and Iversen to include, for scintillation counting, the preoptic area in their critical hypothalamic sample. It is commonly recognized that this rostral diencephalic area is a region of great importance in vertebrate thermoregulation.

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We have confirmed the increased rate of disappearance of tritiated norepinephrine (^{3}H-NE) from the hypothalamus at 32°C and have shown that it occurs in both anterior and posterior parts of the hypothalamus, but not in the preoptic area (*1*). Further responses obtained from rats exposed to 9°C, however, differed from those reported earlier. Instead of becoming hypothermic, the rats maintained normal rectal temperatures and, in addition, exhibited a significantly increased rate of disappearance of ^{3}H-NE from the hypothalamus. Again, both anterior and posterior parts of the hypothalamus were involved, but not the preoptic area (*1*). It appears, therefore, that the rats in the earlier experiments failed to thermoregulate effectively in the cold, and this was associated with a failure of hypothalamic NE to increase its rate of turnover. An increased turnover of hypothalamic NE is now obtained regularly in experiments at 9°C as well as in those at 32°C.

These results are consistent with the information presented by Feldberg and Lotti (*2*) and Myers and Yaksh (*3*) concerning the responses of rats to intraventricular injections of NE. Both groups of authors showed that NE could cause a marked hypothermia in doses only two and one-half to four times those required to produce a hyperthermic response. We therefore do not accept Myers' dismissal of the hypothermic responses to NE and suggest that hypothalamic NE may be involved in the responses to the rat to both heat and cold. In this connection, it would be interesting to know how the rat's rectal temperature responds to intraventricular NE when the animals are placed in either a warm or cool environment.

Myers' conclusion that "it is difficult to imagine that the [increased rate of] decline of ^{3}H-NE from the whole hypothalamus could be attributed solely to an alteration in environmental temperature" suggests that his concept of what constitutes a thermoregulatory response is somewhat narrower than ours. His list of "other effects" which may be influenced by environmental temperature and which may involve changes in NE turnover in fact describes a pattern of behavioral changes which assists the animal in reducing heat production and, in the case of the thirst drive, in maintaining its water balance during increased salivation in the heat (*4*). Such behavioral responses to heat may be just as important as vasodilation in reducing hyperthermia. There is also no evidence in the rat that either the preoptic or anterior hypothalamic area, or both, are the only sites of action of intraventricular NE in the temperature responses to the amine. Our results, indeed, suggest that NE in both anterior and posterior parts of the hypothalamus is involved in the responses to both heat and cold. Thus, it is not clear that the role of NE in the central regulation of body temperature can be regarded quite so simply as a "relationship between a single function and a specific chemical change."

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Histochemical Fluorescence as an Index of Spread of Centrally Applied Neurochemicals

Routtenberg *et al.* (*1*) used fluorescence of biogenic amines to trace the movement of carbachol, norepinephrine, and dopamine from cannulas implanted in the caudate nuclei and septal areas of freely moving rats. They concluded that their results (i) "support the view that the ventricle transports chemicals applied to brain tissue," and (ii) "are clearly relevant to discussions of widespread behavioral effects of neurochemicals applied to the brain." We question the validity and generality of these conclusions.

Routtenberg *et al.* tested three neurochemicals but obtained clear results with only one, dopamine. Their technique was inappropriate for a study of the diffusion of carbachol, and their inability to obtain any significant effect with norepinephrine is described as "somewhat puzzling." Yet the studies which have demonstrated behavioral effects of centrally applied neurochemicals have largely used carbachol and norepinephrine, not dopamine (*2*, *3*).

The criticism made by Routtenberg *et al.* (*1*) of the generalization to other substances of findings regarding diffusion of microinjected dyes (*4*) is thus applicable to their own work, since they used the diffusion pattern of one substance as an index of the diffusion of two other structurally different substances. Their data seem insufficient to support their generalized conclusions.

The amount of crystalline chemical used by Routtenberg *et al.* is estimated at approximately 10 μg. The amounts used in the studies of behavioral effects typically range from 0.5 to 5.0 μg, and in some of the most extensive work 1.0 to 3.0 μg was used (*2*). Grossman reports optimum behavioral effects with less than 0.5 μg of carbachol (*2*). Thus, Routtenberg *et al.* have introduced at least twice the usual dose, and up to 20 times as much, a procedure which must result in greatly increased osmotic pressure and diffusion of the injected substance. Coury, who used 1.0 to 3.0 μg (*2*), reports nonresponsive loci within 0.25 mm of responsive loci, for neurochemicals applied by an extendible cannula. Booth, applying solutions of neurochemicals via very fine cannulas (modified 27-gauge needles), reports distances between effective and ineffective sites between animals of as little as 100 μm (*4*). These data support the view that, within the usual dose ranges, diffusion through brain tissue is not a critical factor.

Although their conclusions include, at least by implication, the studies in which solutions of neurochemicals instead of crystals have been used, Routtenberg *et al.* do not specifically deal with these studies. Typically, the stimulus solutions are prepared as isotonic with 0.9 percent saline and contain a range of extremely low concentrations of the neurochemical, such as 0.5 to 72.0×10^{-4} mole/liter (*3*). Injected volume is usually 0.5 or 1.0 μl, giving a dose range of 0.5 to 72.0×10^{-10} mole. For carbachol, this represents a quantitative range of 0.009 to 1.300 μg, and maximum behavioral effects, as compared to those from placebo injections of isotonic saline, are usually obtained in the lower third of this

range. Thus in these studies change in osmotic pressure is a controlled variable, which is not the case in the study by Routtenberg *et al.*

Thus, the amounts of neurochemical in solutions used in studies of behavioral effects are from 10 to 1000 times smaller than those used by Routtenberg *et al.* Yet these studies have replicated and extended the findings of the behavioral studies when crystals are used. The a priori assumption often made in the past, that solutions will diffuse further than crystals, seems to us to carry little weight, since it ignores the fact that crystalline substances placed in the brain must dissolve in the endogenous fluid before they can have any effect. The valid comparison then, is between the likely diffusion patterns of isotonic solutions of low concentration and those of the high and uncontrolled concentrations resulting from dissolving crystals.

Fisher and Levitt (*5*) have called for careful consideration of the possibility of ventricular involvement in studies of central chemical stimulation on the basis of appropriate experimentation. It seems to us that Routtenberg *et al.* have failed to employ the appropriate experimental procedures to demonstrate convincingly such a mechanism for the neurochemicals most directly concerned.

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3 March 1969

In a combined fluorescence histochemical and autoradiographic study of the localization of chemicals applied to subcortical structures (*1*), we found that the conclusions drawn in our earlier report (*2*) do not require modification. We report here, therefore, only those data directly relevant to the argument of Montgomery and Singer. Consideration of these data leads to the conclusion that the spread of chemicals in brain from the site of the cannula tip proceeds for considerably greater distances than the 100 μm suggested by Montgomery and Singer, and that this spread is seen with norepinephrine as well as dopamine, with low as well as high doses, and with liquid application in which osmolality is controlled.

Procedures for implantation and preparation of the albino rat for injection have been described (*2, 3*). Of particular interest (*1*) was the application to the caudate nucleus of 1 μg of *dl*-norepinephrine hydrochloride contained in 1 μl with osmolality adjusted to 314 milliosmols. Ten minutes elapsed between the time of injection and decapitation. The spherical diffusion (*2*) obtained with procedure is shown in Fig. 1. There is, in this case, fluorescence extending no less than 0.5 mm from the probe site, which suggests that a sphere of chemical spreading from the cannula may be at least 1 mm in diameter. There was also movement of chemical up the shaft of the cannula, as well as axon-related movement (*2*).

Norepinephrine did move considerable distances from the probe site and thus appears to behave, in this respect, no differently from dopamine (*2*). Thus, the spread of chemical cannot be considered related only to dopamine, as Montgomery and Singer suggested, since similar effects appeared with injections of liquid norepinephrine. In addition, the amount of norepinephrine used was less than that which gave the optimum effects on feeding in the dose-response study of Miller *et al.* (*4*). Hence our results cannot be dismissed on the basis of dosage, since considerable spread of chemical occurred even when the dosage was less than that used to give optimum behavioral effects. Finally, since the injection was delivered in a controlled osmotic vehicle, it is likely that the high osmotic pressure associated with crystalline applicaton does not represent a major contributing cause to the spread of chemical from the probe site.

Concerning the original suggestion by Routtenberg (*5*) that the ventricular system may mediate carbachol-induced drinking, we note the reply to this suggestion by Fisher and Levitt (*6*) that drinking can be obtained by application of carbachol to the lateral ventricle. Negative results (*7*) seem less compelling than positive ones and may be attributed to difficulties with method (*8*). The importance of the ventricles in mediating behavioral effects of chemical stimulation has been supported by Baxter (*9*) in his investigation of carbachol-elicited emotional behavior in the cat.

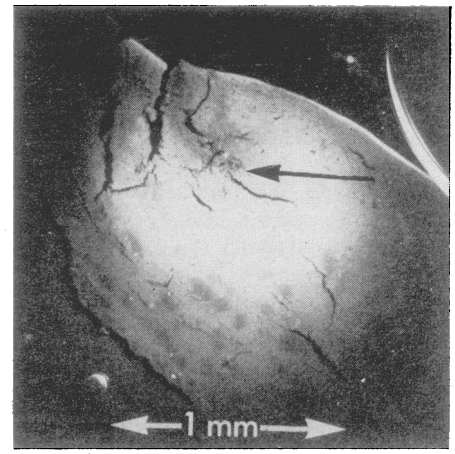

Fig. 1. Fluorescence photomicrograph demonstrating diffusion of 1 μg of *dl*-norepinephrine locally applied in 1 μl to the left caudate nucleus of a female Sprague-Dawley rat (301 g). *dl*-Norepinephrine (*dl*-Arterenol, Sigma) was dissolved in 150 m*M* NaCl. The *p*H was adjusted to 7.2 with 0.2*M* NaOH. The osmolality of the final solution, estimated from freezing point depression (Advanced osmometer) was 314 milliosmols. Black arrow points to tip of cannula.

Our own view has been that inadequate attention has been paid to the methodological aspects of chemical stimulation of the brain (*10*). The issue of the extent of spread (*1, 2, 5*) represents one of the complexities that has until recently been virtually disregarded, although it should be of concern to investigators who employ this technique.

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Histochemical Fluorescence as an Index of Spread of Centrally Applied Neurochemicals

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