tissue-specificity of the 2-mercaptoethanol effects was observed. In whole tissue, the normal liver NAD(P)-dependent and NAD and NAD(P)-dependent hepatoma aldehyde dehydrogenase were affected. No effect was demonstrable on any TCDD-treated liver ALDH nor on normal liver NAD-dependent ALDH. In subcellular fractions, the specificity was even more restricted; only the NAD(P)-dependent hepatoma ALDHs were affected. That TCDD-treated liver disulfiram inhibition was neither potentiated nor blocked by 2-mercaptoethanol indicates that 2-mercaptoethanol was not interacting with hepatoma isozyme IV, but with one or both of the mM K_m NAD(P)-dependent isozymes, II or III.

We are currently unable to explain fully the differing effects of 2-mercaptoethanol on the disulfiram sensitivity of the NAD(P)-dependent hepatoma aldehyde dehydrogenases in whole tissue as opposed to subcellular fractions. It is difficult to directly compare the results obtained in whole tissue preparations and subcellular fractions due to the great differences in specific activity observed [1, 2, 14], and to the possible aggregation and subsequent inactivation of aldehyde dehydrogenase that may occur in whole tissue preparations [3, 14]. TCDD-treated liver ALDHs, however, did not show similar opposing responses to 2-mercaptoethanol and the normal liver potentiation of whole tissue was not apparent in subcellular fractions. These observations suggest that the 2-mercaptoethanol effects were due to variable accessibility of sulfhydral groups in hepatoma isozymes III and perhaps IV to disulfiram caused by 2-mercaptoethanol, perhaps in association with the procedures used to prepare whole tissue homogenates and subcellular fractions. Although this may be further evidence of inherent differences in the ALDHs of the various tissues, confirmation will require comparison of the disulfiram sensitivities of purified preparations of the isozymes involved.

In summary, disulfiram inhibition *in vitro* is shown to be a useful tool in distinguishing among the various aldehyde dehydrogenase isozymes of normal rat liver, rat hepatomas, and xenobiotic-treated rat liver. The NAD(P)-dependent aldehyde dehydrogenases, as well as the NAD-dependent isozymes, are significantly affected by disulfiram. A significant interaction between sulfhydral reagents and certain aldehyde dehydrogenases, especially in rat hepatomas, is demonstrable.

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Calcium and frequency-dependent release of norepinephrine

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Brown and Gillespie [1] showed that the release of norepinephrine (NE) from the cat spleen increased with increasing stimulation frequency, up to 30 Hz. NE output was about 5-fold greater at 30 than at 10 Hz; above 30 Hz the output progressively declined. Although the exact mechanism responsible for the increase in NE output with stimulation frequency is not clear, Kirpekar *et al.* [2] proposed that facilitation of transmitter release with increase in stimulation frequency may be a reflection of a greater accumulation of calcium in the nerve terminals during a train of pulses at a high frequency as compared to a low frequency. If calcium plays such a role in the frequency–output relationship, then changing the extracellular concentration of calcium ions should modify the frequency-dependent NE release, i.e. at low extracellular calcium concentrations the output at the lower frequency of stimulation should be much more reduced than the output at the higher frequency. Conversely, at high extracellular calcium concentrations the output should be preferentially enhanced at low as compared to high frequencies. We report here the effect of calcium on the frequency–NE output relationship in the perfused spleen of the cat.

Cats were anesthesized with ether, followed by chloralose (60 mg/kg, i.v.). The cats were then given iproniazid (20 mg/kg, i.v.) to inhibit monoamine oxidase. After 30 min the spleens were isolated and perfused *in situ* with oxygenated Krebs-bicarbonate (Krebs) solution at a rate of about 7 ml/min at 35°, as described previously [3]. Control venous perfusate samples were collected for 2 min before nerve stimulation, for 2 min during stimulation at 5 and 30 Hz, and for 4 min during stimulation at 1 Hz. To label the endogenous stores of NE with [^{3}H]-NE, a single injection of 200 μCi of [^{3}H]-NE (10.43 Ci/mole) was made into the femoral vein; the spleen perfusion was started 30 min after the injection. The splenic nerves were stimulated with supramaximal monophasic rectangular pulses of 1–2 msec duration at 1, 5 and 30 Hz for a total of 200 stimuli.

The spleens were perfused with Krebs solution for 30 min before nerve stimulation. The nerves were stimulated at 1, 5 and 30 Hz at intervals of about 15 min in normal Krebs solution, and then in low- or high-calcium solution. The order of perfusion with different calcium solutions was reversed, so that in some experiments the first perfusion was with low calcium. Perfusion with high-calcium solution was usually done during the last period. To prepare high-calcium (10 mM) solution, KH_2PO_4 and $NaHCO_3$ were

omitted, and Tris buffer (5 mM) was added. $NaHCO_3$ was replaced by a corresponding amount of NaCl. The pH of the final solution was adjusted to 7.4 with HCl (1 N), and the solution was bubbled with 100% O_2.

NE contents of the venous samples were determined by the trihydroxyindole procedure [4]. Standard solutions of NE were analyzed concurrently, with recoveries ranging from 70 to 90 per cent. [^{3}H]-NE content was determined by measuring radioactivity after adding 0.5 ml of an alumina-extracted sample to 10 ml of scintillation fluid. Background activity of the same volume of solution in the absence of stimulation was deducted from the total output during stimulation, to determine [^{3}H]-NE release due to nerve stimulation.

Figure 1 shows the relationship between NE output and the stimulation frequency during perfusion of the spleen with different calcium concentrations. In normal Krebs solution containing 2.5 mM calcium, the output at 30 Hz was about four and eight times greater than the outputs at 5 and 1 Hz respectively. The output at 5 Hz was twice as large as the output at 1 Hz ($P < 0.001$).

If the calcium concentration of the perfusion medium was reduced to 0.25 mM, and thereby reducing the NE (or [^{3}H]-NE) output, the frequency–output relationship was not only maintained but greatly exaggerated. The output at 30 Hz was about 11-fold greater than the output at 5 Hz and 30-fold greater than that at 1 HZ (Fig. 1). The relative outputs at 1, 5 and 30 Hz were also determined on the basis of the release of [^{3}H]-NE from three spleens that had been prelabelled with radioactive NE. The results of the [^{3}H]-NE release were directly comparable to the outputs determining endogenous NE release. It also should be pointed out that lowering the calcium concentration of the perfusion medium reduced the outputs of NE and [^{3}H]-NE at all frequencies, but the reduction was much more marked at 1 and 5 Hz. On increasing the calcium concentration to 10 mM, the outputs at 1 and 5 Hz were dramatically increased and were almost comparable to that at 30 Hz. The output at 30 Hz was not significantly different from the output at 1 Hz. The steep frequency–output relationship that is observed in normal (2.5 mM) or low calcium solutions almost disappeared. Moreover, the difference between the outputs at 1 and 5 Hz in normal Krebs solution also disappeared in high calcium solution.

Experiments reported in this paper suggest that calcium may play an important role in modulating the changes in transmitter release from adrenergic nerve terminals of the cat spleen produced by different frequencies of stimulation. In spleens perfused with Krebs solution containing 2.5 mM calcium, the output per stimulus at 30 Hz was about 8-fold greater than the output at 1 Hz. On lowering the calcium concentration to 0.25 mM, this relationship was even further exaggerated, and the output at 30 Hz became nearly 30 to 40-fold greater than the output at 1 Hz. This relationship was more or less reversed in 10 mM calcium, and the outputs at 1, 5 and 30 Hz became almost comparable to each other. The reversal is mainly due to the fact that the outputs at 1 and 5 Hz were preferentially enhanced as compared to the outputs at 30 Hz. In fact, the output at 30 Hz in high-calcium solution was not significantly different from the output in normal Krebs solution at this frequency. These results, therefore, suggest that the increase in release of NE with the increase in stimulation frequency is probably related to the greater availability of calcium to the sympathetic nerve terminals during stimulation at high

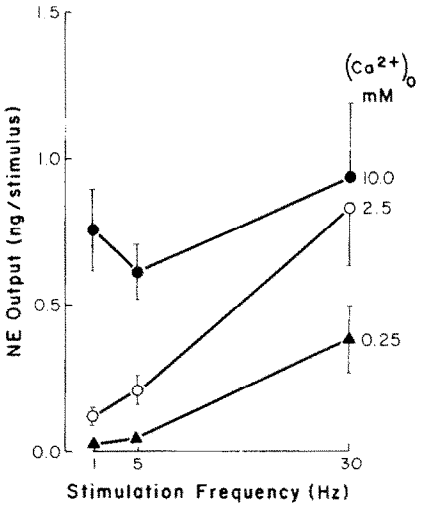

Fig. 1. Effect of calcium on the release of NE elicited by nerve stimulation in the perfused cat spleen. The splenic nerves were stimulated at 1, 5 and 30 Hz in the presence of different extracellular calcium concentrations. Data are means ± S.E. (vertical lines) of three to eight experiments. When the standard error was smaller than the size of the symbol, it was not drawn.

than at low frequencies. StJärne [5] showed that secretion of [^{3}H]-NE from guinea pig vas deferens was completely dependent on external calcium concentration up to 8 mM only in the presence of an alpha-adrenergic blocking agent. Since the present experiments were done in spleens without blockade of alpha-receptors, the role played by the negative feedback mechanism in the frequency-dependent secretory response remains to be elucidated.

Some possible explanations for the facilitating effect of frequency on transmitter release, and hence on calcium entry, may be as follows: (a) each action potential in the sympathetic nerve terminal, as in axons of C fibres [6], may be followed by a transient hyperpolarization (positive after-potential). It is conceivable that the degree of hyperpolarization present at the onset of each action potential is greater in the case of higher frequencies of stimulation than in lower frequencies. If this were the case, and if the extent of hyperpolarization determines the number of available calcium gates to be opened during an action potential (as in cardiac muscle), then at higher frequencies there might be more calcium influx per action potential and therefore more NE released per stimulus, or (b) facilitation of release with increase in frequency may simply reflect a greater accumulation of calcium in the nerve terminals on the time average during a train of pulses at a high frequency as compared to a low frequency.

In conclusion, we have shown that facilitation of NE release with stimulation frequency depends on the level of extracellular calcium concentrations and that, using appropriate calcium concentrations, the frequency response can be modified.

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Induction of uterine peroxidase by hexestrol analogues

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The rapid increase in the activity of peroxidase (EC 1.11.1.7) in the uterus following treatment of ovariectomized or immature rats with estrogen [1, 2] has led to the proposal that this enzyme may be a useful specific marker for those tissues which respond to estrogen [3–5]. Recently, we described a biochemical assay for estrogens based on the correlation between the estrogenic activity of a number of steroids and their abilities to induce peroxidase in the immature rat uterus *in vivo* [6]. The results gave a more appropriate measure of the physiological characteristics of the steroids than those based on binding affinity to specific receptors. This system has also been used to determine whether the catechol estrogens have any antiestrogenic properties [7]. It was therefore considered of interest to extend these studies to hexestrol [meso-3:4-di(*p*-hydroxyphenyl) hexane] and its derivatives and to look for antagonism of peroxidase induction by a structural analogue of hexestrol.

Materials. Most of the compounds tested were from the collection of estrogens synthesized by Mr. W. Lawson at the Courtauld Institute of Biochemistry, London [8–10] and were provided by Dr. W. Coulson. 2-Hydroxyestradiol-17β was a gift of the Cancer Chemotherapy National Service Center, Bethesda, MD. Hexestrol was purchased from the Sigma Chemical Co., St. Louis, MO. [4-^{14}C]Estradiol (52 mCi/mmole) from Amersham, Oakville, Ontario, was shown by chromatography and autoradiography to be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 2–3 mCi/mmole and kept at 4° in the dark as a stock solution in ethanol (1 mg/ml). The diethyl ether used for extraction was free of peroxides.

Preparation of uterine extracts. Immature female Holtzman rats weighing 70–95 g were given a priming dose of estradiol (5 μg in 0.2 ml sesame oil) subcutaneously on day 1 and the test compounds in oil on day 5, before killing the animals 18 hr later. The uteri were dissected free of adhering fat, blotted, and weighed. The tissue was then cut into small pieces and homogenized in 5 ml of 0.1 M sodium phosphate buffer, pH 7.0, in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted to 10 ml with buffer and centrifuged at 100,000 *g* (4°) for 30 min. After washing with buffer, the sediment was rehomogenized in the appropriate amount of 1.2 M NaCl to give a 5% (w/v) homogenate. It was centrifuged again at 100,000 *g* for 30 min, and portions of the supernatant fraction were used for the subsequent enzyme assays.

Determination of peroxidase activity. The uterine extract (1 ml), derived from 25 mg wet wt of tissue, was incubated for 30 min or 1 hr at 38° with [4-^{14}C]estradiol (1.8 μM), 2,4-dichlorophenol (0.25 mM), H_2O_2 (0.25 mM) and bovine serum albumin (10 mg) in 0.1 M sodium phosphate buffer, pH 7.4; total volume was 4 ml. After incubation, the medium was extracted with ether (3 × 1 vol.) and the radioactivity in the aqueous fraction was determined by scintillation counting as described previously [11]. Peroxidase activity was also determined by a more direct method using guaiacol as substrate [12]. The reaction mixture (3 ml) contained guaiacol (13 mM) and H_2O_2 (0.33 mM) in 0.01 M sodium phosphate buffer (pH 7.0) and 1.0 ml of the fraction containing peroxidase. The linear increase in absorbance at 470 nm resulting from the oxidation of guaiacol was then followed at 25° in a Beckman model 25 recording spectrophotometer.

Results and discussion. A good correlation was obtained between the estrogenic activity of a series of hexestrol analogues and their ability to induce peroxidase in immature rat uteri (Table 1). This extends our previous studies with steroids related to estradiol [6]. The compounds to be tested were arranged in order of decreasing potency in bringing about vaginal cornification in ovariectomized rats [8–10]; their uterotrophic effect was also determined. Peroxidase activity was measured by the conversion of [4-^{14}C]estradiol to water-soluble products and by the oxidation of guaiacol, two methods that have been shown previously [6] to give comparable results.

With the exception of XII, the compounds chosen for testing had intact *p*-hydroxyl groups in the aromatic ring because their replacement by other groups is known to cause a marked decrease in estrogenic potency [9], as well as in binding affinity to receptors in the uterus [13]. Polar substituents in the side-chain abolished estrogenic activity (Table 2). Diethylstilbestrol epoxide, however, had been found previously to retain much of the activity of its parent compound [14, 15].

One of the objectives of these studies was to determine whether a natural estrogen such as 2-hydroxyestradiol-17β, which has been shown previously [7] to inhibit the action of estradiol, would show similar antagonism toward a synthetic estrogen and whether hexestrol analogues might act as anti-estrogens. For the latter purpose, 3,3′-difluorobutestrol (VI), having weak estrogenic activity, appeared to be the best candidate by analogy with anti-estrogens such as nafoxidine (U-11,/100A) and CI-628 which, in high doses, are estrogenic and able to induce uterine peroxidase [16, 17]. Analogue VI, however, showed no anti-estrogenic activity at any of the doses tested, even though 2-hydroxyestradiol-17β at a dose of 100 μg/rat decreased significantly the response of the animals to hexestrol (1 μg/rat) (Table 3). Neither compound affected uterine weight, but this dissociation of effects on weight increase and peroxidase induction in the uterus was also observed when 2-hydroxyestradiol was administered concurrently with estradiol [7].

The increase in peroxidase activity in the immature rat uterus is a slightly less sensitive index of estrogenic potency than the increase in uterine weight, but nevertheless, it provides a useful alternative assay. It has the potential to yield additional information about the mechanism of estrogen action, particularly in distinguishing between the early inductive phase and the later phase of true growth [18, 19]. It is possible that the lack of an inhibitory effect on the estradiol-induced uterine weight increase by 2-hydroxyes-