ment with the results of Hakala and co-workers (*12*), who have shown that the K_m of beef heart lactate dehydrogenase is doubled in the range 25° to 35°C.

The ratio between the enzyme reaction rates in 0.33 m*M* and 10 m*M* pyruvate has been taken as characteristic of the heart (H) and muscle (M) types of lactate dehydrogenase; tissues of the H-type giving values between 2.2 and 4.0 and those of the M-type values below 2.0 (*3, 6, 7*). Our results give an average value for erythrocytes, heart extracts, and kidney extracts of 2.8 at 25°C and 2.5 at 37°C and for liver and skeletal muscle extract values of 1.2 at 25°C and 1.1 at 37°C.

Our results offer confirmation that the response of tissue lactate dehydrogenase activity toward increasing pyruvate concentration at both 25° and 37°C can be used as an indication of whether a particular tissue contains more of the faster or slower moving lactate dehydrogenase isoenzymes. The assertion of Vesell (*4*) that the total activity of tissue lactate dehydrogenase does not retain the characteristics of the activity of individual isoenzymes with respect to substrate inhibition phenomena is definitely not confirmed by our observations.

The similarities in the behavior of tissue extracts towards pyruvate which have been described by Vesell have been attributed by him to the presence of sufficient amounts of the substrate-sensitive isoenzymes in all tissues to render the total lactate dehydrogenase activity subject to the same degree of inhibition (*4*). We have, however, shown that tissues which contain relatively large amounts of the slowest moving isoenzymes are inhibited to a significantly lesser degree than tissues which contain predominantly faster moving isoenzymes. At 25°C and 10 m*M* pyruvate we have found approximately 65 and 45 percent inhibition respectively with heart and liver extracts. Under these conditions of temperature and substrate concentration, Vessel obtained 75 percent inhibition in both cases. We consider that our own findings correlate quite closely with what could be expected from the known percentage concentration of each of the isoenzymes present in the extracts.

From the results described by Vesell (*4*) it would appear that the pyruvate inhibition of heart extracts is more similar to LDH-1 than the pyruvate inhibition of liver extracts is to LDH-5. In the same way the behavior of purified LDH-1 at 20°C does not appear to be significantly different from that of LDH-1 at 37°C, whereas the behavior of purified LDH-5 appears markedly temperature dependent (*4*). This would seem to imply that Vesell's findings might conceivably reflect instability of LDH-5 in his whole homogenates and purified enzyme preparations rather than an increased sensitivity to pyruvate. We are otherwise at a loss to explain why his findings with tissue homogenates differ from our own. We admit that Vesell prepared homogenates in a Waring Blendor, centrifuged them at 15,000*g* for 1 hour and presumably used undiluted homogenate in the assay, in which he also used phosphate buffer and $1 \times 10^{-4}M$ $NADH_2$. Conceivably these differences in technique could be responsible for the observed discrepancies between our findings, but we do not believe this is the case.

It would appear, therefore, that at present there is insufficient evidence to warrant the suggestion that the kinetic properties in vitro of lactate dehydrogenase from crude human tissue extracts cannot be correlated with the distribution of isoenzymes and their possible metabolic role.

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Release of Catecholamines and Specific Protein from Adrenal Glands

Abstract. *Rabbit antiserums directed against a purified protein obtained from the catecholamine storage vesicles of the adrenal medulla of the cow were used to study the mechanism of secretion from the adrenal medulla. During secretion evoked by acetylcholine in isolated perfused adrenal glands, catecholamines and the specific intravesicular protein were released in relative amounts comparable to those present in the intact gland. Thus catecholamines are apparently released from the storage vesicles by a process resembling reverse pinocytosis.*

Adrenaline and noradrenaline are released from the adrenal medulla in response to nerve stimulation or to perfusion with acetylcholine and other secretagogues. There is evidence (*1, 2*) that the catecholamines which are stored in intracellular vesicles are secreted from these vesicles directly to the exterior of the cell by a process resembling "reverse pinocytosis." This evidence is based on (i) electron microscopy and (ii) observations that, during stimulated secretion, the ratio of the quantity of catecholamines to that of adenine nucleotides in the perfusate was similar to that in the intact storage vesicles. We have obtained further evidence to support this mechanism by measuring the amounts of catecholamines and a specific intravesicular protein released during stimulation and then comparing the ratios of these compounds in the perfusates to the ratios of the amounts in intact vesicles. Banks and Helle (*3*) have recently reported that this protein was present in perfusates of isolated glands during stimulated secretion but was not detectable by Ouchterlony analysis during the resting period.

The major soluble protein component of the catecholamine storage vesicles can be readily purified on diethylaminoethyl (DEAE) cellulose and Sephadex G-200 (*4*). Rabbit antiserums were prepared by immunization with the purified protein in complete Freund's adjuvant. The purified protein showed a

single component by immunoelectrophoresis. However, the crude protein fraction obtained upon lysis of the vesicles with water showed that each of the antiserums contained antibodies directed against one to three very minor contaminants present in the purified protein. Antiserums used in these studies initially contained antibodies directed only against the purified protein and a minor contaminant migrating slightly from the starting position in immunoelectrophoresis.

The antibody to this contaminant was removed by adsorption with a protein fraction eluted from the DEAE-cellulose column immediately preceding the major protein peak. This small protein peak contained dopamine-β-oxidase (dopamine hydroxylase) activity. The dopamine-β-oxidase activity was removed almost completely by incubation with the antiserum, followed by centrifugation of the resultant antigen-antibody precipitate. The purified protein obtained from Sephadex G-200 also contained dopamine-β-oxidase, but almost all the enzyme could be removed by chromatographing the protein again on DEAE cellulose. Our data were obtained with antiserums purified with the dopamine-β-oxidase fraction. These antiserums were monospecific for the purified protein, as demonstrated by the absence of the contaminant peak by immunoelectrophoresis and by the micro-complement fixation technique of Wasserman and Levine (*5*). The antigen could not be detected in extracts of heart, kidney, spleen, sympathetic nerves, and adrenal cortex of the cow by immunoelectrophoresis.

Beef adrenal glands were obtained from a local abattoir 20 to 30 minutes after the animals had been killed. Immediately upon removal, the adrenal glands were cannulated through the adrenal vein, flushed with ice-cold Locke's solution (154 m*M* NaCl, 5.6 m*M* KCl; 2.2 m*M* $CaCl_2$, 2.15 m*M* Na_2HPO_4, 0.85 m*M* NaH_2PO_4, 10 m*M* glucose), and transported to the laboratory in ice. The glands were then perfused with oxygenated Locke's solution for 45 minutes prior to the start of the experiment. Stimulation was evoked by changing the perfusion medium to Locke's solution containing the secretagogue, either acetylcholine or nicotine, each at 10^{-5} g/ml for 2 minutes. Experiments were carried out at room temperature, and at 30° and 33°C. The perfusion rate, except where indicated, was 4 ml/min. Samples were collected at 2-minute intervals, centrifuged to remove cells, and assayed for catecholamines by the trihydroxyindole method (*6*) and for the specific antigenic protein by microcomplement fixation (*5*). Total protein in the perfusate was measured either by the method of Lowry (*7*) or by the ninhydrin method (see *8*).

Table 1. Catecholamines and antigenic protein in perfusates and homogenates of adrenal glands. The perfusion rate was 4 ml/min. The figures for the catecholamines (CA) and protein (P) represent net increases above the base level of secretion. The catecholamines are expressed as micrograms of free base. The CA/P for the adrenal glands are the averages of all fractions.

Experiment	Conditions		Perfusates			Adrenal gland CA/P
	Secretagogue	Temp. (°C)	CA (μg/min)	P (μg/min)	CA/P	
1a	ACh	23 *	108	40	2.7	
b	ACh	23	143	37	3.9	
2a	ACh	23	77	34	2.3	1.7
b	ACh	23 †	111	44	2.5	
3a	ACh	23	98	45	2.2	1.2
b	ACh	23 ‡	23	24	1.0	
4a	ACh	23	103	32	3.2	1.5
b	ACh	23 §	63	27	2.3	
5a	ACh	23	63	27	2.3	1.4
b	ACh	23	44	30	1.5	
6a	ACh	30	96	56	1.7	1.1
b	ACh	30	96	62	1.5	
7a	ACh	23	32	17	1.9	1.1
b	ACh	23	50	31	1.6	
8a	ACh	33	63	35	1.8	1.3
b	Nicotine	33	131	84	1.6	
		Average ± S. D.			2.1 ± 0.8	1.3 ± 0.3

* Perfusion rate 2 m/min. † After 30 minutes perfusion with $10^{-5}M$ diisopropyl fluorophosphate in Locke's solution. ‡ After 20 minutes prefusion with $5 \times 10^{-5}M$ cocaine in Locke's solution. § After 20 minutes perfusion with $1 \times 10^{-5}M$ cocaine in Locke's solution.

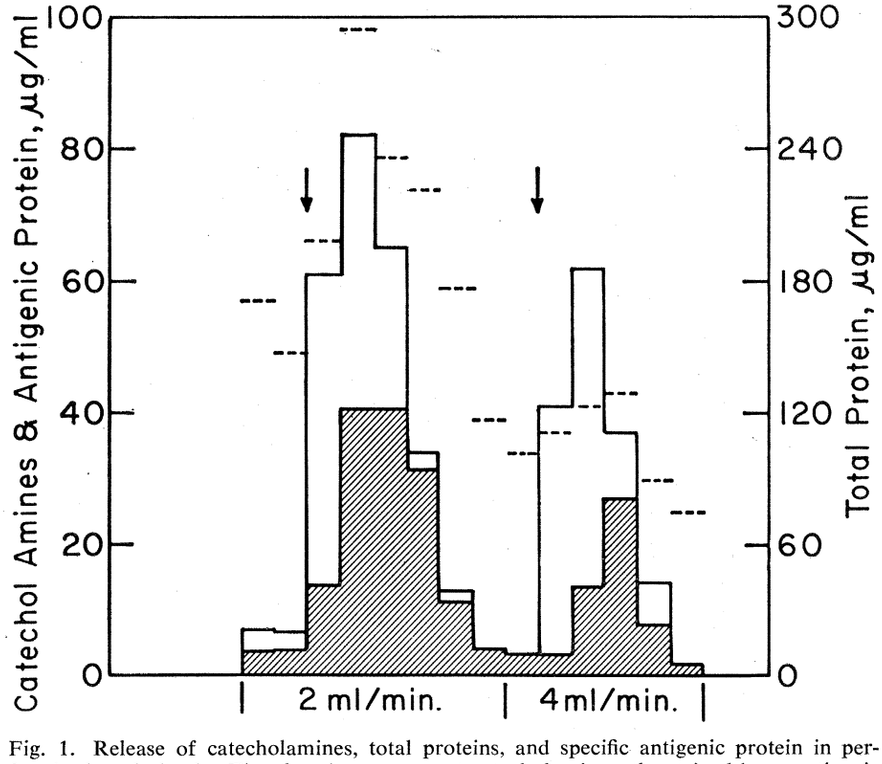

Fig. 1. Release of catecholamines, total proteins, and specific antigenic protein in perfused adrenal glands. The clear bars represent catecholamines; the striped bars, antigenic protein; the horizontal broken lines, total protein. The arrows indicate the addition of acetylcholine to the perfusion medium for a 2-minute period. Each bar represents a 2-minute period.

At the end of the perfusion, the medulla of the perfused gland and the contralateral gland, kept in ice since removal from the animal, were dissected from the cortex and homogenized in 0.25*M* sucrose. The homogenate was separated into the following fractions by differential centrifugation: low-speed (800*g*) supernatant and low-speed sediment, and high-speed (25,000*g*) supernatant and high-speed sediment which contained the storage vesicles. Portions of each fraction and of the total homogenate were assayed for catecholamines, total protein, and specific antigenic protein. The ratio of catecholamines to antigenic protein in all of these fractions was constant within the limits of error. When the high-speed sediment was suspended in 0.25*M* sucrose and sedimented through a sucrose density gradient, 84 percent of the catecholamines and 86 percent of the antigenic protein in the high-speed sediment were recovered in the fractions containing the storage vesicles (see *9*).

During the first part of one perfusion experiment, the perfusion rate was 2 ml/min; in the second part the rate was 4 ml/min (Fig. 1). Because of the relatively slow washout of both catecholamines and protein, the total amounts of catecholamines and protein secreted in the 8-minute period, starting with the injection of acetylcholine (ACh), were measured. After the first injection of acetylcholine the rate of catecholamine secretion increased from a resting level of 13 to an average of 120 μg/min. The specific antigenic protein increased from a resting level of 7 to 46 μg/min. There was a 9-fold net increase in catecholamine secretion and a 5.5-fold increase in antigenic protein.

During the second period of stimulation there were net increases in the secretion rates of 143 μg/min and 37 μg/min, respectively, of catecholamines and antigenic protein. There were also increases in total protein secreted during the stimulation period. During the first stimulation period the rate of protein secretion rose from a resting level of 264 to 476 μg/min. The antigenic protein was 2 percent of the total protein released during the resting period, 8 percent of the total protein released during the stimulation period, and 19 percent of the net increase. During the second stimulation period the total protein released rose from a resting level of 360 to 455 μg/min. The increase in antigenic protein was again much greater relative to the total protein, rising from 4.2 percent of the total protein released during the resting period to 8 percent of the total protein released during stimulation; and it represented 38 percent of the net increase of protein secretion. The protein released during stimulation had neither dopamine-β-oxidase activity (*10*) nor *N*-phenylethanolamine-*N*-methyltransferase activity (*11*).

Table 1 summarizes the results obtained from eight different glands. The data are net increases above the resting levels for catecholamines and protein. The ratio of catecholamines to protein in the perfusates was higher than that found in the glands. However, since the assay for the antigenic protein was made with a serial dilution technique having an inherent error of $\pm$50 to 100 percent, the results indicate that within the limits of error of the assay, catecholamines and the specific antigenic protein are secreted in approximately the same relative amounts in which they are associated in the storage vesicles. When the purified protein was perfused through the adrenal gland, 100 percent of it was recovered, indicating that (i) the protein was not absorbed by the gland and (ii) no appreciable degradation had occurred.

The ratio of catecholamines to protein in the perfusates was higher (Table 1) than that in the glands with one exception. Although the difference in a single perfusion was within experimental error, the consistent trend may be significant. The apparent lower recoveries of protein may be due to incomplete release of protein from the storage vesicle or incomplete diffusion of the released protein into the capillary bed.

The appearance of the protein in the perfusates lags behind the appearance of the catecholamines (Fig. 1). This finding has been generally true in all of our experiments. The lag in the appearance of the protein may be due to (i) extracellular release of the protein and a slower diffusion of the protein across the capillaries, or (ii) intracellular release of the protein and a slower diffusion rate out of the medulla cell or across the capillaries, or a combination of both.

The data reported here are consistent with either an intracellular or an extracellular release mechanism. However, when taken together with the reports of Douglas *et al.* (*1*), it would seem unlikely that catecholamines, adenine nucleotides, and proteins—if released intracellularly—would diffuse across the cell membrane in nearly the same relative amounts as found in the intact vesicles.

The presence in the perfusates of other specific proteins of adrenal medullary cells was also sought. The observation that phenylethanolamine-N-methyltransferase, an enzyme present in the soluble fraction of cell homogenates, could not be found in the perfusates would indicate that, if the membrane does become permeable to the antigenic protein, this permeability is highly selective. Dopamine-β-oxidase could not be detected in the cell debris obtained upon centrifugation of the perfusates or in the concentrated perfusates by either enzymatic assay or immunoelectrophoresis. Since this enzyme is associated with the granule membrane (*12*), its apparent absence in the perfusates argues against the proposal that secretion occurs by extrusion of the entire granule into the extracellular space (*13*).

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