

References and Notes

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20. We regret that Antelman did not consider his personal communication concerning methodological detail as advice. Our table 1 (8) was meant to demonstrate that tail-pinch behavior in our hands was similar to that reported by others and as such implicitly acknowledged the existence of prior studies. We did not mean to suggest that our dose of haloperidol was the same as that used by Antelman and his colleagues.

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Limitations in Identifying Neurotransmitters Within Neurons by Fluorescent Histochemistry Techniques

Grace and Bunney (1) purport positively to identify neurons as dopaminergic by intracellularly injecting L-dopa and subsequently inducing fluorescence in those cells by treating the tissue with glyoxylic acid. This method is based on the assumption that "only the dopamine reacts with formaldehyde vapor or glyoxylic acid to form fluorescent compounds" (1). On the contrary, our studies (2) show that L-dopa itself fluoresces

when treated with the SPG (sucrose, phosphate buffer, and glyoxylic acid) method of de la Torre and Surgeon (3), and that the emission maximum of L-dopa is exactly the same as that of dopamine (Fig. 1). Furthermore, the products of the same biosynthetic pathway, epinephrine and norepinephrine, also fluoresce at the same $\lambda_{\max}$ when treated with glyoxylic acid. Additionally, L-dopa fluoresces if the tissue is treated by the

paraformaldehyde (FGS) method (4). In this case the spectral emission of the L-dopa fluorophore is also very similar to that of the dopamine fluorophore. Therefore, it appears that neither the SPG nor the FGS methods of fluorescent histochemistry allow one unequivocally to distinguish the L-dopa fluorophore from the dopamine fluorophore.

The implication of Grace and Bunney that lack of fluorescence in the nondopaminergic cells of the zona reticulata is evidence that L-dopa is not the glyoxylic acid reactant is open to question on at least two counts. The 10 to 30 minutes allowed to elapse before the animals were killed would certainly be sufficient time for degradation of the L-dopa by either the monoamine oxidase or catechol-O-methyltransferase pathways (5). There is no assurance in the report (1) that the amount of L-dopa injected into zona reticulata cells was comparable to that iontophoresed into the zona compacta of the substantia nigra. In view of the fact that L-dopa does fluoresce, careful and exact controls must be used. One would have to examine considerably more data than were given on the controls before one could reach any conclusions concerning the ability to identify dopaminergic neurons after intracellular injection of L-dopa.

The identification of putative transmitters on the basis of their fluorescence has been a problem for some time (6). When treated by any number of methods, monoamines, as well as many amino acids, fluoresce. Even tyrosine, the precursor of monoamines, has a fluorescent spectrum that would be difficult to

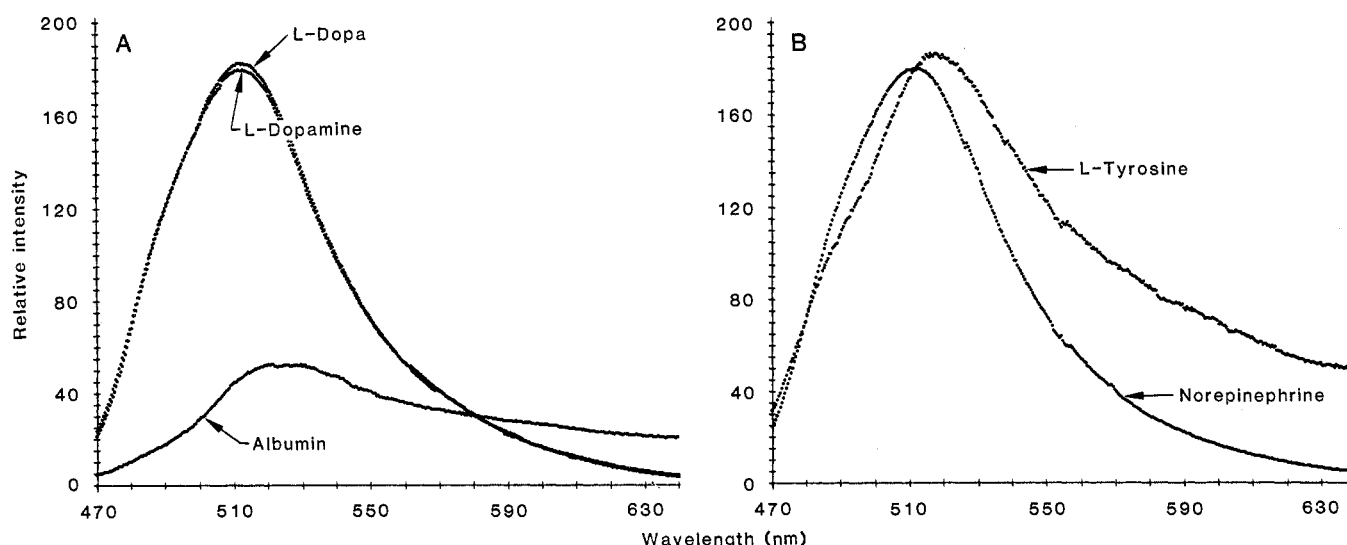


Fig. 1. Fluorescence emission spectra of L-dopa, dopamine, tyrosine, and norepinephrine in dried albumin droplets on glass microscope slides. Epifluorescent illumination was monitored and recorded as described (2). These are uncorrected records that are representative of at least three similar samples. The albumin was the only record for which we had to set the microspectrophotometer calibration at high gain. Hence, the relative intensities of the samples and the albumin are not accurately represented. The fluorescence of the monoamines is at least 1000 times that of albumin. Readings for each sample were taken every 0.44 nm and the point plotted is an average of ten readings taken at each 0.44-nm interval.

discern visually, from the final products formed, from norepinephrine (Fig. 1B), dopamine, or L-dopa. The collaborative evidence of the workers cited (1) as well as the electrophysiological data presented support the contention of Grace and Bunney that the cells in the zona compacta of the substantia nigra are good dopaminergic candidates. Thus, although the cells injected with L-dopa may well be dopaminergic, the methodologies cannot be relied upon to demonstrate this conclusively. These cells might merely be demonstrating the accumulation of L-dopa injected into their cytoplasm.

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2. For each substance we examined, a $10^{-3}M$ solution was made by dissolving the drug in a solvent that consisted of 25 percent albumin and 25 percent glycerin in distilled water. A droplet each of the albumin solvent (control) and the albumin plus the sample was placed on either end of a glass microscope slide. The two droplets were immediately treated with a single drop (approximately 0.05 ml as measured from a 20-gauge needle and 3-ml syringe) of SPG solution (3). The slides were then dried and processed exactly as described by de la Torre and Surgeon (3). Cover slips were placed on the slides with mineral oil and the fluorescence was viewed under a Zeiss epifluorescent microscope equipped with a 50-W mercury lamp. The substances were excited through a BG-12 excitation filter and FT466 and LP478 barrier filters. The fluorescence was monitored through a Nanometrics Nanospec/10S UV-VIS-NIR microspectrophotometer attached to the camera tube of the microscope. The microspectrophotometer was controlled via linkages to a Nanometrics wavelength programmer and a programmed Hewlett-Packard 9815A calculator to scan and plot the emission spectrum of the fluorophore automatically on a Hewlett-Packard 9871A printer.
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Keenan and Koopowitz (1) question the adequacy of methods used to establish the precise neurochemical identity of neurons from which electrophysiological recordings are made. Indeed, some investigators have recently reported recording from neurochemically identified cells when, in fact, no adequate identification had been made. However, we cannot agree with the arguments raised against our proof (2) of intracellular recordings from identified dopamine (DA)-containing neurons.

Keenan and Koopowitz argue that since L-dopa fluoresces in their experiments any cell we injected would fluoresce. However, caution must be observed when one extrapolates from data obtained in vitro in an extremely simplified solution to results obtained in vivo from chemicals injected into a complex intracellular environment in a living animal. It is not clear to us what is being measured (1). The emission maximum obtained (1) for DA, norepinephrine, and L-dopa (510 nM) is significantly higher [indeed, high enough to be confused with the characteristic serotonin yellow fluorescence of 520 nM (3)] than results obtained by others using microspectrofluorometry in brain slices or protein models [460 to 480 nM (3), the characteristic yellow-green catecholamine fluorescence we obtained with L-dopa injection]. Catecholamines and their precursors are susceptible to autooxidation at a pH above 4.0; thus the procedure used by Keenan and Koopowitz for exposing these compounds, without the protection of the cellular environment, to a pH of 7.4 at 80°C for 5 minutes in order to react them with glyoxylic acid could result in alterations of their chemical properties and may explain this discrepancy.

Keenan and Koopowitz also express concern that for some reason the amount of L-dopa injected into non-DA-containing zona reticulata (ZR) cells was inadequate in contrast to the injections into DA-containing cells. As we stated (2), the non-DA-containing ZR cells are larger and thus easier to maintain in a healthy state during intracellular recording than are the smaller and hence less stable DA neurons. Thus, the larger ejection currents and longer ejection times possible with ZR cells assured that a comparatively greater amount of L-dopa was injected into these cells than into the DA neurons. [Indeed, we have obtained consistently better results with Lucifer yellow injected into these neurons than into the DA cells (4).] Despite these favorable experimental conditions not one fluorescent non-DA-containing ZR cell was obtained. In studies in which comparatively large amounts of L-dopa were ejected extracellularly in non-DA areas (5), no neuronal uptake was noted upon processing for fluorescence histochemistry.

The argument that the time elapsed before the rats were killed could have led to catabolism of the L-dopa in the non-DA cells seems unreasonable, since DA cells contain much higher concentrations of monoamine oxidase than non-DA cells (6). Thus the DA cells would be

expected to show less fluorescence after L-dopa injection than the non-DA cells if this were the only factor operating. Since any catecholamine not taken up into granules intraneuronally is metabolized by monoamine oxidase (7), this protective granular DA uptake is another identifying characteristic of catecholamine-containing neurons. Furthermore, our fluorescing neurons *cannot* be epinephrine- or norepinephrine-containing cells, because the synthetic enzyme for these neurotransmitters (dopamine- β -hydroxylase) is not present in this brain region (8). Thus, we believe our controls were adequate for this demonstration.

Nevertheless, we have used two additional histochemical procedures to increase fluorescence in intracellularly recorded DA neurons to further confirm the identity of these cells as dopaminergic. These were: (i) activation of the rate-limiting DA synthetic enzyme by intracellular injection of minute amounts of the pteridine cofactor, and (ii) intracellular injection of colchicine to increase the DA content of the DA neuron recorded. Neither the cofactor nor colchicine demonstrates fluorescence at the catecholamine emission wavelengths in vitro or in vivo. Therefore, these substances only augment fluorescence in cells already containing the biochemical machinery necessary to produce catecholamines which fluoresce with the glyoxylic acid technique [that is, in the substantia nigra, DA cells (4)].

Thus, in our view, our L-dopa results are sufficient identification of these cells as dopaminergic. This is confirmed by our other histochemical and electrophysiological methods.

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