temperature for 15 min with 10 μM nicotine or 50 μM veratridine and for 15 and 30 min with 20 μM Ionomycin. All experiments were carried out using either duplicate or triplicate wells. At the end of the incubation period, the media were removed and saved for assay of DBH and CA [14]. In these studies, the total CA content was determined as equivalents of adrenaline. All DBH assays were carried out in duplicate using 10 μM *p*-hydroxymercuribenzoate to inactivate endogenous inhibitors. The cells were lysed by adding 1 ml of distilled water and freezing. After thawing, 1 ml of 0.3 M NaCl was added and the cells were scraped into an all glass Potter–Elvehjem homogenizer. After homogenization, the cells were subjected to two cycles of freezing and thawing and then centrifuged at 100,000 *g* for 1 hr. The supernatant fraction was decanted and saved, and the pellet was washed twice with 0.15 M NaCl. The washes were combined with the initial lysate and the pellet was resuspended for CA and DBH assay. Less than 1 per cent of the total CA content was present in the pellet after the second wash. Lactic dehydrogenase activity in the media and lysates was determined as described by Bergmeyer *et al.* [15]. The sucrose medium was composed of 9.75% (w/v) sucrose, 2.2 mM $CaCl_2$, 5 mM 4-(2-hydroxyethyl)-1-pipercazine-ethanesulfonic acid (HEPES) and 10 mM glucose adjusted to pH 7.4 with KOH. The composition of Locke's solution and sources of chemical were reported previously [14]. Ionomycin is a new antibiotic which has been characterized as a calcium ionophore [16] and was supplied by Squibb Institute for Medical Research, Princeton, NJ.

Results and discussion

The results of this study are presented in Table 1 and Fig 1. First let us consider the studies carried out in Locke's solution. In each of three different cell preparations (A, B, C) in which experiments were carried out in triplicate, there were no significant differences upon stimulation with nicotine between the DBH:CA ratios secreted into the medium and those which were present in the soluble fractions of lysed cells, nor was there a significant difference between the mean of the DBH:CA ratios secreted in all three preparations and that obtained in the soluble fraction of the lysed cells. Similar results were obtained when veratridine or Ionomycin was used to stimulate secretion in several different cell preparations. In each preparation stimulated with Ionomycin, secretion was measured after 15 and 30 min in duplicate wells.

The responses to nicotine and Ionomycin in sucrose medium were entirely similar to the responses in Locke's solution. In each of three preparations in which triplicate experiments were performed with nicotine, there were no significant differences between the ratios of DBH:CA which were secreted and those present in the soluble fraction of lysed cells. In the experiments in which Ionomycin was the secretagogue there was again no significant difference between the means of the secreted DBH:CA ratio and that present in the soluble fraction of cell lysates. It is also pertinent to note that there were no differences between the ratios of DBH:CA released in Locke's solution and those released in Ca^{2+}–sucrose medium with either nicotine or Ionomycin as the secretagogue.

The possibility that some of the DBH and catecholamines were released as a result of cell lysis was investigated by determining the release of lactic dehydrogenase (LDH) as a cytoplasmic marker. Under each of the conditions in which we studied secretion and in at least two different cell preparations for each condition, we could not detect the release of LDH into the medium. Even when we extended the period of study for up to 1 hr with nicotine and Ionomycin we could not detect release of the enzyme. The amount of LDH that was released into the soluble fraction of cells lysed as described in Methods was sufficient to enable us to detect at least 2 per cent of the enzyme. Perlman *et al.* [17] have reported that 10 μM Ionomycin causes some lysis of rat pheochromocytoma cells, but on a per cent basis the amounts of CA released were 3-fold greater than the amount of LDH. Possible explanations for the observed discrepancy between the resistance of bovine adrenal medulla cells and rat pheochromocytoma cells to lysis by Ionomycin are tissue and species differences. Even when our secretion studies were carried out at 37° we found no significant release of LDH by Ionomycin.

Figure 1 shows the correlation between the percent of total soluble DBH released and the percent of total soluble catecholamines released for each of the conditions employed. The correlation coefficient for the regression line calculated by the method least squares was 0.84 with an S_{yx} value of ± 1.40. The per cent of the total DBH present as soluble DBH reported in Table 1 (36 ± 1) is somewhat lower than that previously reported for fresh bovine glands [4, 18, 19]. This may be due to loss of soluble DBH and retention of membrane DBH due to low levels of secretion during preparation of the isolated cells or during maintenance of the cells in culture.

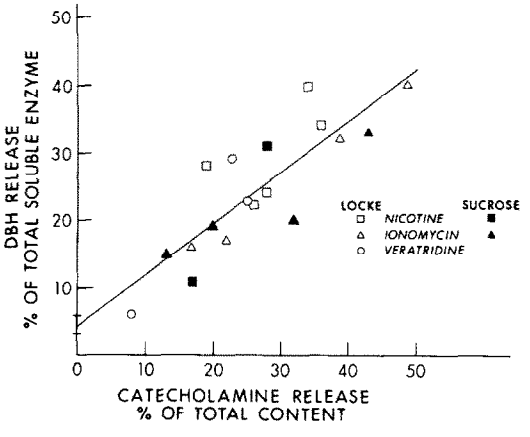

Fig. 1. Correlation between the secretion of dopamine-β-hydroxylase and catecholamines by adrenal medulla cells stimulated as follows: (□) 10 μM nicotine, Locke's solution; (■) 10 μM nicotine, sucrose medium; (○) 50 μM veratridine, Locke's solution; (△) 20 μM Ionomycin, Locke's solution; and (▲) 20 μM Ionomycin, sucrose medium.

Table 1. Dopamine-β-hydroxylase and catecholamine content in the media and lysates of stimulated cells*

	Media			Lysate				
	DBH†	CA‡	DBH:CA§	DBH†	CA‡	DBH:CA§	Membrane DBH†	Soluble DBH‖ (% of total)
				Locke's solution				
Nicotine								
A	8.6 ± 0.5	12.4 ± 0.5	6.8 ± 0.4	26.6 ± 1.7	31.7 ± 1.6	8.5 ± 1.1	78 ± 5.5	31
B	13.2 ± 0.6	12.1 ± 1.0	10.7 ± 0.1	19.6 ± 1.2	23.7 ± 0.7	8.4 ± 0.2	47 ± 1.3	41
C	8.4 ± 0.8	10.0 ± 1.0	8.4 ± 1.8	21.2 ± 1.0	17.6 ± 1.2	12.1 ± 0.2	46 ± 1.0	39
	Average ± S.E.		8.6 ± 1.1			9.9 ± 1.2		
Veratridine								
A	10.8 ± 0.5	9.0 ± 0.6	11.7 ± 1.0	26.0 ± 1.7	29.7 ± 1.0	8.0 ± 1.0	71 ± 11.0	34
C	1.3	2.0	6.5	20.9	23.9	8.6	49	31
E	10.1 ± 0.5	10.4 ± 0.4	9.6 ± 0.8	34.0 ± 2.4	30.9 ± 1.3	9.6 ± 0.8	64 ± 3.5	42
	Average ± S.E.		9.3 ± 1.5			8.7 ± 0.5		
Ionomycin								
C¶	4.4	5.2	8.4	21.0	18.8	11.1	36	37
C**	11.3	12.4	9.1	17.0	19.8	8.6	40	35
D¶	12.3	12.1	10.2	66.3	58.0	11.4	136	37
D**	27.5	26.4	10.4	58.4	41.0	14.2	153	35
	Average ± S.E.		9.5 ± 0.5			11.3 ± 1.2		
				Sucrose medium				
Nicotine								
A	5.1 ± 0.4	8.0 ± 0.8	6.5 ± 0.3	41.4 ± 3.0	40.3 ± 2.3	10.2 ± 0.4	71 ± 8.0	40
B	11.1 ± 0.7	8.8 ± 0.3	12.7 ± 1.0	25.1 ± 1.8	22.5 ± 1.1	11.1 ± 0.3	48 ± 1.2	43
E	10.5 ± 0.6	9.8 ± 0.3	10.7 ± 0.3	38.0 ± 2.0	29.3 ± 1.8	12.7 ± 0.7	69 ± 2.4	43
	Average ± S.E.		10.0 ± 1.8			11.3 ± 0.7		
Ionomycin								
B¶	3.5	4.2	8.3	19.8	28.0	7.1	45	40
B**	5.5	7.4	7.5	23.3	29.0	8.0	42	36
C¶	5.6	9.3	6.0	22.4	19.4	11.5	38	33
C**	10.6	14.3	7.4	21.4	19.0	11.3	45	37
	Average ± S.E.		7.3 ± 0.5			9.5 ± 1.1		36 ± 1

* Letters on the left-hand margin refer to different cell preparations. Standard errors were calculated for experiments done in triplicate. All other values are the average of duplicate determinations all of which were within 10 per cent of each other. Control values (DBH and CA in the medium in the absence of secretagogue) were subtracted from the stimulated samples. In Locke's solution basal release was 3–5 per cent of the total soluble content of each component, while in sucrose media basal release was 8–10 per cent of the total soluble content. See Fig. 1 for per cent of total content released during stimulation.

† Expressed in nmoles octopamine formed · hr^{-1} · $(1.2 \times 10^6$ cells$)^{-1}$.

‡ Expressed in nmoles adrenaline/1.2×10^6 cells.

§ Calculated as 10 × DBH/CA.

‖ Calculated as media DBH + lysate DBH/media DBH + lysate DBH + membrane DBH.

¶ Fifteen minute secretion period.

** Thirty minute secretion period.

Our results support earlier findings that soluble DBH and CA are secreted in the same relative amounts as found in the cell [6, 7, 11–13] and suggest that, in those studies which show considerably less DBH than expected, there may be loss of DBH activity through inactivation or poor recovery of the released enzyme. The poor recovery of secreted DBH in perfused tissues may be largely due to delayed outflow of the enzyme. During stimulation, CA and DBH are secreted into the synaptic cleft of innervated tissue or into the intercellular spaces of the adrenal gland. Catecholamines can diffuse across the capillaries into the bloodstream while diffusion of DBH across the capillaries is limited by the size of the molecule. Several reports indicate that a large fraction of the secreted DBH enters the blood via lymphatic channels [20–22]. In earlier studies from our laboratory, there was only a slight lag between the appearance of CA and soluble proteins of chromaffin vesicles in the effluents from stimulated perfused adrenal glands [6, 23]. Much longer delays have been reported between the appearance of CA and DBH in perfusates from sympathetically innervated tissues [24, 25], and it has been shown that the lag in DBH outflow could be decreased markedly by replacing NaCl in the perfused Tyrode's solution with urea [26].

In conclusion, our data show that stimulation of adrenal medulla cells with nicotine, veratridine or Ionomycin in Locke's solution results in the parallel release of CA and DBH. With all three secretagogues the ratio of DBH:CA secreted into the medium was the same as that obtained in the soluble fraction of lysed cells. When the cells were stimulated with nicotine or Ionomycin in a medium in which NaCl was replaced with osmotically equivalent sucrose, the results were identical to those obtained upon stimulation in Locke's solution.

Departments of Pharmacology and Biochemistry
Duke University Medical Center
Durham, NC 27710, U.S.A.

FREEMAN H. LEDBETTER
NORMAN KIRSHNER*

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*Address all correspondence to: Dr. Norman Kirshner, Department of Pharmacology, Duke University Medical Center, P.O. Box 3813, Durham, NC 27710, U.S.A.

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The binding of gold to human albumin *in vitro*. Intrinsic association constants at physiological conditions

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Although gold in the form of thio-complexes has been successfully used in the treatment of rheumatoid arthritis for many years, important aspects of their pharmacology and the mode of action of these compounds are still unknown.

The first real quantitative information concerning the binding of gold to plasma proteins *in vitro* were given by Mason [1]. He used ultrafiltration and found that sodium aurothiomalate was bound to human albumin at a single site with a fairly high affinity constant and also at several sites of lower affinity.

In the present study the binding of sodium aurothiosulphate—another gold compound widely used in the treatment of rheumatoid arthritis—to human albumin was investigated at physiological conditions with regard to albumin concentration, pH, temperature and ionic strength. The purpose of determining the binding constants of aurothiosulphate to human albumin was; to compare the degree of binding of this gold compound with that of aurothiomalate; to get more information concerning the binding mechanism of gold to albumin; and to attempt to get a more valuable parameter of monitoring the patients during chrysotherapy. This parameter may well be the nonprotein-bound gold concentration, since it is conceivable, that this concentration correlates with therapeutic as well as toxic effects. In order to calculate the 'free' gold concentration, reliable values for the binding constants are needed.

Aurothiosulphate was found to be strongly bound to the dialysis membrane used. The following experiments were, therefore, performed using an equilibrium dialysis system with the great advantages that partial absorption to the membrane does not influence the results [2].

Materials and methods

Materials. The albumin preparation used was purified, lyophilised human albumin (Behringwerke AG, Marburg, West Germany). The albumin preparation fulfilled the criteria for purity specified in [3]. Crossed-immunoelectrophoresis [4] performed against rabbit antihuman serum (DAKO, Copenhagen, Denmark) showed that no peaks attributable to other proteins than albumin were detectable.